

# NON-CODING RNAs IN GASTROINTESTINAL AND GYNECOLOGICAL CANCERS: NEW INSIGHTS INTO THE MECHANISMS OF CANCER THERAPEUTIC RESISTANCE

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# NON-CODING RNAs IN GASTROINTESTINAL AND GYNECOLOGICAL CANCERS: NEW INSIGHTS INTO THE MECHANISMS OF CANCER THERAPEUTIC RESISTANCE

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# Editorial: Non-Coding RNAs in Gastrointestinal and Gynecological Cancers: New Insights Into the Mechanisms of Cancer Therapeutic Resistance

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## Editorial on the Research Topic

### Non-Coding RNAs in Gastrointestinal and Gynecological Cancers: New Insights into the Mechanisms of Cancer Therapeutic Resistance

## BACKGROUND

Chemoresistance is mediated by a variety of molecular mechanisms, including inhibition of drug influx, increased drug efflux, evasion of cell apoptosis, enhanced repair of DNA damage, induction of epithelial-mesenchymal transition (EMT), activation of cancer stem-like features, induction of autophagy, alterations in cancer metabolism, mutations of drug targets, and dynamic interactions between tumor cells and their surrounding microenvironment (Holohan et al., 2013). Tumor cells can become resistant to chemotherapeutic agents through a combination of different mechanisms (Vasan et al., 2019). Non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), have been implicated in chemoresistance (Wang et al., 2019).

In this research topic, 21 papers (including thirteen reviews and eight original research articles) that have shed light on the therapeutic resistance mechanisms controlled by non-coding RNAs in human gastrointestinal and gynecological cancers were chosen. The latest findings presented in this Research Topic add to our understanding of the mechanisms by which non-coding RNAs govern cancer cell resistance to chemotherapy, and may also point to meaningful molecular targets that could be utilized to eliminate chemoresistance and increase the survival of cancer patients.

## MIRNA AND CHEMORESISTANCE

Tumor cells frequently have a high rate of glucose metabolism, and aerobic glycolysis has been linked to chemoresistance in tumors (Marcucci and Rumio, 2021). Hexokinase 2 (HK2), an

enzyme that catalyzes the first committed step of glucose metabolism, has additional non-enzymatic roles in contributing to tumor chemoresistance (Dong et al., 2021). Yang et al. now report that HK2 is highly expressed in cervical cancer tissues, and its overexpression is correlated with poor patient survival. The authors found that HK2-knockdown cells formed fewer spheres than control cells, and knocking down HK2 markedly increased the susceptibility of cervical cancer cells to cisplatin. Furthermore, downregulation of the tumor suppressor miR-148a is essential for HK2 overexpression. Upregulation of miR-148a impairs the sphere-forming ability of cervical cancer cells and reduces cisplatin resistance. Hence, the combination of cisplatin and miR-148a mimic may be an effective strategy for preventing cancer stem cell-like properties and overcoming cisplatin resistance in cervical cancer patients.

## LNCRNA AND CHEMORESISTANCE

ATP binding cassette transporter proteins are well known for their contributions to chemoresistance by removing anti-cancer drugs from cancer cells. ATP Binding Cassette Subfamily C Member 10 (ABCC10, also known as MRP-7) plays a major role in this process (Szakács et al., 2006). A study by Huang et al. revealed that MRP-7 is expressed at high levels in drug-resistant endometrial cancer cell lines, and MRP-7 overexpression confers paclitaxel resistance to endometrial cancer cells while also promoting cell invasiveness *in vitro*. The authors further discovered that miR-98 is a key upstream suppressor of MRP-7 and that miR-98 directly targets *MRP-7* mRNA to repress MRP-7 expression. As a result, forced expression of miR-98 significantly reverses paclitaxel resistance and reduces the invasion of endometrial cancer cells. The authors found that lncRNA NEAT1 elevates MRP-7 levels by decreasing miR-98 expression. Thus, NEAT1/miR-98/MRP-7 signaling may be exploited as a critical therapeutic target to overcome paclitaxel resistance in endometrial cancer.

In endometrial cancer, DCST1-AS1 was discovered as an oncogenic lncRNA that promotes cancer progression by regulating the miR-665/HOXB5 pathway and the miR-873-5p/CADM1 pathway (Wang et al.).

Using an RNA binding protein immunoprecipitation assay, Trujano-Camacho et al. report that lncRNA HOTAIR interacts with  $\beta$ -catenin in the cervical cancer cell line HeLa. Their results demonstrate that the interaction between HOTAIR and  $\beta$ -catenin might hinder ICRT14 (a novel potent inhibitor of the Wnt/ $\beta$ -catenin pathway) from having anti-tumor effects on cell viability. The authors conclude that HOTAIR knockdown synergizes with ICRT14 to cause necrotic death in HeLa cells.

Qian et al. describe that lncRNA MALT1 is highly expressed in colorectal cancer tissues and cell lines, and MALT1 induces the proliferation and migration of colorectal cancer cells. Mechanistically, MALT1 promotes colorectal cancer progression via the miR-375/miR-365a-3p/NF- $\kappa$ B axis. They propose that targeting MALT1 might be an attractive treatment option for colorectal cancer.

## CIRCRNA AND CANCER PROGRESSION

Li et al. report that circDNMT1, a circular RNA overexpressed in gastric cancer, can sponge miR-576-3p and subsequently elevate the levels of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) in gastric cancer cells. By regulating the miR-576-3p/HIF-1 $\alpha$  axis, circDNMT1 promotes gastric cancer cell proliferation, migration, invasion, and glycolysis.

## COMPUTATIONAL STUDIES OF NCRNAS

An original article by Wang et al. highlighted the importance of an immune-related lncRNA signature in predicting the prognosis of patients with colon cancer. Ding et al. confirmed that the expression of RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 was increased in endometrial cancer tissues and that this 3-lncRNA signature was closely associated with histological subtype and advanced clinical stage in endometrial cancer patients. Their results indicate that this new lncRNA model might be useful for predicting the prognosis of patients with endometrial cancer.

## REVIEWS

13 review articles focus on the recent advances in the ncRNA research field and summarize the function and mechanisms of miRNAs, circRNAs, and lncRNAs in the pathogenesis of gastrointestinal and gynecological cancers. Zhao et al. outline the important impacts of miR-30d-5p on many types of malignancies, indicating that miR-30d-5p has broad potential as a diagnostic and therapeutic target for tumors. Ma et al. discuss the potential use of circRNAs as biomarkers or therapeutic agents for gynecological cancers. Wang et al. summarize the mechanisms underlying the involvement of circRNAs in modulating gastrointestinal cancer chemoresistance. Furthermore, Ghafouri-Fard et al. summarize the tumor suppressor role for a circRNA circITCH in various cancers. All these studies improve our understanding of the underlying mechanisms of ncRNAs and may aid in the development of new therapies for gastrointestinal and gynecological cancers.

## SUMMARY

Taken together, the discovery of novel non-coding RNAs involved in the regulation of glycolysis or drug efflux has revealed fresh insights into the molecular basis of chemoresistance in gynecological cancers. Aberrant regulation of the miRNA-mRNA pathway, or the lncRNA/circRNA-miRNA-mRNA ceRNA network, contributes to chemoresistance in gastrointestinal and gynecological cancer cells. Notably, lncRNAs can interact with RNA-binding proteins and some RNA-binding proteins have critical roles in circRNA biogenesis. Further research on the interplay between ncRNAs and their molecular partners might provide opportunities for more

effective strategies to prevent or overcome chemoresistance in gastrointestinal and gynecological cancer patients.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# A Comprehensive Overview of circRNAs: Emerging Biomarkers and Potential Therapeutics in Gynecological Cancers

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Circular RNA (circRNA) is a highly conserved, stable and abundant non-coding RNA (ncRNA). Also, some circRNAs play an essential part in the progression of human cancers. CircRNA is different from traditional linear RNA. CircRNA has a closed circular structure, so it is resistant to exonuclease-mediated degradation and is more stable than linear RNA. Numerous studies have found that many circRNAs can act as a microRNA (miRNA) sponge, interact with RNA-binding proteins, regulate gene transcription, affect alternative splicing and be translated into proteins. Recently, some studies have also indicated that circRNA participates in the progression of gynecological cancers. In addition, circRNA can act as a promising biomarker for the diagnosis of gynecological tumors. Additionally, they can also play a key role in the prognosis of gynecological tumors. Furthermore, to our delight, circRNA may be a potential therapeutic target in gynecological cancers and widely used in clinical practice. This article reviews the functions and related molecular mechanisms of circRNAs in gynecological tumors, and discusses their potential as biomarkers for diagnostic and prognostic and therapeutic targets for gynecological cancers.

**Keywords:** circular RNA, expression, biomarkers, therapeutics, gynecological cancers

## INTRODUCTION

Circular RNA (circRNA) is a unique class of RNA that unlike other RNAs forms a covalently closed loop, generally thought to be non-coding, which as a result of improved sequencing strategies has recently gained renewed interest among scientists (Hsu and Coca-Prados, 1979; Ashwal-Fluss et al., 2014). Many scientists believe that circRNA is a widely distributed and diverse small endogenous RNA with multiple regulatory functions. According to the synthesis mechanism based on the location of the genome splice junction from which circRNAs originate, circRNAs are classified into four major types: exonic circRNAs (ecircRNAs), intronic circRNAs, exon-intron circRNAs (EIciRNAs), and intergenic circRNAs (Dong Y. et al., 2017). Unlike linear RNA, circRNA is a covalently closed single-stranded circular transcript without 5'cap or 3'poly(A) tail. This special structure makes the circRNA a stable, conserved, highly abundant RNA that is dynamically expressed in specific tissues through a unique process (Nicolet et al., 2018). Since circRNA has the advantages of cell- type-, tissue- and developmental stage-specific expression, and cancer cells have diverse expression profiles of the different types of circRNA, circRNA can also be used to classify and identify different tumor types (Athanasiadis et al., 2004; Salzman et al., 2013; Zhang et al., 2017; Nicolet et al., 2018; Smid et al., 2019). In recent years, many studies have indicated

that circRNA is related to a variety of human diseases, including cardiovascular diseases (Hansen et al., 2013; Dong et al., 2017; Zhang et al., 2017; Smid et al., 2019), neurological diseases (Liu et al., 2017; Kristensen et al., 2019), and other diseases (Li P. et al., 2015; Wang et al., 2015). In addition, recently, it has been found that the development of certain tumors is affected by functional circRNA-mediated regulatory networks in various ways, such as acting as a microRNA (miRNA) “sponge” and regulating the function of miRNA target genes (Hansen et al., 2013). Besides, it has been reported that certain circRNAs can bind with specific RNA binding proteins (RBPs), thereby affecting the function of the parental genes and alternative splicing (Liu et al., 2017; Zeng et al., 2017; Kristensen et al., 2019). Interestingly, increasing evidence shows that circRNA can encode proteins/peptides involved in cancer pathogenesis and progression (Legnini et al., 2017; Ye et al., 2018; Zheng et al., 2019). The unique characteristics and biological functions of circRNAs indicate that circRNA has the potential to be a promising biomarker for the diagnosis and prognosis of various diseases, as well as a therapeutic target (Kun-Peng et al., 2018; Zhou et al., 2019).

## THE CHARACTERISTICS OF CircRNA

Accumulating evidence shows that circRNAs are ubiquitously distributed in eukaryotic cells, and have important characteristics and multiple biological functions, which make circRNAs the focus of interest in many scientific research fields, including ncRNA (Dong R. et al., 2017). (1) Since, unlike linear RNA, circRNA is a covalently closed loop with no 5' end cap or 3' poly (A) tail structure, it is not easily degraded by exonuclease and is more stable than linear RNA (Suzuki and Tsukahara, 2014). (2) CircRNAs are more diverse and abundant than their linear mRNA analogs. The expression of circRNA is often cell-type-, tissue- and developmental stage- specific (Patop et al., 2019; Huang et al., 2020). (3) Most circRNAs are evolutionarily conserved among different species, at both their sequence level and their pattern of expression (Jeck et al., 2012; Memczak et al., 2013; Salzman et al., 2013; Haddad and Lorenzen, 2019). (4) Except for intronic circRNAs that are sequestered in the nucleus, most ecircRNAs are exported to the cytoplasm in a size-dependent manner during their biogenesis (Huang et al., 2018). These characteristics of circRNAs indicate that they may play vital roles at both the transcriptional level and the post-transcriptional level, and may be useful in disease diagnosis.

## BIOGENESIS OF CircRNAs

CircRNA is produced through a circularization process involving a canonical spliceosome-mediated precursor mRNA (pre-mRNA) back-splicing mechanism (Meng et al., 2017), which connects a downstream splice donor site (3' splice site) to an upstream acceptor splice site (5' splice site), and is modulated by RBPs and intronic complementary sequences. However, unlike in canonical (linear) splicing, in back-splicing the canonical

cis-acting splicing regulatory elements and trans-acting splicing factors have different or even opposite activity. As briefly mentioned above, based on the synthesis of circRNAs from different locations of the genome splice junction from which circRNAs originate, circRNAs can be categorized into four types: ecircRNAs, intronic circRNAs, EIciRNAs, and intergenic circRNAs (Chen, 2016). Although, at present, the precise mechanism of circRNA biogenesis remains unclear, advances in sequencing technology, especially RNA-seq, have led the identification and characterization of numerous circRNAs, that resulted in significant progress regarding their biogenesis (Meng et al., 2017). It is known that ecircRNAs lack intronic sequences, are the most abundant type of circRNAs, predominantly localize to the cytoplasm and are formed by the reverse covalent binding of the splice donor site and the splice acceptor site of the pre-mRNA. Intronic circRNAs contain only intron sequences and include five subtypes, namely circular intronic RNAs (ciRNAs), excised group I introns, excised group II introns, excised tRNA introns, and intron lariats. EIciRNAs contain both intronic and exonic sequences as they are concurrently circularized by exons and introns, likely in similar manner to ecircRNAs. Intergenic circRNAs are another non-exonic circRNA type derived from the genomic interval between two genes and formed by two intronic circRNA fragments (ICFs) flanked by GT-AG splicing signals. As a result, three major models of the formation of ecircRNA or EIciRNA through a back-splicing mechanism have been proposed, namely lariat-driven circularization (exon skipping), intron pairing-driven circularization, and resplicing- or RBP-driven circularization (Ebbesen et al., 2016).

The lariat-driven circularization model is associated with exon skipping, in which one or more exons of the transcript are skipped, generating a lariat consisting of both exons and introns. Then, the introns are removed to produce an ecircRNA. However, in some cases the introns are retained between the encircled and results in the formation of EIciRNA (Kristensen et al., 2019). In addition, if the activity of the debranching enzymes that control these lariat introns is reduced, these lariat introns escape subsequent degradation to form ciRNA through intron cyclization. The intron pairing-driven circularization (direct back-splicing) model (Zhang et al., 2014), the major pathway of ecircRNA production, is mostly associated with the pairing of flanking intronic complementary motifs (Alu elements) of the pre-mRNA, which induces circularization to form ecircRNA after the removal of introns. If there are inverted repeats, other than Alu elements, circRNA can also be generated via direct base-pairing of these inverted repeats. However, unlike ecircRNA, ciRNA formation is dependent on a conserved sequence near both sides of the spliceosome. CiRNA biogenesis relies on a 7-nt GU-rich element near the 5' splice site and an 11-nt C-rich element near the branch point site (Petkovic and Müller, 2015). During direct back-splicing, the two elements bind into a lariat-like intermediate, containing the excised exons and introns, and are spliced by the spliceosome, generating lariats that undergo 3' tail degradation, ultimately resulting in the production of ciRNAs (Petkovic and Müller, 2015). The RBP-driven circularization model is another biogenesis pathway to generate circRNAs by direct back-splicing driven by RBPs.

Certain proteins, including quaking (QKI) and muscleblind like splicing regulator 1 (MBNL1, also termed MBL) proteins can bind to specific flanking intronic sequence motifs on linear pre-mRNA sequences and act as RBPs to link flanking introns, thus promoting circularization and subsequent circRNA generation (Conn et al., 2015). The process resembles the intron pairing-driven circularization model, except that in this case, after binding to specific putative binding sites, RBPs like MBNL1 dimerizes and brings the introns together, which leads to the circularization of the pre-mRNA and production (Figure 1).

## THE FUNCTIONS OF CircRNAs

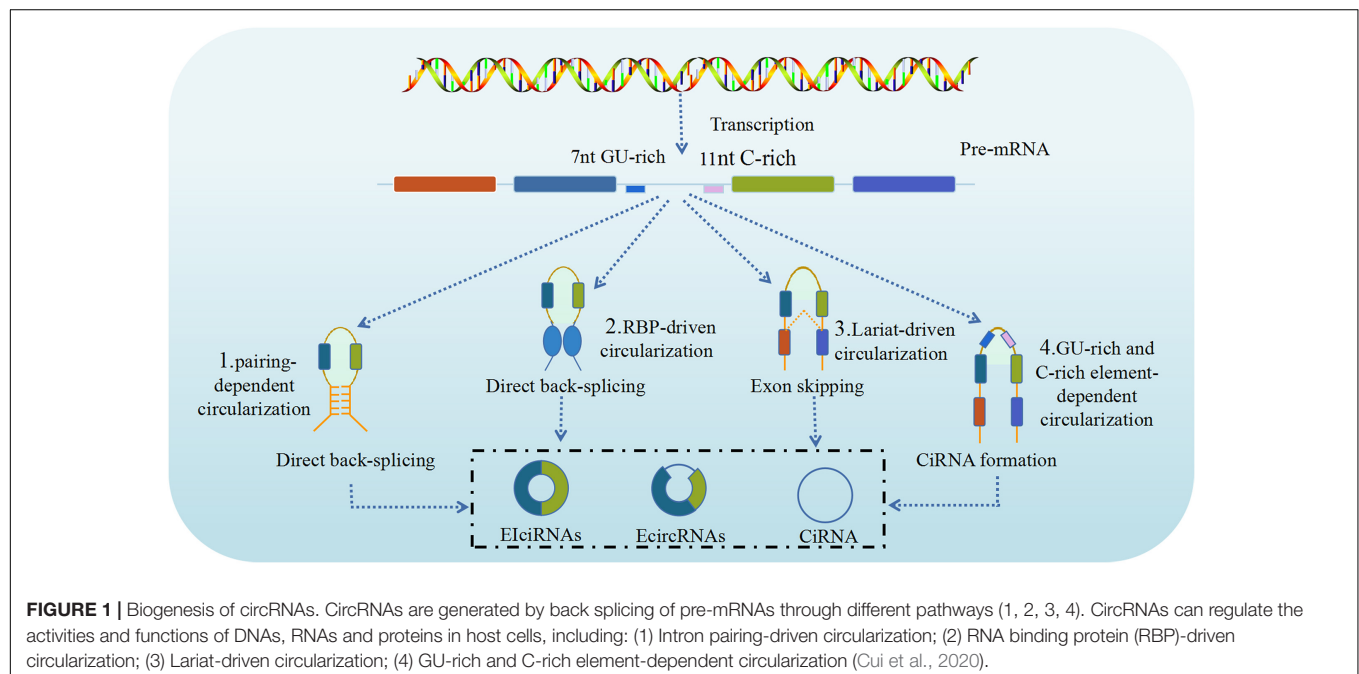
It is well established that circRNAs can act as miRNA sponges by competing for miRNA binding sites, and diminish the effect of miRNA-mediated regulatory activities. The best characterized circRNA with miRNA sponge function, the human cerebellar degeneration related protein 1 antisense (CDR1as, also termed as ciRS-7) circRNA acts as a sponge for miR-671 and miR-7 to inhibit their expression *in vivo* (Piwecka et al., 2017). Experiments overexpressing CDR1as circRNA and using CDR1as gene knockdown demonstrated that overexpression of CDR1as circRNA increases the expression of miRNA targets, whereas its knockdown had the opposite effect, indicating that this circRNA be crucial for normal neuronal development. This type of *in vivo* models helps us gain a deeper understanding of the complex functions of circRNA in gynecological tumors. In human tumor cells, circRNA the following five possible functions in regulating different molecular pathways have been identified: miRNA sponging, protein binding, regulation of gene transcription or splicing, and translation of proteins or peptides (Figure 2).

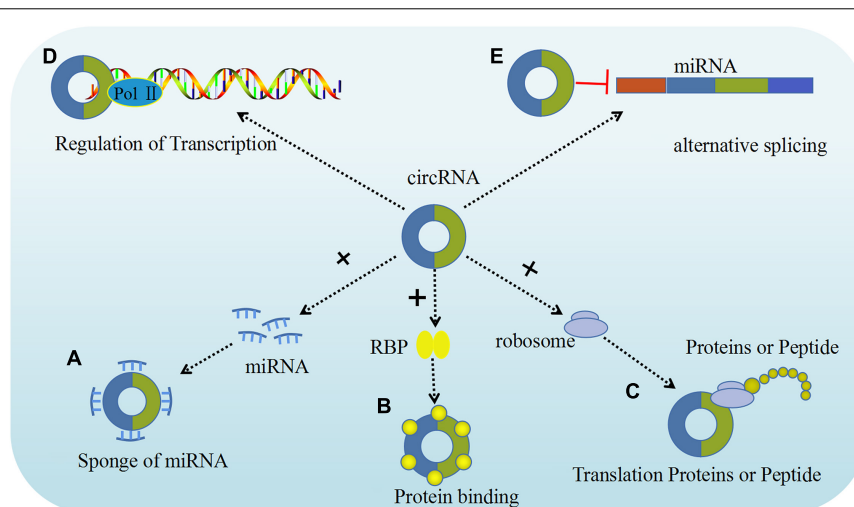
## MicroRNA (miRNA) SPONGING

Growing evidence has confirmed that circRNA regulates gene expression through competitive binding with miRNA, leading to its designation as a “miRNA sponge” (Chen et al., 2018). For example, the upregulation of circ\_0103552 induced oncogenic activity in breast cancer cells, partly by directly sponging miR-1236, and is accompanied by a poor prognosis (Yang et al., 2019). Other findings indicate that circRNAs regulate the development of ovarian tumors through a variety of mechanisms, among which miRNA sponging is the most prominent. After being synthesized in the nucleus, circRNAs are transported to the cytoplasm where they mainly act as a miRNA sponge, while other circRNAs can interact with miRNA to transcribe or post-transcriptionally regulate gene expression (Chen, 2016). Recently, a study indicated that hsa\_circ\_0061140 is highly expressed in ovarian cancer (OC) cell. In addition, circ-FOXO1 becomes a competitive endogenous circRNA by binding to miR-370 (Chen et al., 2018; Yang et al., 2019). Several studies showed that circ-ITCH suppresses the Wnt/ $\beta$ -catenin pathway and thus its dysregulation is involved in the progression of various cancers (Hu C. et al., 2018). Circ-HIPK3 plays an important role in cancers by sponging multiple miRNAs, thereby promoting miRNA-124 and circFoxo3-mediated inhibition of the growth and survival of cancer cells (Teng et al., 2019) (Table 1).

## CircRNAs INTERACT WITH AND BIND TO PROTEINS

Like linear RNAs, recent studies have shown that some circRNAs can also be used as RBP sponge to isolate the RBP by hiding the binding site of a specific protein, and





**FIGURE 2 |** The functions of circRNAs. Five major functions of circRNAs have been identified, including: **(A)** Sponge of miRNA; **(B)** Proteins binding; **(C)** Translation proteins or peptide; **(D)** Regulation of transcription; **(E)** Alternative splicing (Yang et al., 2020).

**TABLE 1 |** The expression and mechanisms of circRNAs in cervical cancer.

Circular RNAs	Cancers expression	Clinical samples or target gene	Mechanisms	References
has_circ_0018289 _ CC Up		35 pairs of cervical cancer tissue compared with adjacent normal tissue and cell lines	Hsa_circ_0018289 promote cervical cancer proliferation, migration and invasion via sponging miR-497.	Gao et al., 2017
circMTO1 CC Up	S100A1	HeLa and SiHa cells	Increase S100A1 expression by sponging miR-6983	Wright et al., 2009
hsa_circ_0000515 CC Up	ELK1	Hela, U14, SiHa, CaSki	Increase ELK1 expression by sponging miR-326	

competitively bind the protein and reduce protein activity. Some circRNAs, such as ciRS-7 and sex-determining region Y (SRY) circRNA (circ-SRY), can trigger the linearization and AGO2-mediated degradation of ciRS-7, which enables the release of the absorbed miRNA molecules and inhibit mRNA transcription (Capel et al., 1993). In addition, Du et al. (2016) found that circ-Foxo3 can competitively bind to p53 and mouse double minutes 2 homolog (MDM2), thereby decreasing the interaction between Foxo3 and MDM2 and enhancing Foxo3 activity, promoting MDM-induced p53 ubiquitination and degradation, ultimately leading to cell apoptosis (Huang et al., 2015; Li F. et al., 2015; Du et al., 2016; Wan et al., 2016). Circ-MBNL1 can bind to muscleblind like splicing regulator 1 (MBNL1, also termed MBL) protein; some studies have shown that due to the feedback loop

mechanism, the biosynthesis of circ-MBNL1 will be affected by the level of MBNL1, and the high level of MBNL1 protein will also affect the process of converting MBNL1 pre-mRNA into circ-MBNL1 (Ashwal-Fluss et al., 2014). Furthermore, current evidence indicates that circRNAs can participate in the regulation of cancer pathogenesis by combining with various partners such as RBPs, including QBP, AGO, POL II, MBNL1, and other RBPs (Ashwal-Fluss et al., 2014; Du et al., 2016). For example, QKI interaction with circRNAs may lead to the regulation of epithelial-mesenchymal transition (EMT) during cancer progression. In addition, recent findings indicate that many circRNAs interact with HuR (a type of RBP) in cervical cancer (CC) cells. In addition, Circ-PABPN1 can bind to HuR, and HuR and circ-PABPN1 compete with PABPN1 mRNA (Dudekula et al., 2016). Despite extensive research on circRNAs, many remain unknown, and they may play key roles in cancer and could serve as biomarkers. According to these findings, it is safe to say that circRNAs have multiple functions in tumor development and progression.

## REGULATION OF TRANSCRIPTION AND ALTERNATIVE SPLICING

Most circRNAs are located in the cytoplasm and regulate gene transcription. However, recent studies have reported that an EICiRNA accumulates at a transcription site and promotes the expression of the parental gene (Zhang et al., 2013). Knockout of a pair of EICiRNAs can inhibit the transcription of their host gene. Some EICiRNAs can interact with U1snRNPs (U1 small nuclear ribonucleoproteins), and the EICiRNA-U1 snRNP complex binds to RNA Pol II on the promoter of its parent gene, thereby enhancing gene expression. Once such RNA-RNA interaction is blocked, it destroys the interaction between EICiRNA and RNA



Pol II and reduces the transcription of their host genes (Li P. et al., 2015). Moreover, certain circRNAs can compete with their linear counterparts against canonical pre-mRNA splicing, thus suppressing the expression of their host gene (Zhang et al., 2013; Wang et al., 2017). Cyclization and splicing compete with each other, leading to ecircRNAs to play a role in alternative splicing (Ji et al., 2019). Once back splicing occurs, it splices out internal exons, leading to alternative splicing. ElciRNA may be isolated from the translation start site, and thus truncated linear mRNA cannot be translated.

## TRANSLATING PROTEINS OR PEPTIDE

Although circRNAs are considered to be non-coding RNAs, accumulating data indicate that certain circRNAs are also translated into proteins. According to reports, circCTNNB1 produces a CTNNB1 isoform (i.e.,  $\beta$ -catenin) containing 370 amino acids, which was found to be able to antagonize the GSK3 $\beta$ -induced  $\beta$ -catenin phosphorylation, thereby stabilizing the degradation of full-length  $\beta$ -catenin (Wang et al., 2015). In addition, recently numerous studies have shown that cyclic lncRNA-PINT may be translated into peptides to inhibit the proliferation of glioblastoma cells and the translation extension of oncogenes by capturing PAF1c (Abe et al., 2015). Unlike linear RNAs, some circRNAs prevent ribosomes from being recognized and translated into protein due to the lack of the 7-methylguanosine cap structure and poly(A) tail (Pamudurti et al., 2017). However, to date, almost no circRNA has been found to be translated into protein, so it is necessary to conduct further research in this field.

## THE EXPRESSION, AND RELATED MECHANISMS OF CircRNAs IN GYNECOLOGICAL CANCERS

### Expression of CircRNAs in Cervical Cancer

CC is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women worldwide in 2018 (Cohen et al., 2019). Human papilloma virus (HPV) infection is positively correlated with the occurrence and development of CC (Cohen et al., 2019). Recent study analyzed the expression of circRNAs in CC tissues. The analysis found that 45 different circRNAs were prominently expressed in CC tissues compared to control tissue samples, among which hsa\_circ-0018289 was the most prominent. They found that knocking out this particular circRNA inhibited the proliferation and invasion of tumor cells. In addition, they also ascertained that hsa\_circ\_0018289 acts as a miRNA sponge and directly binds to miRNA-497 (Gao et al., 2017). Zheng et al. (2018) analyzed the circRNA expression profiles of CaSki CC cell line transfected with E7 sicirc-RNA and identified 526 abnormally expressed circRNAs. Subsequent bioinformatics analysis revealed that these HPV16/18-type E7 and E6 oncoproteins were necessary for the transformation

and maintenance of the malignant phenotype of CC cells (Cai et al., 2018; Zheng et al., 2018). Chen et al. (2019) showed that the direct interaction between Circ-MTO1 and miR-6893 could rescue the invasion, migration and chemotherapeutic resistance of CC cells regulated by MTO1. Previous studies have shown that S100A1 promotes the progression of OC and hepatocellular carcinoma and circMTO1 was found to act as a sponge of miR-6893 to elevate the expression level of S100A1 in clinical CC (Wright et al., 2009). Moreover, Tang et al. (2019) reported that hsa\_circ\_0000515 was highly expressed in CC tissues and cells. Furthermore, hsa\_circ\_0000515 acts as a sponge of miR-326 and enhances ELK1 expression. ELK1, a transcription factor that belongs to the ETS family and ternary complex factor (TCF) subfamily, was found to play key roles in the regulation of cellular growth, differentiation, and survival (Dun and Gao, 2019). Inhibition of ELK1 inhibited cell cycle and promoted apoptosis. Thus, hsa\_circ\_0000515 plays a tumor promoting role in CC via hsa\_circ\_0000515/miR-326/ELK1 regulatory axis. Last but not least, they also suggested that hsa\_circ\_0000515 may be a promising target for CC treatment (Table 1).

### CircRNAs Suppress Tumor Progression

Currently, several circRNAs have been found to play different roles in a variety of cancer types. In cancer research, one of the best studied circRNAs is generated from the tumor suppressor gene FOXO3, a member of the forkhead family of transcription factors that has been found to induce apoptosis of cancer cells through a variety of biological processes. Circ-FOXO3 indirectly increases the expression of the linear mRNA of its host gene by binding to p53 and MDM2. The increase in circ-Foxo3 expression reduces the interaction between FOXO3 and MDM2, leading to the release of FOXO3, thereby increasing its activity, ultimately leading to increased apoptosis (Du et al., 2016; Kong et al., 2020). Hsa\_circ\_0001445 (also known as circ-SMARCA5) acts as a sponge for miR-620 and inhibits the progression of CC tumors, since its expression in CC cells is downregulated and its overexpression inhibits their proliferation, invasion and migration (Dai et al., 2018). Recently, circ-CLK3 was identified as a new type of circRNA overexpressed in CC. It has also been shown that inhibiting miR-320a and preventing its ability to inhibit the expression of the FoxM1 transcription factor promote cell proliferation, epidermal-mesenchymal transformation (EMT) and invasion of CC cells (211) (Hong et al., 2019) (Table 4).

### CircRNAs Promote Tumor Progression

Recently, a reported research study has shown that a new type of circRNA, namely circ-SLC26A4, is highly expressed in CC (Ji et al., 2020). Knockout of circ-SLC26A4 *in vitro* and *in vivo* was shown to inhibit tumor proliferation and invasion. Moreover, circ-SLC26A4 was found to target the 3'UTR of HOXA7 mRNA and sponge miR-1287-5p. Therefore, circ-SLC26A4 acts as a sponge of miR-1287-5p to promote the expression of HOXA7, which may potentially lead to new CC treatment strategies. In addition, circAMOTL1, which acts as a sponge for miR-485-5p, was found to enhance the expression of AMOTL1 (Ou et al., 2020). Moreover, Gao et al. (2017) found

45 upregulated circRNAs by analyzing 35 CC patients. Like other circRNAs, hsa\_circ\_0018289 exhibited carcinogenic effects on the progression of CC (He et al., 2020). Also, hsa\_circ\_0018289 was found to act as a sponge for miR-497, leading to increased cell proliferation of CC cells (He et al., 2020). Additionally, Mao et al. (2019) found that circ-EIF4G2 is overexpressed in CC cells and binds to miR-218, and at the same time, both can promote the expression of HOXA1 and enhance the progression of CC. Liu J. et al. (2018) showed that circ\_8924 is overexpressed in CC cells and can bind to members of the miR-518d-5p/519-5p family. Therefore, they can promote the expression of the chromobox 8 (CBX8) axis (Liu J. et al., 2018) (Table 4).

## CIRCULAR RNAs AND OVARIAN CANCER

### Expression of CircRNAs in Ovarian Cancer

OC was one of the major causes of cancer death in women worldwide in 2020 (Siegel et al., 2020). Signaling pathways and dysfunction of cellular mechanisms significantly influence the progression and invasion of OC. The correlation and association between the regulation of circRNAs and OC have received much research interest. Ahmed et al. (2016) was the first to analyze circRNA expression in clinical ovarian tumors and found numerous differentially expressed circRNAs in tumor samples. They also detected differences in the miRNA and circRNA expression levels between primary and metastatic ovarian tumors. Regarding RNA exonuclease resistance and circRNAs stability, primary tumors were distinguished from metastatic lesions rather than mRNAs. Notably, in ovarian metastases, signaling pathways, such as STAT, AKT, NF- $\kappa$ B, TGF- $\beta$ , ILK, HGF, and VEGF are commonly activated to produce linear RNA and angiogenesis signaling pathways that are negatively expressed in circRNAs. Hu J. et al. (2018) have demonstrated that circ-ITCH acts as a sponge for miR-145 to increase RASA1 expression and inhibit progression of OC cells. The miRNA sponge cycle in cells is a candidate for cancer diagnosis and treatment based on RNA. In contrast to the downregulated circ-ITCH expression, studies have indicated that the abnormal expression of circRNA in OC can play an oncogenic role (Hu J. et al., 2018). Recently, VPS13C-hsa\_circ\_001567 was shown to be able to promote the invasion and migration of OC cells, as well as enhance their invasion and migration ability. It was also shown that VPS13C-hsa\_circ\_001567 is involved in the VPS13C-hsa\_circ\_001567-miR-370- FOXM1 axis playing a role as the competing endogenous RNA (ceRNA) of the miR-370 sponge, thereby increasing the level of FOXM1 (Bao et al., 2019). This finding indicates that different circRNAs play opposite roles in the regulation of the biological behavior of OC cells by acting as tumor suppressor genes or oncogenes (Table 2).

### CircRNAs Suppress Tumor Progression

Previously, a number of studies showed that CDR1as can promote cancer in the various human cancer processes. Clinical

**TABLE 2 |** The expression and mechanisms of circRNAs in ovarian cancer.

Circular RNAs	Cancer expression target gene	Clinical samples or cell lines	Mechanisms	References
circITCH OC down	RASA1	20 paired OC tissues and adjacent normal tissues; CAO3 and SKOV-3 cells	Increases FOXK2 expression by sponging miR-93-5p	Hu J. et al., 2018
VPS13C OC Down - has_circ_001567	FOXM11	20 paired OC tissues and adjacent normal tissues; SKOV-3 and OV-1063 cells	Increases N-cadherin expression And decreases E-cadherin expression	Bao et al., 2019
circ_0061140 OC Up	FOXM1	SKOV-3 and A2780 cells	Promotes EMT by increasing FOXM1 expression via sponging miR-370	Chen et al., 2018; Zou et al., 2018
circPLEKHM3 OC Down	BRCA1 DNAJB6KLF4	5 OC tissues and 5 normal ovarian epithelial tissues; A2780 and MDAH2274 cells	Inactivates the PI3K/AKT and Wnt/ $\beta$ -catenin pathways via promoting BRCA1, DNAJB6a and KLF4 expression by sponging miR-9	Zhang et al., 2019

studies have indicated that the expression level of CDR1as has a positive correlation with poor clinical prognosis, and it is a key prognostic indicator for lung cancer (Chen et al., 2019). However, some studies have shown that CDR1as is downregulated in OC tissues. In addition, CDR1as inhibits the function of OC cells by binding to miR-135b-5p. Other studies have shown that circ\_100395 has a lower expression level in OC tissues than the corresponding non-cancerous tissues (Li et al., 2020). Furthermore, OC patients with a lower expression of circ\_0078607 are more likely to have better prognosis. Therefore, circ\_0078607 may be a crucial molecular target for patients with ovarian cancer. Also, circ\_0078607 can function as a molecular sponge for miR-518a-5p, which promotes the inhibitory effect of miRNA on the expression of Fas, which is a membrane protein belonging to the tumor necrosis factor receptor superfamily (Zhang et al., 2020). Fas interacts with receptor Fas ligand (FasL), which induces a cascade of death signals and ultimately leads to cell apoptosis. According to the bioinformatics analysis results, Fas is the direct target of miR-518a-5p, thus we hypothesize that circ\_0078607 acts as a sponge for miR-518a-5p and can increase the expression of Fas, thereby inhibiting the proliferation and invasion of OC cells and promoting their apoptosis. Accordingly, the circ\_0078607/miR-518a5p/Fas axis can promote new aspects of the treatment of ovarian cancer patients (Zhang et al., 2020). Zhang et al. (2019)



showed that Circ-*PLEKHM3* downregulation can promote the progression of ovarian cancer cells; EMT induction promotes tumor metastasis; and Circ-*PLEKHM3* upregulation can play the opposite role. Circ-*PLEKHM3* can act as a miR-9 sponge and since the ceRNA enhances the inhibitory effects of *BRCA1*, *DNAJB6*, and *KLF4* on miR-9 target genes. The circ-*PLEKHM3*-miR-9 axis promotes the occurrence and metastasis of OC. Circ-*PLEKHM3* expression is downregulated in OC. Patients with a low level of circ-*PLEKHM3* have a poor prognosis (Zhang et al., 2019). Chen et al. (2018) found that *hsa\_circ\_0061140* is overexpressed in OC cell lines. And a Knockout of this gene can inhibit the progression of OC cells *in vivo* and *in vitro* by inhibiting the expression of fork head box M1 (*FOXM1*) by sponging miR-370 (Chen et al., 2018; Zou et al., 2018) (Table 4).

## CircRNAs Promote Tumor Progression

Zong et al. (2019) analyzed 79 cases of epithelial ovarian cancer tissue and 13 cases of normal ovarian tissue and measured the expression level of circ-*WHSC1*. Their results reveal that circ-*WHSC1* is significantly overexpressed in OC and is significantly correlated with the degree of differentiation, which indicates that circ-*WHSC1* may be significantly correlated with the progression of OC. Moreover, circ-*WHSC1* was upregulated, while the expression of miR-1182 and miR-145 was upregulated in OC. The high expression of circ-*WHSC1* may promote cell proliferation and inhibit cell apoptosis. Furthermore, circ-*WHSC1* acts as a sponge for miR-1182 and miR-145 to enhance the expression of *MUC1* and *hTERT*. In other words, Circ-*WHSC1* promotes the occurrence and development of OC through the miR-1182 and miR-145/*MUC1* and *hTERT* axis, providing a promising target for the treatment of OC. Liu et al. (2020a) found that circ-*GFRA1* is highly expressed in OC. The downregulation of circ-*GFRA1* inhibits cell proliferation and invasion and induces apoptosis. Additionally, circ-*GFRA1* can play an important role by acting as a sponge for miR-449a. Therefore, circ-*GFRA1* may be a potential diagnostic biomarker, and a therapeutic target for OC (Liu et al., 2019). Finally, Xie et al. (2019) conducted a series of experiments to demonstrate that circ-*EPSTI1* acts as a sponge for miR-942 to increase the expression of *EPSTI1*. In addition, another study showed that circ-*EPSTI1* inhibited the occurrence and development of cancers, and induced apoptosis in OC, thus confirming its carcinogenic effect (Xie et al., 2019) (Table 4).

## CIRCULAR RNAs AND ENDOMETRIAL CANCER

### Expression of CircRNAs in Endometrial Cancer

Endometrial cancer (EC) is a common gynecological tumor in women all over the world. CircRNAs are highly conserved and stable non-coding RNAs, which have recently attracted considerable attention due to their potential function in cancer development (Li et al., 2018). They have multiple miRNA binding sites, which mediate their activity by competitively binding to target miRNAs, thereby inhibiting transcription of

downstream genes (Patop and Kadener, 2018). However, the mechanism by which circRNAs regulate gene expression in EC remains unclear. Studies have shown that circ\_0067835 is significantly increased in EC. Recently, circ\_0067835 was found to be positively correlated with *HMGA1* in EC (Liu et al., 2020a). *HMGA1* is predicted to be a downstream target of miR-324-5p in EC and is identified as an important prognostic biomarker for EC. Circ\_0067835 acts as a sponge for miR-324-5p to induce the expression of *HMGA1*. Accordingly, circ\_0067835 can compete with miR-324-5p, leading to the overexpression of *HMGA1*, thereby inducing the progression of EC. In addition, the expression of *hsa\_circ\_0002577* was found to be significantly upregulated in EC tissues, and its high expression was associated with advanced FIGO staging, lymph node metastasis and low overall survival rate of EC patients. Knockout of *hsa\_circ\_0002577* significantly reduced the proliferation, and migration of EC cells *in vitro*, and reduced tumor growth *in vivo*. Therefore, *hsa\_circ\_0002577* can play a vital role through the *hsa\_circ\_0002577*/miR-197/*CTNND1*/Wnt/ $\beta$ -catenin signaling pathway, which represents a new therapeutic option for developing EC therapeutics. Increasing evidence shows that by sequestering miRNAs, circRNAs play a key role in regulating gene expression (Shen et al., 2019). Circ\_0109046 and *HMGA2* were upregulated in EC tissues and cells, while miR-136 was downregulated. *HMGA2* promotes tumor progression in gynecological cancers. Shi et al. (2020b) proposed that circ\_0109046 acts as a sponge for miR-136 to enhance the expression of *HMGA2*, indicating that circ\_0109046 may be a promising target for EC treatment. In addition, studies have shown that the expression level of Circ-*PUM1* in EC tissues is significantly higher than that of normal tissues. The upregulation of circ-*PUM1* promotes the proliferation, migration and invasion of EC cells. After knocking out circ-*PUM1*, the tumorigenic ability of EC cells is reduced. Circ-*PUM1* can compete with miR-136, leading to the up-regulation of *NOTCH3*, thereby promoting EC development (Zong et al., 2020) (Table 3).

### CircRNAs Promote Tumor Progression

Some previous studies have shown that circ-*WHSC1* is highly expressed in (EC) and promotes EC. Liu et al. (2020d) collected a total of 26 normal endometrial tissues and 32 EC tissues, which were confirmed pathologically. They found that overexpression of circ-*WHSC1* promoted cell proliferation, migration and invasion, and inhibited apoptosis (Liu et al., 2020d). In addition, it has been reported that circ-*WHSC1* acts as a sponge for miR-136 and targets *NPM1* mRNA to negatively regulate the progression of EC. *NPM1* is highly expressed in EC and has a positive correlation with the clinical stage and histological grade of EC. A recent study in 28 tumor samples and adjacent normal tissues of EC patients showed that circ-*TNFRSF21* rescues the *MAPK13*-*ATF2* signaling pathway activity by acting as a miR-1227 sponge in EC cells. The high expression level of circ-*TNFRSF21* promoted EC cell growth, cell cycle progression and *in vivo* tumor growth. Thus, circ-*TNFRSF21*-miR-1227-*MAPK13*/*ATF2* axis may be a promising target in EC treatment (Liu et al., 2020b). Liu et al. (2020b) showed that *hsa\_circ\_0061140* acts as a sponge for miR-149-5p and as an oncogenic circRNA in EC. In addition, it has

**TABLE 3 |** The expression and mechanisms of circRNAs in ovarian cancer.

Circular RNAs	Cancer expression target gene	Clinical samples or cell lines	Mechanisms	References
circ_0067835 EC Up	HMGA1	10 Endometrial cancer tissues and 10 normal endometrial tissues; HEC1-B and RL95-2 cells	Increases HMGA1 expression by sponging miR-324-5p	Liu et al., 2020a
hsa_circ_0002577 EC Up	CTNND1	36 paired EC tissues and adjacent normal endometrial tissues; ECC-1 and HEC-1-A cells	Increases Wnt/ $\beta$ -catenin pathways via promoting CTNND1 expression by sponging miR-197	Shen et al., 2019
circ_0109046 EC Up	HMGA2	44 Endometrial cancer tissues and 44 normal endometrial tissues; HEC1-A, KLE and Ishikawa cells	Increases HMGA2 expression by sponging miR-136	Shi et al., 2020a
circPUM1 EC Up	NOTCH3	69 paired EC tissues and adjacent normal endometrial tissues; HEC-1B CELLS and Ishikawa human endometrial carcinoma cells	Increases NF- $\kappa$ B/MMP2 expression by sponging miR-615-5p/miR-6753-5p	Zong et al., 2020

also been confirmed that STAT3 is a downstream target of miR-149-5p. Therefore, hsa-circ\_0061140 promotes the progression and migration of EC by regulating miR-149-5p and STAT3 (Liu et al., 2020c) (Table 4).

## THE BIOMARKERS AND THERAPEUTIC TARGETS OF CircRNAs IN GYNECOLOGICAL CANCERS

### CircRNAs as Promising Biomarkers of Cervical Cancer Prognosis

Compared with paired normal tissues, circ\_0067934 is highly expressed in CC tissues and cell lines. The upregulation of circ\_0067934 is also related to positive lymphatic metastasis of CC patients, which indicates that it can be used as a promising biomarker for CC metastasis. Compared with adjacent normal tissues, circ\_0018289 is highly expressed in CC tissues, and is strongly associated with decreased overall survival rate (He et al., 2020). This suggests that circ\_0018289 may be

a promising biomarker for prognostic evaluation. Moreover, it has been shown that hsa\_circ\_0023404 is overexpressed in CC tissues and cell lines (Zhang et al., 2018). This finding indicates that the overall survival rate of patients with high expression level of hsa\_circ\_0023404 is lower than that of patients with low expression level of hsa\_circ\_0023404. The possible related mechanism is that hsa\_circ\_0023404 sponges miR-136. Also, hsa\_circ\_0023404 induces the activation of the related protein Yes-associated protein (YAP) pathway, which leads to the progression of CC (Zhang et al., 2018). Recently, Song et al. (2019) found that hsa\_circ\_101996 is upregulated in CC and is related to tumor stage, size, lymph node metastasis and poor prognosis. Moreover, circ\_0067934 sponges miR-545 and is correlated with advanced cancer, lymph node metastasis and metastasis, as well as poor prognosis (Hu C. et al., 2018; Song et al., 2019). In addition, circ-ATP8A2 is also upregulated in CC cells, and the circATP8A2/miR-433/EGFR axis plays a critical role in the progression of CC (Mao et al., 2019) (Figure 3).

### CircRNAs as Potential Therapeutic Targets in Cervical Cancer

A very recent study has shown that has\_circ\_0000745 is overexpressed in CC patients, and its high expression is correlated with poor tumor differentiation and vascular invasion. Knocking out hsa\_circ\_0000745, upregulated the expression of cadherin 1, which can inhibit the progression of CC cells, indicating that this circRNA could be an ideal target candidate for the development of CC therapy (Jiao et al., 2019). Other studies have shown that post-transcriptionally circ-ATP8A2 acts as a sponge for miR-433 to inhibit the expression of epidermal growth factor receptor (EGFR). Studies in a nude mouse model revealed that the knockout of circ\_0000285 can clearly inhibit the occurrence and migration of CC. In other words, circ\_0000285 can promote the progression and metastasis of CC (Chen et al., 2019). Other studies found that the expression of circRNA\_8924 in CC tissue samples was significantly higher than that in adjacent non-tumor tissue samples, and was correlated with FIGO staging, myometrial invasion, and tumor size (Liu J. et al., 2018). Also, circRNA\_8924 was found to act as a sponge for miR518d-5p/519-5p to elevate CBX8 expression and inhibit progression of CC cells and promote apoptosis of CC cells (Figure 3).

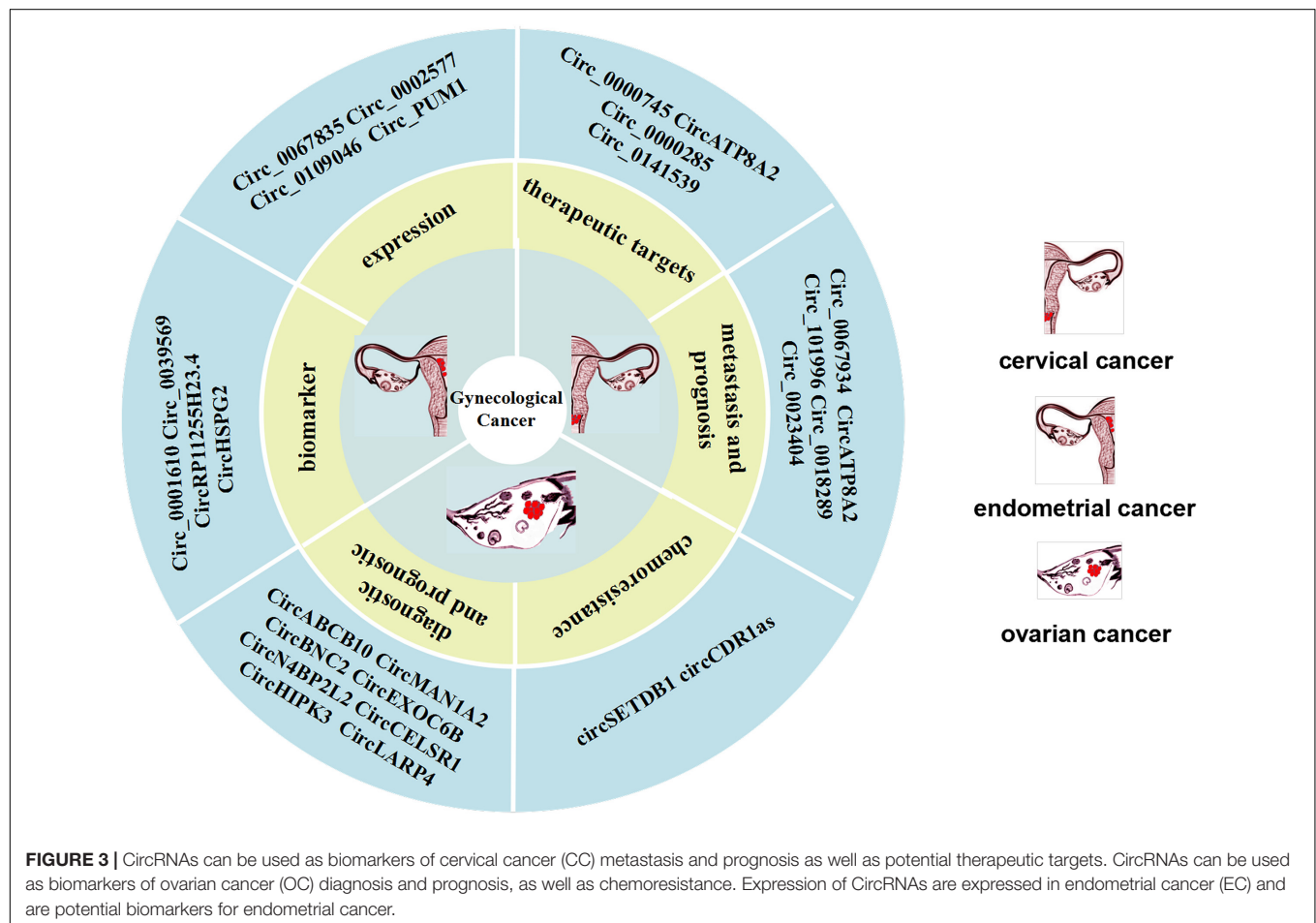
### CircRNAs as Biomarkers of Ovarian Cancer Diagnostic and Prognostic CircRNAs as Novel Serum Biomarkers of Ovarian Cancer Diagnostic and Prognostic

CircRNAs can reflect the progression of cancer in clinical practice and can be used to predict the prognosis of OC patients, thus it has the potential to act as a biomarker. Research on circulating cell-free circRNA extracted from blood is a hot topic in the field of diagnostic biomarkers (Chen et al., 2019; Fan et al., 2019). For example, Fan et al. (2019) collected 414 serum samples, including samples from 121 healthy controls, and found that circMAN1A2 was significantly upregulated in the serum samples of patients with NPC, oral cancer, thyroid cancer, ovarian cancer, and lung cancer and had good clinical

**TABLE 4 |** CircRNAs suppress and promote gynecological tumors progression.

Circular RNAs	Gynecological cancers	miRNA	Cancer expression target gene	Function	References
hsa_circ_0001445 (circ-SMARCA5)	CC	miR-620	—	—	Dai et al., 2018
circ-CLK3	CC	miR-320a	FoxM1	—	Dai et al., 2018
circ-SLC26A4	CC	miR-1287-5p	HOXA7	+	Ji et al., 2020
circAMOTL1	CC	miR-485-5p	AMOTL1	+	Ou et al., 2020
circ-EIF4G2	CC	miR-218	HOXA1	+	Mao et al., 2019
circ_8924	CC	miR-518d-5p/519-5p	CBX8	+	Liu J. et al., 2018
circCDR1as	OC	miR-135b-5p	—	—	Chen et al., 2019
circ_0078607	OC	miR-518a-5p	Fas	—	Zhang et al., 2020
circ-PLEKHM3	OC	miR-9	BRCA1, DNAJB6, KLF4	—	Zhang et al., 2019
hsa_circ_0061140	OC	miR-370	FOXN1	—	Chen et al., 2018; Zou et al., 2018
circ-WHSC1	OC	miR-1182 miR-145	MUC1/hTERT	+	Zong et al., 2019
circ-GFRA1	OC	miR-449a	—	+	Liu et al., 2020a
circEPSTI1	OC	miR-942	EPSTI1	+	Xie et al., 2019
circWHSC1	EC	miR-136	NPM1	+	Liu et al., 2020a
circTNFRSF21	EC	miR-1227	MAPK13/ATF2	+	Liu et al., 2020a
hsa_circ_0061140	EC	miR-149-5p	STAT3	+	Liu et al., 2020a

Promote tumor progression (+); suppress tumor progression (—).



diagnostic value. Thus, they suggested that circMAN1A2 could be a serum biomarker for malignant tumors, providing important insights into diagnostic approaches for malignant tumors (Fan

et al., 2019). In addition, the circulating circ-ABCB10 can also serve as diagnostic biomarkers of OC, based on the finding that the high expression level of circ-ABCB10 is correlated with

the lower overall survival rate of OC patients (Chen et al., 2019) (Figure 3).

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In addition, Ning et al. (2018) found that the circ-EXOC6B, circ-N4BP2L2 and circ-CELSR1, are clearly dysregulated in OC tissues, and thus may represent promising OC biomarkers. It is of great importance to evaluate the prognosis of doctors of the OC patients. It has been reported that circ-BNC2 can distinguish early stage ovarian cancer patients from benign and healthy subjects, and may be a specific diagnostic biomarker for OC (Hu et al., 2019). The low expression of circ-EXOC6B and circ-N4BP2L2 in OC patients is closely related to the overall survival rate and disease-free survival rate. However, it has been demonstrated that several circRNAs can suppress tumors in OC patients and their prognosis evaluated. The Circ-HIPK3 was found to be overexpressed in OC tissues and its high expression was significantly correlated with lymph node infiltration, FIGO stage and poor prognosis in OC patients (Liu N. et al., 2018). Recent studies have shown that the expression of circ-LARP4 is significantly downregulated in OC tissues, and is correlated with FIGO stage, progression and lymph node metastasis. Therefore, circ-LARP4 may be a promising marker for predicting the prognosis of OC patients (Zou et al., 2018) (Figure 3).

### CircRNAs in Ovarian Cancer Chemoresistance

Chemoresistance and recurrence of OC have become a burden for effective management of the disease. It has been reported that there are many subtypes of cancer associated with chemoresistance and a circRNA. Patients with OC often develop chemotherapy resistance, which makes it unlikely to achieve satisfactory treatment outcomes with chemotherapy. Wang et al. (2019) found that the higher circ-SETDB1 expression level in serum is significantly correlated with lymph node metastasis and clinical stages, and can be used as an important indicator to distinguish patients from healthy subjects. It should be noted that in that study the circ-SETDB1 serum expression level of patients with primary chemotherapy resistance was significantly increased, indicating that serum circ-SETDB1 is likely an important indicator of the OC chemotherapy response and recurrence. Zhao et al. (2019) compared cisplatin-resistant and sensitive OC tissues at the expression level of circRNAs and found that expression level of CDR1as in tissues and cells from patients with cisplatin resistance was generally low (Zhao et al., 2019). In other studies, an inhibitory effect of CDR1as on OC cells. These studies showed that CDR1as can enhance the sensitivity of OC to platinum through the miR-1270/SCAI signaling pathway (Figure 3).

### CircRNAs as Potential Biomarker in Endometrial Cancer

In recent years, circRNAs have been used as a promising biomarker and the mechanism of action of certain circRNAs

in EC cells and tissues have been studied. Numerous studies have shown that there are 75,928 dysregulated circRNAs in EC cells. There are 62,167 circRNAs expressed in EC cells, which are significantly upregulated or downregulated compared to their expression in normal endometrial tissue (Ye et al., 2019). Both Hsa\_circ\_0039569 and hsa\_circ\_0001610 are relatively unique circRNAs. Although their expression levels have nothing to do with myometrial invasion, grade 1–2 EC, lymph node metastasis, tumor size, FIGO stage, and patient age, they are closely correlated with tumor differentiation. Some studies indicate that the hsa\_circ\_0039569 interacts with the hsa-miR-542-3p/hsa-let-7c-5p axis, which has a low expression level in grade 3 EC. In other words, hsa\_circ\_0039569 inhibits the expression of hsa-miR-542-3p/hsa-let-7c-5p, which may be a promising biomarker in EC (Xu et al., 2018). According to reported research, RP11255H23.4 and HSPG2 are expressed in normal endometrial tissues, but not in EC tissues (Shi et al., 2020a). Moreover, the corresponding miRNA expression level is also elevated state in normal tissues, which indicates that circRNAs can competitively combine with related miRNAs to promote the progression of EC. Together the above research results showed that circRNAs may be promising biomarkers and therapeutic targets for the diagnosis of EC (Figure 3).

## CONCLUSION

In conclusion, as described in this review, dysregulation of the expression of circRNA genes is considered to be one of the main mechanisms driving tumorigenesis and progression. A large amount of research data indicates that circRNAs have important roles in the progression of various cancers. The latest research shows that abnormal circRNA expression can have an important impact on the occurrence and development of gynecological cancers through a miRNA sponge mechanism. EICRNAs are very stable, so they have great potential for application in the diagnosis or therapeutic intervention of tumors. Furthermore, certain circRNAs may be ideal for the treatment of tumors. With the advances and application of high-throughput sequencing technology, researchers have identified increasing an increasing number of circRNAs. However, their role and mechanism in gynecological tumors are still unclear. Moreover, the recent research sample population usually comes from a single research center and the number of samples is relatively small. Furthermore, due to the complexity of the tumor, not many exact functions have been found. Therefore, the use of more readily available clinical sample types (such as serum, urine) will improve the reliability of the research results. At present, most research focuses on the sponge function of circRNAs, and to a certain extent, the research on other functions is ignored. The pathogenesis of tumors is complex and variable, thus, the mechanisms of action of circRNAs in tumors have not been thoroughly studied, and further research on this topic is needed. Based on the conclusions of current clinical and experimental studies, this article summarized the potential of circRNAs as new biomarkers of the diagnosis and treatment of gynecological cancers. However, research on circRNAs in gynecological tumors



is still in the early stages, further research is needed to broaden the application potential of circRNAs.

## AUTHOR CONTRIBUTIONS

YM, LZ, YG, WZ, QZ, and YX performed literature searches and selected the studies and reviews discussed in the manuscript. The first draft of the manuscript was prepared by YM, LZ, YG, WZ, and QZ made subsequent amendments. YX revised the

manuscript. All authors read and approved the final manuscript and contributed to the conception of this review.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# LncRNA DCST1-AS1 Promotes Endometrial Cancer Progression by Modulating the MiR-665/HOXB5 and MiR-873-5p/CADM1 Pathways

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Dysregulation of long noncoding RNA (lncRNA) is implicated in the initiation and progression of various tumors, including endometrial cancer (EC). However, the mechanism of lncRNAs in EC tumorigenesis and progression remains largely unexplored. In this work, we identified a novel lncRNA DC-STAMP domain-containing 1-antisense 1 (DCST1-AS1), which is highly upregulated and correlated with poor survival in EC patients. Overexpression of DCST1-AS1 significantly enhanced EC cell proliferation, colony formation, migration, and invasion *in vitro* and promoted tumor growth of EC *in vivo*. Mechanistically, DCST1-AS1 mediated EC progression by inducing the expression of homeobox B5 (HOXB5) and cell adhesion molecule 1 (CADM1), *via* acting as a competing endogenous RNA for microRNA-665 (miR-665) and microRNA-873-5p (miR-873-5p), respectively. In addition, we found that the expression of miR-665 and miR-873-5p was significantly downregulated, while HOXB5 and CADM1 expression levels were increased in EC tissues. Taken together, our findings support the important role of DCST1-AS1 in EC progression, and DCST1-AS1 may be used as a prognostic biomarker as well as a potential therapeutic target for EC.

**Keywords:** endometrial cancer, long noncoding RNA, DC-STAMP domain-containing 1-antisense 1, microRNA-665, homeobox B5, microRNA-873-5p, cell adhesion molecule 1

## INTRODUCTION

Endometrial cancer (EC) is the most common malignant gynecological cancer in women (1, 2). Most women diagnosed with EC have the early-stage disease and show favorable outcomes (3, 4). However, there is a subset of ECs in which metastasis and recurrences do occur (3, 4).

**Abbreviations:** EC, endometrial cancer; lncRNAs, long noncoding RNAs; DCST1-AS1, DC-STAMP domain-containing 1-antisense 1; HESCs, human normal endometrial stromal cells; NC, negative control.

Clinical outcomes worsen considerably for women diagnosed with clinically aggressive disease (5). Thus, there is an urgent need to develop more effective strategies for the diagnosis and treatment of EC.

Long noncoding RNAs (lncRNAs) represent a large class of nonprotein-coding transcripts larger than 200 nucleotides (6, 7). Numerous lncRNAs are aberrantly expressed in a broad spectrum of cancers, and they play important roles in regulating gene expression (6, 7). lncRNAs could act as guides to promote or inhibit transcription and as scaffolds by interacting with chromatin-modifying complexes (8). Furthermore, lncRNAs function as competing endogenous RNAs (ceRNAs) to sponge microRNAs (miRNAs), indirectly modulating gene expression (7). In human tumors, lncRNAs are considered as regulators of multiple cancer phenotypes, including tumor cell proliferation, motility, invasion, and metastasis (9). For example, lncRNA small nucleolar RNA host gene 5 (SNHG5) sponged miR-25-3p to enhance B-cell translocation gene 2 (BTG2) expression, thereby repressing EC cell proliferation (10). lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) was found to promote EC cell growth and invasion through its interaction with miR-144-3p or targeting miR-361 (11, 12). Additionally, lncRNA titin-antisense RNA1 (TTN-AS1) and lncRNA taurine-upregulated gene 1 (TUG1) were demonstrated to promote EC progression by sponging miRNAs (13, 14). Furthermore, another lncRNA steroid receptor RNA activator (SRA) was considered to promote EC progression by activating the Wnt signaling (15).

lncRNA DC-STAMP domain-containing 1-antisense 1 (DCST1-AS1) was overexpressed in various tumor types, including breast cancer, glioblastoma, cervical cancer, and hepatocellular carcinoma (16–19). Elevated expression of DCST1-AS1 regulates cancer progression by sponging different miRNAs and influencing the downstream signaling pathways (16–19). However, our current understanding of DCST1-AS1 in EC is still limited. Recently, DCST1-AS1 was demonstrated to promote EC cell invasion by sponging miR-92a-3p and upregulating the expression of Notch1 (20). The function and mechanism of DCST1-AS1 involved in regulating EC progression remain unexplored.

In this study, we predicted that DCST1-AS1 potentially interacts with microRNA-665 (miR-665) and microRNA-873-5p (miR-873-5p), which play important roles in a series of cancers acting as either oncogene or repressor (21–23). Our study demonstrated that DCST1-AS1 was upregulated in EC tissues and its expression was associated with worse patient outcomes. DCST1-AS1 could promote EC progression by binding with miR-665 and miR-873-5p and inducing the expression of homeobox B5 (HOXB5) and cell adhesion molecule 1 (CADM1), respectively. Therefore, we revealed a novel mechanism by which lncRNA DCST1-AS1 facilitated EC proliferation *via* the miR-665/HOXB5 axis and the miR-873-5p/CADM1 axis. Thus, these signaling pathways might be potential targets for developing therapeutic strategies for the treatment of EC.

## MATERIALS AND METHODS

### Patient Tissue Specimens

Seventy pairs of human EC tissues and corresponding adjacent normal tissues were obtained from patients treated in People's Hospital of Rizhao. All patients signed the informed consent form for the use of samples, and this study was approved by the Ethical Committee on Scientific Research of People's Hospital of Rizhao. All tissue samples were immediately frozen in liquid nitrogen after surgical removal and stored at  $-80^{\circ}\text{C}$ .

### Cell Culture and Transfection

Four human EC cell lines (HEC-1A, HEC-1B, RL-95-2, and JEC) and human normal endometrial stromal cells (HESCs) were obtained from the ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  incubator. Two small interfering RNAs (siRNAs) targeting lncRNA DCST1-AS1, negative control siRNA (si-NC), miR-665 mimic, miR-873-5p mimic, control mimic (miR-NC), miR-665 inhibitor, miR-873-5p inhibitor, control inhibitor, pcDNA-HOXB5, pcDNA-CADM1, and empty vector (pcDNA3.1) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Briefly, EC cells were seeded into six-well plates at 70% cell confluence. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. After transfection, EC cells were cultured for the indicated time and subjected to the following experiments.

### RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from frozen tissues or cell lines using TRIzol reagent (Invitrogen), and then was reverse transcribed into complementary (cDNA) using PrimerScript RT reagent (TaKaRa, Dalian, China). The quantitative real-time PCR (qRT-PCR) assay was conducted using SYBR Green Mix (TaKaRa) on a Bio-Rad system. GAPDH was used as the internal control. The primer sequences for qRT-PCR are listed in **Table 1**.

### Cell Counting Kit-8 Assay

Cell viability experiment was conducted using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were transfected and seeded into 96-well plates. Twenty-four, 48, and 72 h later, the CCK-8 solution was added to each well and incubated for 4 h at  $37^{\circ}\text{C}$ . The absorbance of each well was measured at 450 nm wavelength using a microplate reader (PerkinElmer, Shanghai, China). All assays were performed in triplicate.

### Colony Formation Assay

EC cells were seeded on six-well plates and maintained in the culture media at  $37^{\circ}\text{C}$  for 2 weeks. Then, cells were fixed with 4% paraformaldehyde for 10 min and stained using 0.1% crystal

**TABLE 1 |** The primers sequence for qRT-PCR assay.

Gene	Forward	Reverse
<i>LncRNA DCST1-AS1</i>	TTCGTCTGGTCCCAATGTGTGG	AAGCAGGACGAGTAAACCAACC
<i>U6</i>	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTGCAT
<i>miR-665</i>	GCCGAGACCAGGAGGCUGA	CTCAACTGGTGTGCGTGGA
<i>miR-873-5p</i>	GCAGGAACUUGUGAGUCUCCU	AGGAGACUCACAAAGUUCUGC
<i>GADPH</i>	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG
<i>HOXB5</i>	TGCATCGCTATAATTCATT	GCCTCGTCTATTTCGGTGA
<i>CADM1</i>	TCAACACGCCGTACTGTCTG	GTGGGAGGAGGGATAGTTGTG

violet solution for 30 min at room temperature. The number of colonies was counted using ImageJ software.

## Cell Migration and Invasion Assay

The transfected cells were seeded in the top chamber in 200  $\mu$ l of serum-free DMEM medium (Gibco) with Matrigel (BD Biosciences, San Jose, CA, USA), and a complete medium (750  $\mu$ L) containing 10% FBS was added to the lower chamber. After 24 or 48 h, the migrated or invaded cells were fixed and stained with crystal for 15 min at room temperature. Five random fields per well were observed, and the cells were counted under the microscope.

## Flow Cytometry Analysis

The apoptosis of EC cells was examined using an Annexin V-FITC/propidium iodide (PI) staining assay (BD Biosciences). After washing with cold PBS, the cells were resuspended in binding buffer followed by staining with Annexin V-FITC/PI at room temperature for 15 min in the dark. Apoptotic cells were evaluated by a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences). All experiments were performed in triplicate.

## Luciferase Reporter Assay

The wild-type (WT) fragments of DCST1-AS1 3'-untranslated region (UTR), HOXB5 3'-UTR, and CADM1 3'-UTR were synthesized and cloned into pGL3 vector (Promega Corporation, Madison, WI, USA).

Mutations (MUT) of the miRNA-binding sites in the DCST1-AS1, HOXB5 3'-UTR, and CADM1 3'-UTR were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Then, miR-665 mimic, miR-873-5p mimic, or control mimic was co-transfected with the indicated reporter plasmids into EC cells using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturer.

## Tumor Xenograft Studies

Animal experiments were approved by the Institutional Animal Care and Use Committee of the People's Hospital of Rizhao. Four- to 6-week-old female nude mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and were randomly divided into two groups for further study. We established stable EC lines using shRNA against lncDCST1-AS1 or control shRNA. EC cells with

stable knockdown of lncDCST1-AS1 or control cells were injected subcutaneously into the flanks of nude mice. Tumor volume was calculated as length (mm)  $\times$  width<sup>2</sup> (mm<sup>2</sup>)  $\times$  0.5. After 40 days, tumors were harvested and weighed.

## Bioinformatics

The expression level of lncRNA DCST1-AS1, miR-665, miR-873-5p, HOXB5, and CADM1 was examined using the Encyclopedia of RNA Interactomes (ENCORI) database (<http://starbase.sysu.edu.cn/>). The online website tool DIANA-TarBase ([https://carolina.imis.athena-innovation.gr/diana\\_tools/web/index.php](https://carolina.imis.athena-innovation.gr/diana_tools/web/index.php)) was used to predict the interaction between microRNAs, DCST1-AS1, and mRNAs.

## Statistical Analysis

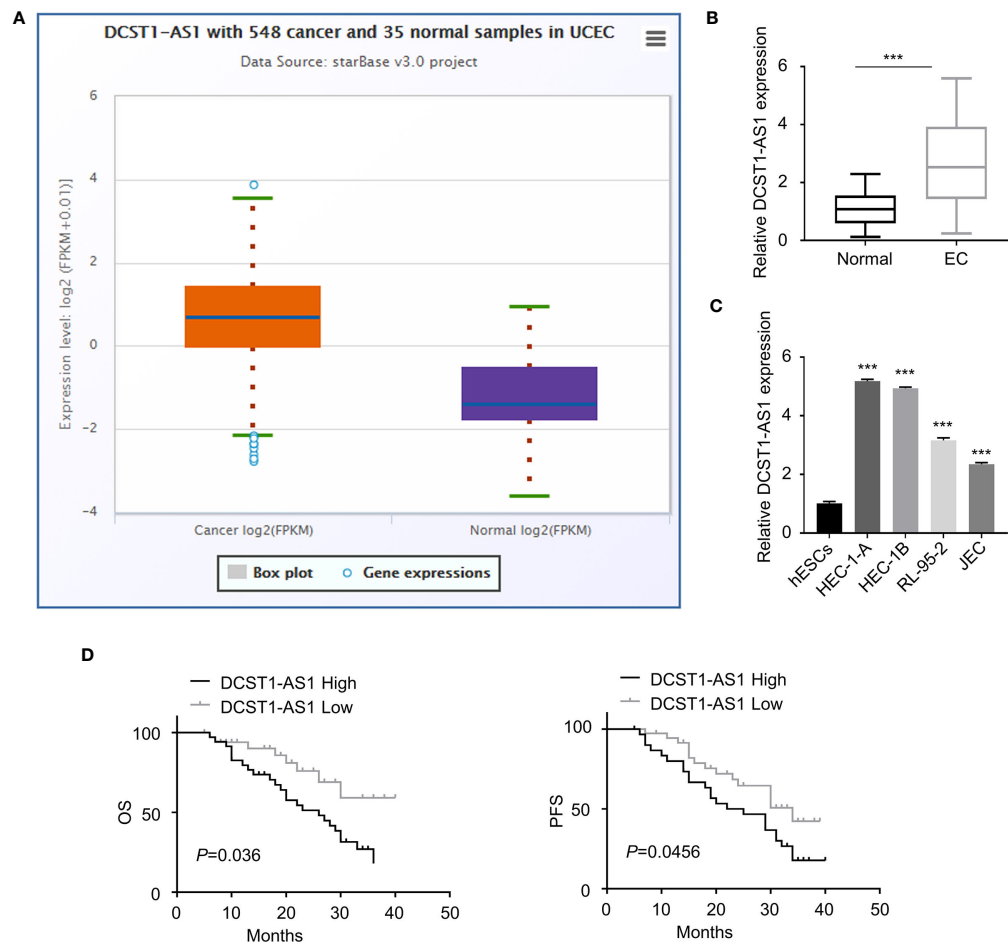
All data were presented as means  $\pm$  standard deviation. Student's *t*-test was used to compare the difference between the two groups. One-way analysis of variance (one-way ANOVA) was used to analyze the differences among multigroups. All of the statistical calculations were performed using GraphPad Prism 5 software (San Diego, CA, USA). *P* < 0.05 was considered statistically significant: \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## RESULTS

### lncRNA DCST1-AS1 Is Overexpressed in EC Tissues and Cell Lines

We first compared the expression of lncRNA DCST1-AS1 in normal endometrial tissues (*n* = 35) and EC tissues (*n* = 548) using the online database ENCORI. As shown in **Figure 1A**, lncRNA DCST1-AS1 was significantly upregulated in EC tissues compared with normal endometrium tissues. Our qRT-PCR analysis of lncRNA DCST1-AS1 expression from 70 pairs of EC tissues and adjacent normal tissues showed that lncRNA DCST1-AS1 was overexpressed in EC patients (**Figure 1B**). Consistently, the expression of lncRNA DCST1-AS1 was significantly higher in EC cell lines than that in human normal endometrial stromal HESC cells (**Figure 1C**). Furthermore, 70 EC patients were classified into lncRNA DCST1-AS1 low and lncRNA DCST1-AS1 high groups. The results revealed that the expression of DCST1-AS1 was not related to the age and gender of the patients, but higher expression of DCST1-AS1 was significantly associated with larger tumor size, advanced TNM stage, and lymph node metastasis (**Table 2**).





**FIGURE 1** | LncRNA DC-STAMP domain-containing 1-antisense 1 (DCST1-AS1) expression is overexpressed in endometrial cancer (EC) tissues and cell lines. **(A)** The expression of lncRNA DCST1-AS1 in 548 EC tissues and 35 normal endometrium tissues (ENCORI database). **(B)** qRT-PCR analysis of DCST1-AS1 expression in 70 EC tissues and matched normal tissues. **(C)** Comparison of lncRNA DCST1-AS1 expression in the indicated EC cell lines and human normal endometrial stromal HESC cells using the qRT-PCR assay. **(D)** Kaplan-Meier curves for overall survival (OS) (left) and progression-free survival (PFS) (right) in EC patients with low or high DCST1-AS1 expression. \*\*\* $P < 0.001$ .

Survival analysis using the Kaplan-Meier (KM)-plotter database suggested that those patients with high DCST1-AS1 expression had worse overall survival (OS) and progression-free survival (PFS) than those with low DCST1-AS1 expression (Figure 1D). All these results suggested that DCST1-AS1 is overexpressed in EC and correlated with poor clinical outcomes.

### Depletion of LncRNA DCST1-AS1 Inhibits EC Cell Proliferation, Migration, and Invasion

To investigate the role of lncRNA DCST1-AS1 in EC, we silenced the expression of lncRNA DCST1-AS1 in EC cell lines using siRNAs and examined the effect of DCST1-AS1 knockdown on EC cell proliferation, migration, and invasion. First, we confirmed the transfection efficiency by qRT-PCR analysis (Figure 2A). Then, CCK-8, colony formation, migration, and invasion assays suggested significant inhibition of proliferation,

growth, migration, and invasion of EC cells transfected with DCST1-AS1 siRNA compared with those transfected with control siRNA (Figures 2B-E). Moreover, silencing of lncRNA DCST1-AS1 enhanced cell apoptosis (Figure 2F). Together, these results suggested that lncRNA DCST1-AS1 functions as an oncogenic lncRNA in EC.

### LncRNA DCST1-AS1 Acts as a Sponge for miR-665 and miR-873-5p

To explore how DCST1-AS1 exerts its function, we predicted miRNAs that can bind with DCST1-AS1 using the online database. The results showed that DCST1-AS1 contains the putative binding sites for miR-665 and miR-873-5p (Figure 3A). Using luciferase reporter assays, we showed that overexpression of miR-665 and miR-873-5p reduced the luciferase activity of the wild-type DCST1-AS1 reporter gene, but not the mutant DCST1-AS1 reporter gene (Figures 3B, C).

**TABLE 2 |** Correlation between lncRNA DCST1-AS1 expression and clinicopathological factors in EC.

Factor		DCST1-AS1 expression		P-value
		Low (n = 35)	High (n = 35)	
Age	≤50	15	19	0.3388
	>50	20	16	
Sex	Male	0	0	1
	Female	35	35	
Tumor size	≤3 cm	23	13	0.0168
	>3 cm	12	22	
T classification	T1–T2	21	17	0.026
	T3–T4	14	18	
N classification	N0–N1	22	11	0.008
	N2–N3	13	24	
Lymph node metastasis	No	24	15	0.0303
	Yes	11	20	

Furthermore, we found that silencing of lncRNA DCST1-AS1 increased the expression of miR-665 and miR-873-5p (Figures 3D, E). We tested the expression of miR-665 and miR-873-5p in EC tissues and normal tissues using the ENCORI database. We demonstrated that the expression of both miR-665 and miR-873-5p was significantly downregulated in EC samples (Figures 3F, G). The qRT-PCR assays showed that the expression of DCST1-AS1 was negatively correlated with miR-665 and miR-873-5p expression (Figure 3H). These results indicated that DCST1-AS1 directly sponges miR-665 and miR-873-5p in EC cells.

### LncRNA DCST1-AS1 Sponges miR-665 to Upregulate HOXB5 Expression

To find out genes sharing the regulatory role of DCST1-AS1 and miR-665, we predicted the target genes of miR-665 using the DIANA database. Among the predicted genes, HOXB5 could be potentially affected by miR-665 (Figure 4A). Our luciferase reporter assay demonstrated that overexpression of miR-665 suppressed the luciferase activity of the wild-type HOXB5 3'-UTR luciferase reporter, while it failed to inhibit the luciferase activity of the mutant HOXB5 3'-UTR (Figure 4A). Western blot analysis suggested that overexpression of miR-665 inhibited the mRNA and protein expression of HOXB5 (Figure 4B), suggesting that HOXB5 is the direct target gene of miR-665.

Since DCST1-AS1 acts as a sponge of miR-665, we tried to examine whether DCST1-AS1 could regulate HOXB5 expression via miR-665. We transfected EC cells with DCST1-AS1 siRNA (or control siRNA), along with miR-665 inhibitor (or control inhibitor), and investigated the mRNA and protein levels of HOXB5 using qRT-PCR and Western blot assays. As shown in Figures 4C, D, silencing of DCST1-AS1 significantly decreased HOXB5 mRNA and protein expression, whereas inhibition of miR-665 rescued the expression of HOXB5. Our meta-analysis and qRT-PCR assays demonstrated that HOXB5 was

dramatically upregulated in EC tissues compared with normal tissues (Figure 4E). We also detected a negative correlation between miR-665 and HOXB5 expression and a positive correlation between DCST1-AS1 and HOXB5 expression in EC tissues (Figure 4F). These findings suggested that DCST1-AS1 sponges miR-665 to increase HOXB5 expression in EC cells.

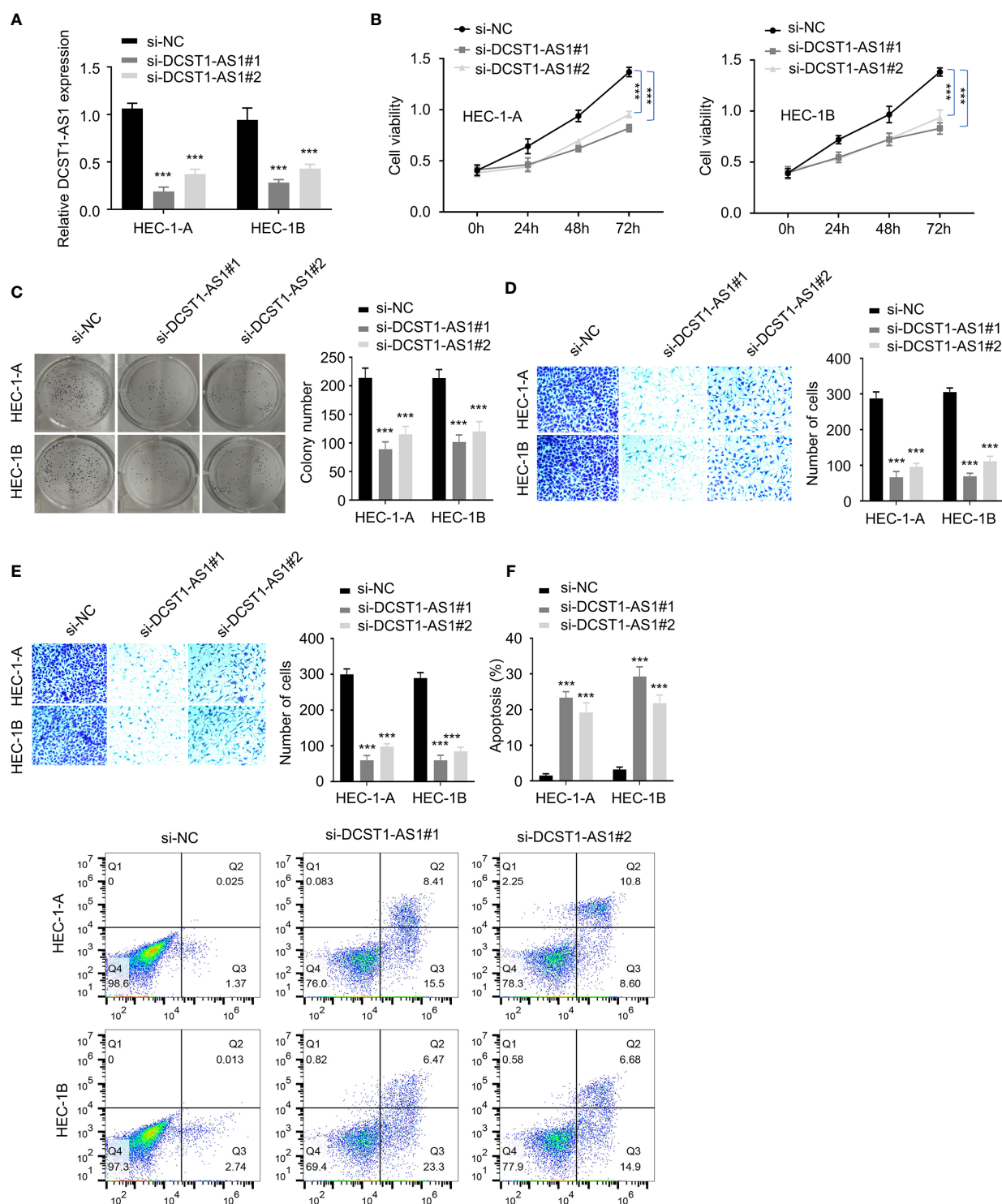
### Knockdown of LncRNA DCST1-AS1 Decreases CADM1 Expression via Sponging miR-873-5p

Similarly, using an online database, we found that CADM1 is a potential target gene of miR-873-5p (Figure 5A). The results from luciferase assays suggested a direct interaction between miR-873-5p and CADM1 3'-UTR (Figure 5A). Furthermore, we found that overexpression of miR-873-5p significantly decreased the mRNA and protein levels of CADM1 (Figure 5B). Our rescue experiments supported that knockdown of DCST1-AS1 downregulated the expression of CADM1 in EC cells, while inhibition of miR-873-5p had the opposite effects (Figures 5C, D). Meanwhile, CADM1 was found to be significantly upregulated in EC tissues (Figures 5E, F). The mRNA level of CADM1 was inversely correlated with miR-873-5p expression, but positively correlated with DCST1-AS1 expression in EC tissues (Figure 5F). These results suggested that DCST1-AS1 induces CADM1 expression in EC cells through sponging miR-873-5p.

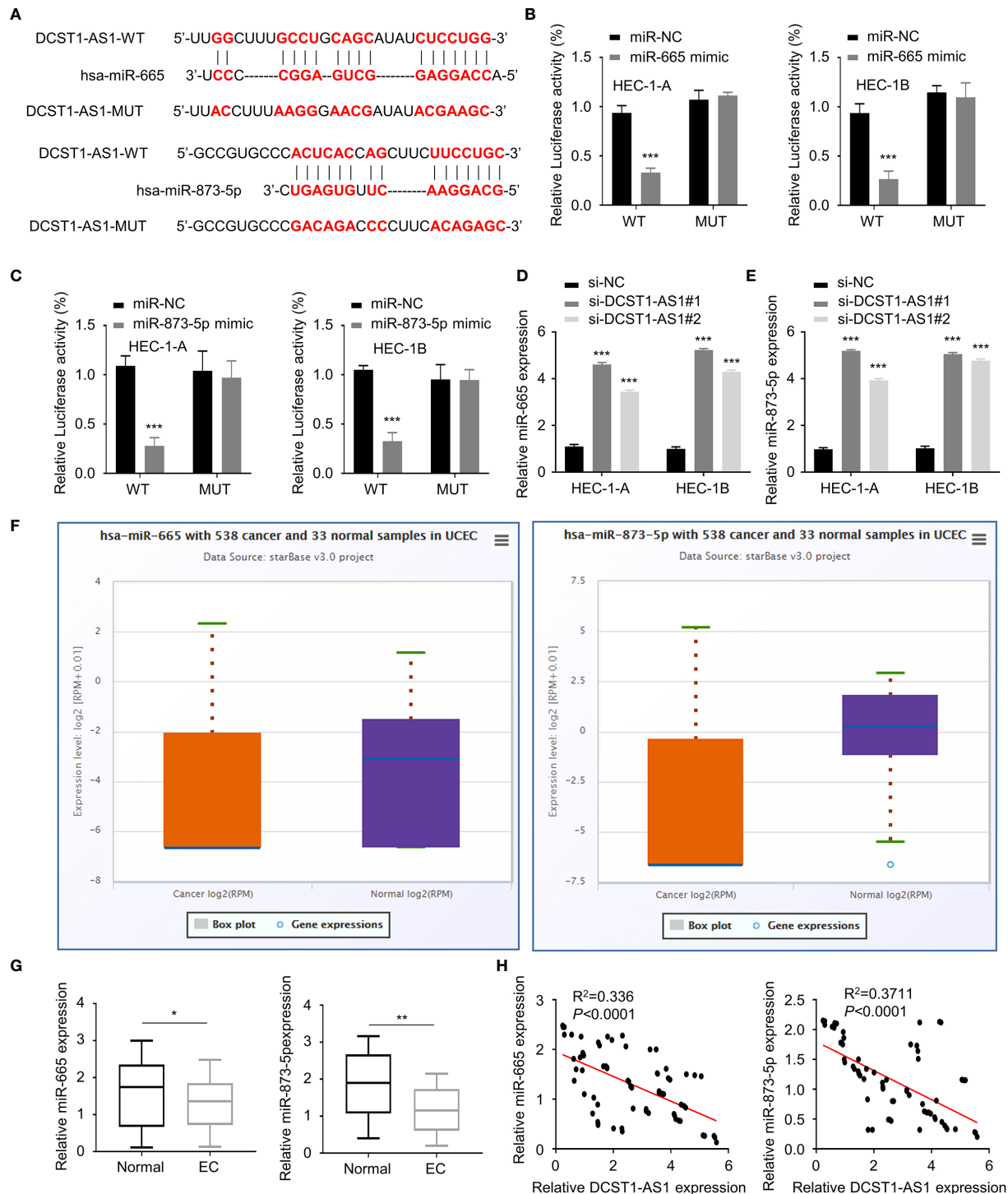
### LncRNA DCST1-AS1 Promotes EC Progression via the miR-665/HOXB5 Signaling

HOXB5, a member of the HOX gene cluster, is a key regulator of developmental processes (24), and it is implicated in multiple human cancers including breast cancer, head and neck cancer, and bladder cancer (25–27). Since we have shown that DCST1-AS1 promotes HOXB5 expression by acting as a sponge for miR-665, we asked whether DCST1-AS1 facilitates EC progression by

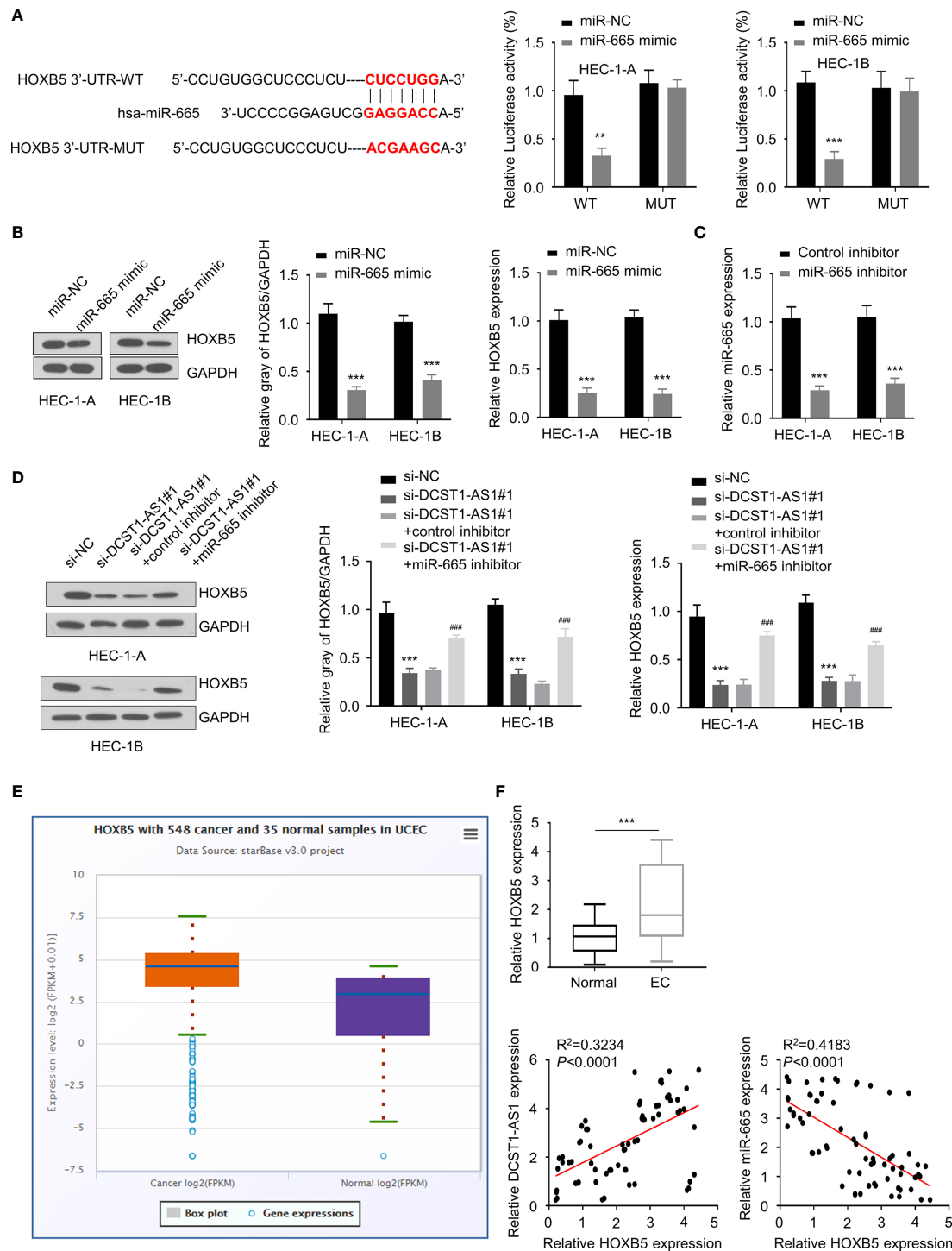




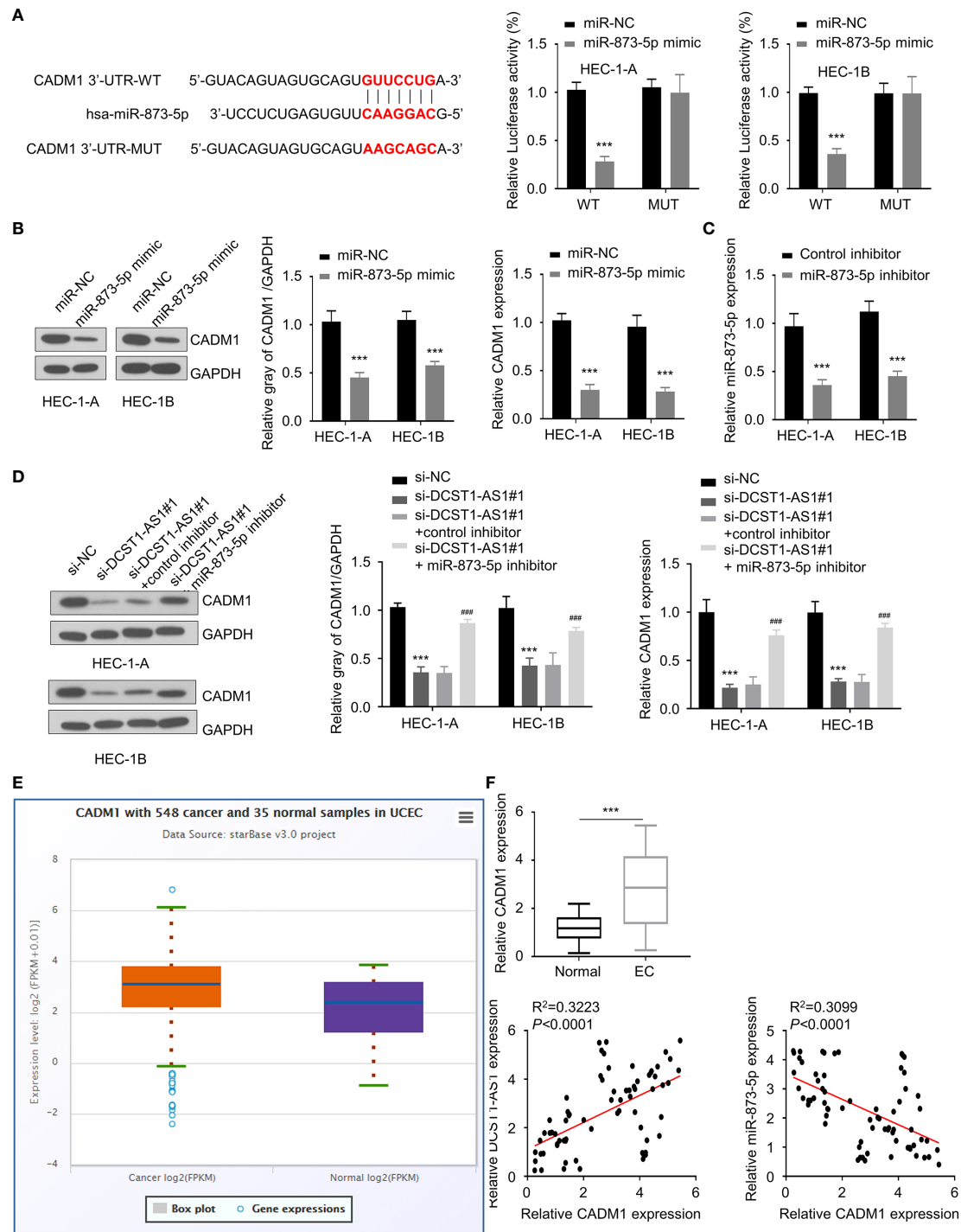
**FIGURE 2 |** Depletion of lncRNA DCST1-AS1 inhibits EC cell proliferation, migration, and invasion. **(A)** The expression of lncRNA DCST1-AS1 was measured by RT-qPCR assay in HEC-1A and HEC-1B cells transfected with DCST1-AS1 siRNA (si-DCST1-AS1) or control siRNA (si-NC). **(B)** Cell Counting Kit-8 (CCK-8) assay showed that depletion of DCST1-AS1 suppressed EC cell proliferation. **(C)** Colony formation assay of EC cells transfected with si-NC or si-DCST1-AS1. **(D, E)** Transwell migration and invasion assay showed that DCST1-AS1 knockdown inhibited EC cell migration **(D)** and invasion **(E)**. **(F)** Flow cytometry assay showed that DCST1-AS1 knockdown increased EC cell apoptosis. \*\*\* $P < 0.001$ .



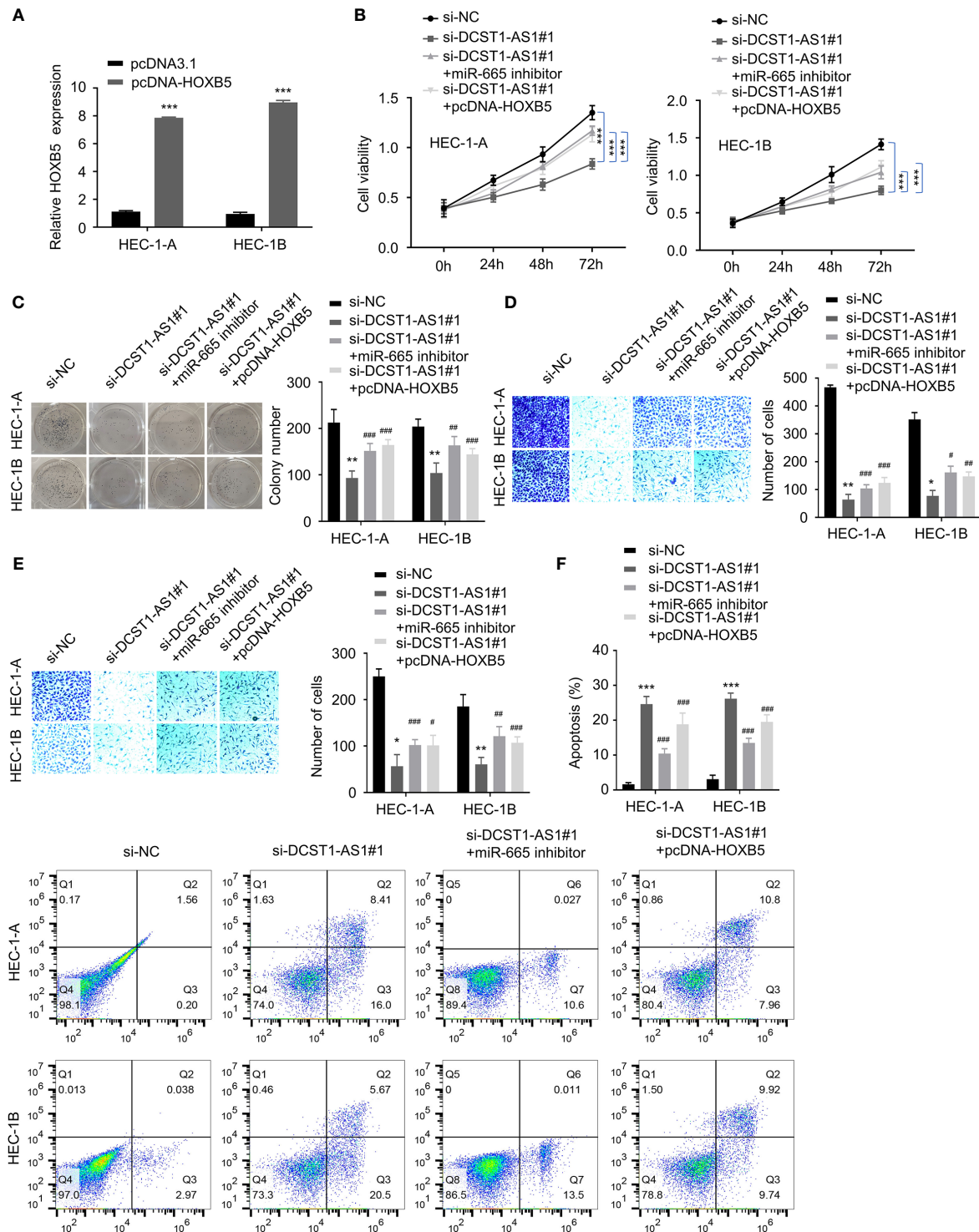
**FIGURE 3 |** LncRNA DCST1-AS1 acts as a sponge for microRNA-665 (miR-665) and microRNA-73-5p (miR-73-5p). **(A)** Predicted and mutant (lower panel) sequence alignments of miR-665 and miR-73-5p with DCST1-AS1. **(B)** Wild-type (WT) DCST1-AS1 and mutant (MUT) DCST1-AS1 luciferase reporters were co-transfected with miR-665 mimic or control mimic (miR-NC). Luciferase activity was detected using luciferase assays. **(C)** Luciferase reporter assays showed that miR-73-5p significantly reduced luciferase activity of WT DCST1-AS1, but mutation of the miR-73-5p binding site abrogated the inhibitory effects of miR-73-5p. **(D, E)** The levels of miR-665 **(D)** and miR-73-5p **(E)** were tested by RT-qPCR in EC cells with (or without) DCST1-AS1 depletion. **(F)** The expression of miR-665 and miR-73-5p in EC ( $n = 538$ ) and tumor-adjacent normal tissues ( $n = 33$ ) (ENCORI database). **(G)** The levels of miR-665 and miR-73-5p in EC tissues and the adjacent normal tissues were measured using qRT-PCR assay. **(H)** The correlation of DCST1-AS1 and miR-665/miR-73-5p expression was analyzed by Spearman's correlation analysis. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**FIGURE 4 |** LncRNA DCST1-AS1 sponges miR-665 to upregulate homeobox B5 (HOXB5) expression. **(A)** Predicted and mutant (lower panel) sequence alignments of miR-665 with HOXB5 3'-UTR sequence. Luciferase reporter assays showed that miR-665 significantly reduced luciferase activity of WT HOXB5, but mutation of the miR-665 binding site abrogated the inhibitory effects of miR-665 (right panel). **(B)** The mRNA and protein levels of HOXB5 in EC cells transfected with miR-665 mimic or control mimic were examined using qRT-PCR assay and Western blot analysis. **(C)** The expression of miR-665 in EC cells transfected with miR-665 inhibitor or control inhibitor. **(D)** HEC-1A and HEC-1B cells were transfected as indicated, and the mRNA and protein expression of HOXB5 was investigated using qRT-PCR and Western blot analysis. **(E)** Comparison of HOXB5 expression in EC tissues ( $n = 548$ ) and normal tissues ( $n = 35$ ) (ENCORI database). **(F)** The mRNA expression of HOXB5 in 70 pairs of EC and adjacent normal tissues was checked using qRT-PCR assay. Spearman's correlation analysis was used to analyze the relationship between HOXB5 expression and DCST1-AS1 or miR-665 expression.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ .  $^{###}P < 0.001$ , compared with si-DCST1-AS1#1 group.



**FIGURE 5 |** Knockdown of lncRNA DCST1-AS1 decreases cell adhesion molecule 1 (CADM1) expression via sponging miR-773-5p. **(A)** Predicted and mutant (lower panel) sequence alignments of miR-773-5p with CADM1 3'-UTR sequence. Luciferase reporter assays showed that miR-773-5p significantly reduced luciferase activity of WT CADM1, but mutation of the miR-773-5p binding site abrogated the inhibitory effects of miR-773-5p (right panel). **(B)** The mRNA and protein levels of CADM1 in EC cells transfected with miR-773-5p mimic or control mimic were examined using qRT-PCR assay and Western blot analysis. **(C)** The expression of miR-773-5p in EC cells transfected with miR-773-5p inhibitor or control inhibitor. **(D)** HEC-1A and HEC-1B cells were transfected as indicated, and the mRNA and protein expression of CADM1 was investigated using qRT-PCR and Western blot analysis. **(E)** Comparison of CADM1 expression in EC tissues ( $n = 548$ ) and normal tissues ( $n = 35$ ) (ENCORI database). **(F)** The mRNA expression of CADM1 in 70 pairs of EC and adjacent normal tissues was checked using qRT-PCR assay. Spearman's correlation analysis was used to analyze the relationship between CADM1 expression and DCST1-AS1 or miR-773-5p expression.  $***P < 0.001$ .  $****P < 0.001$ , compared with si-DCST1-AS1#1 group.



**FIGURE 6 |** LncRNA DCST1-AS1 promotes EC progression via the miR-665/HOXB5 signaling. **(A)** qRT-PCR analysis of HOXB5 expression in EC cells transfected with HOXB5 overexpression plasmid or control plasmid. **(B)** CCK-8 assay showed that either miR-665 inhibition or HOXB5 overexpression rescued EC cell proliferative reduced by DCST1-AS1 knockdown. **(C)** Colony formation assay of EC cells transfected as indicated. **(D, E)** Transwell migration and invasion assay showed that either miR-665 inhibition or HOXB5 overexpression rescued EC cell migration **(D)** and invasion **(E)** that was suppressed by DCST1-AS1 depletion. **(F)** HEC-1A and HEC-1B cells were transfected as indicated, and cell apoptosis was determined by FACS analysis. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ , compared with si-DCST1-AS1#1 group.



regulating the miR-665/HOXB5 signaling. To this end, we constructed a HOXB5 overexpression plasmid (Figure 6A).

Using CCK-8, colony formation, migration, and invasion assays, we confirmed that silencing of DCST1-AS1 suppressed EC cell proliferation, growth, migration, and invasion, whereas either miR-665 knockdown or overexpression of HOXB5 could reverse the effects of DCST1-AS1 silencing (Figures 6B–E). In addition, depletion of DCST1-AS1 increased EC cell apoptosis; however, cell apoptosis was reduced by the transfection with miR-665 inhibitor or HOXB5 overexpression plasmid (Figure 6F). These data suggested that DCST1-AS1 promotes EC cell proliferation, migration, and invasion and inhibited cell apoptosis, at least in part, by regulating the miR-665/HOXB5 pathway.

## LncRNA DCST1-AS1 Promotes the Malignant Features of EC Cells Through the miR-873-5p/CADM1 Signaling

To explore whether the miR-873-5p/CADM1 axis mediates the function of DCST1-AS1 in EC cells, we constructed a CADM1 overexpression plasmid (Figure 7A). Our rescue experiments revealed that suppression of DCST1-AS1 significantly suppressed EC cell proliferation, migration, and invasion and induced cell apoptosis (Figures 7B–F). In contrast, either knockdown of miR-873-5p or CADM1 overexpression rescued the malignant features of EC cells and reduced cell apoptosis

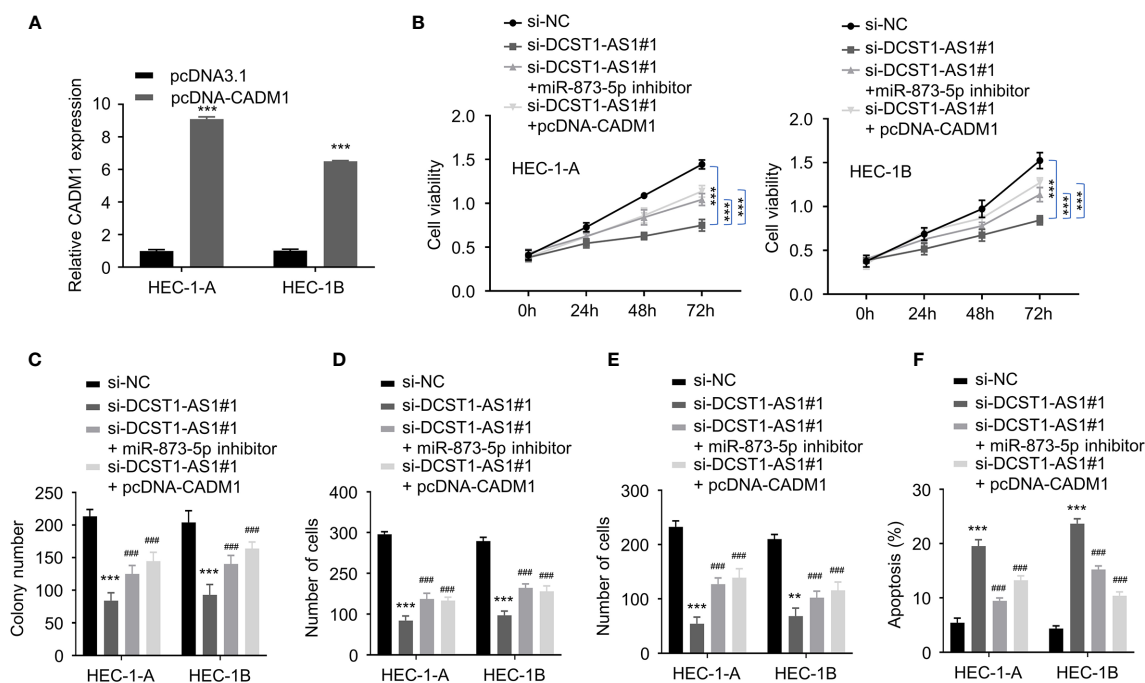
(Figures 7B–F). These data indicated that DCST1-AS1 promotes the aggressive phenotype in EC cells, at least in part, by regulating the miR-873-5p/CADM1 pathway.

## LncRNA DCST1-AS1 Promotes EC Tumor Growth *In Vivo*

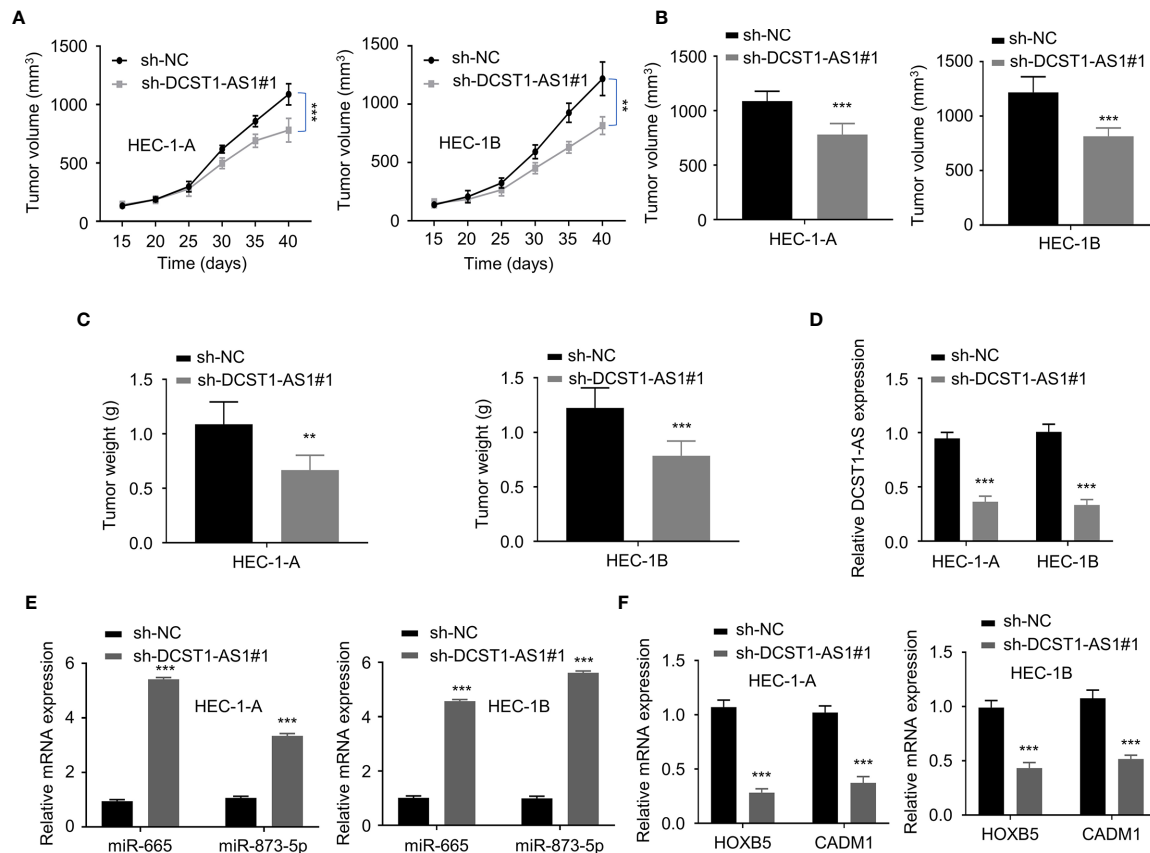
To validate the effects of lncRNA DCST1-AS1 on tumor growth *in vivo*, we injected EC cells transfected with DCST1-AS1 shRNA or control shRNA into nude mice. The results from the tumor xenografts in nude mice showed that the volume and weight of subcutaneous tumors were significantly suppressed by DCST1-AS1 knockdown (Figures 8A–C). Our qRT-PCR analysis of the tumors suggested that knockdown of DCST1-AS1 significantly increased the expression of miR-665 and miR-873-5p, but decreased the expression of HOXB5 and CADM1 (Figures 8D–F). Taken together, our results suggested that DCST1-AS1 promotes EC growth *in vivo*.

## DISCUSSION

LncRNAs are considered as critical epigenetic regulators, and the aberrant expression of lncRNAs contributes to cancer progression (28, 29). The effect of lncRNA DCST1-AS1 has been investigated in multiple human tumors, such as breast



**FIGURE 7 |** LncRNA DCST1-AS1 promotes the malignant features of EC cells through miR-873-5p/CADM1 signaling. (A) qRT-PCR analysis of CADM1 expression in EC cells transfected with CADM1 overexpression plasmid or control plasmid. (B) CCK-8 assay showed that either miR-873-5p inhibition or CADM1 overexpression rescued EC cell proliferative reduced by DCST1-AS1 knockdown. (C) Colony formation assay of EC cells transfected as indicated. (D, E) Transwell migration and invasion assay showed that either miR-873-5p inhibition or CADM1 overexpression rescued EC cell migration (D) and invasion (E) that was suppressed by DCST1-AS1 depletion. (F) HEC-1A and HEC-1B cells were transfected as indicated, and cell apoptosis was determined by FACS analysis. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ### $P < 0.001$ , compared with siDCST1-AS1#1 group.



**FIGURE 8 |** LncRNA DCST1-AS1 promotes EC tumor growth *in vivo*. (A, B) The growth curves (A) and tumor volume (B) after injection of DCST1-AS1 shRNA#1 or control shRNA. (C) The weight of tumors. (D) The expression of DCST1-AS1 was detected in tumors from control shRNA or DCST1-AS1 shRNA#1 group mice by qRT-PCR assay. (E) The expression of miR-665 and miR-873-5p in tumor samples was detected by RT-qPCR assay. (F) The qRT-PCR assay of HOXB5 and CADM1 expression in tumor samples from different groups. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

cancer, cervical cancer, hepatocellular carcinoma, and glioblastoma (17–19, 30, 31). However, the function of DCST1-AS1 in EC is largely unknown. In the present study, we demonstrated that DCST1-AS1 serves as an oncogenic lncRNA in EC (20). Mechanically, we reported that DCST1-AS1 functions as a sponge for miR-665 and miR-873-5p and subsequently upregulates the expression of HOXB5 and CADM1, respectively.

Previous studies have shown that miR-665 could play oncogenic or tumor-suppressive roles in various types of cancer. For instance, miR-665 was shown to act as an oncogene in ovarian cancer by directly inhibiting SRC kinase signaling inhibitor 1 (SRCIN1) expression (32). Elevated expression of miR-665 was associated with poor prognosis in nonsmall cell lung cancer (33). In breast cancer, miR-665 represses the expression of nuclear receptor subfamily 4 group A member 3 (NR4A3) to activate the MAPK/ERK kinase signaling (34). In contrast, the tumor-suppressor role of miR-665 has been also reported. In osteosarcoma, miR-665 regulates Rab23 expression to inhibit tumor invasion and metastasis (35). Additionally, miR-665 suppresses epithelial-mesenchymal transition (EMT) and gastric cancer progression by

targeting cysteine-rich motor neuron protein 1 (CRIM1) (36). However, little is known about the function of miR-665 in EC as well as the association between lncRNA DCST1-AS1 with miR-665. Here, we demonstrated that miR-665 could inhibit the proliferation and invasiveness of EC cells, and the DCST1-AS1/miR-665 axis regulates EC development. Moreover, we screened the target genes of miR-665 and confirmed that miR-665 directly targets HOXB5, which functions as a transcription factor in several cancer types (37). The tumor-promoting roles of HOXB5 have been found in breast cancer, gastric carcinoma, lung cancer, retinoblastoma, and neck squamous cell carcinoma (26, 27, 38–41). In this study, we demonstrated for the first time that HOXB5 could enhance EC cell proliferation and invasiveness. We also revealed that DCST1-AS1 exerts oncogenic functions partly *via* sponging miR-665 and by upregulating HOXB5 expression in EC.

Using the online predicting database, we identified that miR-873-5p is another potential target of DCST1-AS1. Accumulating studies have shown that miR-873-5p represses tumor progression in various human cancers. For example, miR-873-5p was downregulated in colorectal cancer and overexpression of miR-873-5p represses cancer cell migration, invasion, and EMT through targeting ZEB1 (42, 43).



Moreover, miR-873-5p could regulate chemokine (C-X-C motif) ligand 16 (CXCL16) expression to inhibit thyroid cancer progression (44). Besides, miR-873-5p was reported to reduce gastric cancer cell proliferation by mediating hedgehog-Gli signaling (22). In glioblastoma, miR-873-5p repressed IGF2 mRNA-binding protein 1 (IGF2BP1) expression to suppress glioblastoma tumorigenesis (45). MiR-873-5p showed tumor-suppressive effects in esophageal cancer *via* modulating the miR-873/DEC2 axis (46). In contrast, there is also evidence showing that miR-873-5p functions as an oncogene in hepatocellular carcinoma and lung adenocarcinoma (47, 48). In endometrial cancer, miR-873-5p exerts a tumor-suppressor role *via* directly targeting hepatoma-derived growth factor (HDGF) (49). Consistent with this report, we demonstrated that miR-873-5p expression was reduced in EC tissues and further proved that miR-873-5p functions as a key tumor suppressor and a downstream target of DCST1-AS1 in EC cells. CADM1 is a member of single transmembrane glycoproteins that belong to the immunoglobulin superfamily involved in synapse formation and plasticity (50, 51). CADM1 was frequently reported as a tumor suppressor and mostly was abrogated in various cancer types. Loss of CADM1 expression predicted poor prognosis and the development of esophageal cancer and ovarian cancer (52, 53). Also, CADM1 exerts its tumor-suppressor effects in breast cancer, bladder cancer, and ovarian cancer (54–56). However, we revealed that CADM1 is overexpressed in EC and it promotes EC progression.

This study not only advances our understanding of the roles of the DCST1-AS1/miR-665/HOXB5 pathway and DCST1-AS1/

miR-873-5p/CADM1 pathway in EC biology but also provides these signaling pathways as new targets for developing therapy of EC in the future.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee on Scientific Research of People's Hospital of Rizhao. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

PS and JW designed and conducted the experiments. HT, LL, HG, and XW analyzed the data. JW wrote the manuscript and PS revised the manuscript. All authors contributed to the article and approved the submitted version.

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# Using Immune-Related Long Non-coding Ribonucleic Acids to Develop a Novel Prognosis Signature and Predict the Immune Landscape of Colon Cancer

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**Purpose:** This study aimed to construct a novel signature to predict the survival of patients with colon cancer and the associated immune landscape, based on immune-related long noncoding ribonucleic acids (lncRNAs).

**Methods:** Expression profiles of lncRNAs in 457 patients with colon cancer were retrieved from the TCGA database (<https://portal.gdc.cancer.gov>). Differentially expressed (DE) lncRNAs were identified and lncRNA pairs were recognized using Lasso regression and Cox regression analyses. Akaike information criterion (AIC) values of receiver operating characteristic (ROC) curve were calculated to identify the ideal cut-off point for dividing patients into two groups and constructing the prognosis signature. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the expression of LINC02195 and SCARNA9 in colon cancer.

**Results:** We identified 22 lncRNA pairs and patients were divided into high-risk and low-risk groups based on the calculated risk score using these 22 lncRNA pairs. The lncRNA pairs were significantly related to patient survival. Low-risk patients had a significantly longer survival time than high-risk patients ( $p < 0.001$ ). The area under the curve of the signature to predict 5-year survival was 0.951. The risk score correlated with tumor stage, infiltration depth, lymph node metastasis, and distant metastasis. The risk score remained significant after univariate and multivariate Cox regression analyses. A nomogram model to predict patient survival was developed based on the results of Cox regression analysis. Immune cell infiltration status, expression of some immune checkpoint genes, and sensitivity to chemotherapeutics were also related to the risk score. The results of qRT-PCR revealed that LINC02195 and SCARNA9 were significantly upregulated in colon cancer tissues.

**Conclusion:** The constructed prognosis signature showed remarkable efficiency in predicting patient survival, immune cell infiltration status, expression of immune checkpoint genes, and sensitivity to chemotherapeutics.

**Keywords:** immune related long noncoding RNAs, prognosis signature, colon cancer, tumor-infiltrating immune cell, immune checkpoint genes, chemotherapeutics



## INTRODUCTION

Despite the rapid development of medical treatments, the trends in cancer incidence and death rates have been increasing worldwide. Specifically, the incidence and mortality rate of colon cancer are relatively high (Bray et al., 2018). Patients with early colon cancer can be treated by surgery; however, most patients with advanced colon cancer experience cancer recurrence and metastasis, and their 5-year survival rate is lower than 10% (Bhandari et al., 2017; Doonan et al., 2017; Russo et al., 2019). With the development of chemotherapy and targeted medicine, the overall survival rate of patients with colon cancer is significantly higher now than before. In recent years, advances in tumor immune therapy and application of immune checkpoint inhibitors have led to improvements in cancer treatment.

Programmed cell death protein 1 (PD-1), first discovered in 1992, is a 288 amino acid protein expressed on the surface of T cells and related to apoptosis (Ishida et al., 1992). When PD-1 is bound to its ligand programmed cell death ligand 1 (PD-L1), the anti-tumor effect of T cells is inhibited (Kouo et al., 2015). PD-1-blocking antibodies, pembrolizumab and nivolumab, were approved by the United States Food and Drug Administration (FDA) for the treatment of refractory melanoma and advanced non-small cell lung cancer in 2014 and 2015, respectively. Atezolizumab, the first anti-PD-L1 antibody, was approved for the treatment of urothelial cancers in 2016. With the rapid development of tumor immunotherapy, several immune checkpoint inhibitors have been used in the treatment of various types of malignant tumors. According to a recent meta-analysis (He et al., 2020), anti-PD-1 inhibitors have high efficacy and have led to a better prognosis in patients with deficient mismatch repair (dMMR)/microsatellite instability high (MSI-H) metastatic colorectal cancer (mCRC) (dMMR/MSI-H mCRC).

Long non-coding ribonucleic acids (lncRNAs), defined as RNAs longer than 200 nucleotides, are not translated into functional proteins (Iyer et al., 2015). The completely spliced lncRNA is transported into the cytoplasm or other organelles through a mechanism similar to that of mRNA. Once in the cytoplasm, lncRNAs transregulate gene expression at the post-transcriptional level, such as regulating mRNA translation and degradation, or participating in the regulation of intracellular signaling pathways (Statello et al., 2021). Recent studies have shown that lncRNAs not only change the genome or transcriptome, but also modify the immune microenvironment to contribute to the malignant phenotype of cancer. lncRNAs direct the expression of genes related to immune cell activation, leading to tumor immune cell infiltration (Atianand et al., 2017; Chen et al., 2017). Several lncRNAs are differentially expressed (DE) in various types of tumor tissues (Schmitt and Chang, 2016).

In recent years, many researchers have focused on constructing signatures using lncRNAs for predicting prognosis of patients with cancer based on the cancer Genome Atlas (TCGA) database. Qi et al. (2021) identified eight immune-related (ir) lncRNAs and developed a signature for the prognosis of patients with pancreatic adenocarcinoma. Zhou et al. (2021) identified an irlncRNA signature to predict the prognosis,

immune cell infiltration, and immunotherapy response in patients with hepatocellular carcinoma and validated the expression of the six lncRNAs using the quantitative real-time polymerase chain reaction (qRT-PCR) method. Zhang et al. (2021) built a signature based on ten hypoxia-related lncRNAs that showed promising predictive effect for patient prognosis. Ma et al. (2021) identified a metabolism-related lncRNA signature for prediction of risk of recurrence in patients with breast cancer. Compared with using single biomarkers, combinations of two biomarkers lead to much more accurate diagnostic models for cancers (Lv et al., 2020). Moreover, these combinations do not require any specific expression levels or testing methods. However, not many studies have focused on the development of signatures based on lncRNA pairs for diagnosis or survival prediction in patients with colon cancer. We constructed a prognosis signature using irlncRNA pairs, which showed remarkable efficiency in predicting patient survival, immune cell infiltration status, expression of immune checkpoint genes, and sensitivity to chemotherapeutics.

## MATERIALS AND METHODS

### Immune-Related Long Noncoding Ribonucleic Acids Identification and Expression

Expression patterns of irlncRNAs of patients with colon cancer were downloaded from the Genomic Data Commons (GDC) Data Portal.<sup>1</sup> lncRNAs and mRNAs were distinguished using GTF files, which were downloaded from Ensembl.<sup>2</sup> A list of human immune-related genes was prepared using the ImmPort database,<sup>3</sup> a publicly available repository containing up-to-date information on human genes and proteins that are involved in immunity. Co-expression analysis was performed between immune-related genes and all lncRNAs to identify irlncRNAs. The screening criteria used was correlation coefficient > 0.4 and *p*-value < 0.001. *limma* package of R software (version 4.0.3) was used to screen out differentially expressed immune-related lncRNAs (DEirlncRNAs) with criteria of log<sub>2</sub> | fold change (FC)| > 2.5 and *p*-value < 0.01.

### Gene Oncology and Kyoto Encyclopedia of Genes and Genomes Analyses

Pearson correlation coefficient analysis was performed to analyze the relationship between the expression of DEirlncRNAs and mRNAs and the top 10 mRNAs were considered to be associated with lncRNAs. To better understand the biological functions and pathways involved in DEirlncRNAs, Gene Oncology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using *ggplot2*, *Bioconductor*, and *org.Hs.eg.db* R packages. *P*-values and *q*-values < 0.05 were considered statistically significant.

<sup>1</sup><https://portal.gdc.cancer.gov>

<sup>2</sup><http://asia.ensembl.org>

<sup>3</sup><http://www.immport.org>



## Construction of Differentially Expressed Immune-Related Long Non-coding Ribonucleic Acid Pairs

A DElncRNA pair was constructed using two DElncRNAs, for example, lncRNA A and lncRNA B. A 0-or-1 matrix was constructed. If the ratio of lncRNA A to lncRNA B was higher than 1, the expression of the lncRNA pair was defined as 1, otherwise, it was defined as 0. If the proportion of lncRNA pairs with expression defined as 0 or 1 was less than 20% or more than 80%, the pair was considered invalid.

## Clinical Data of Colon Cancer Patients

Clinical information of patients with colon cancer was downloaded from colon adenocarcinoma (COAD) project of TCGA database. Patients with no follow-up or incomplete clinical information were excluded. After selection, 393 cases of colon cancer were included in this study.

## Development and Validation of the Prognosis Signature Using Differentially Expressed Immune-Related Long Non-coding Ribonucleic Acid Pairs

Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis and Cox regression analysis were performed to screen out prognosis-related lncRNA pairs using *survival*, *survminer*, and *glmnet* R packages. lncRNA pairs with  $p < 0.05$  were considered significant. After selection, 22 lncRNA pairs were included to construct the prognosis signature. Risk scores were calculated based on the following formula: Risk score =  $\sum_{i=1}^n \beta_i^* \lambda_i$ , where  $n$  represents the numbers of lncRNA pairs included to construct the signature and  $\beta_i$  and  $\lambda_i$  represent the regression coefficient and expression value of lncRNA pairs, respectively. According to the maximum akaike information criterion (AIC) value of 5-year receiver operating characteristic (ROC) curve, patients were divided into high-risk and low-risk groups. ROC and AUC were used to test the prediction efficiency and compare the constructed signature and other clinical variables. The Kaplan-Meier (KM) method and log rank test were used to evaluate survival differences between high-risk and low-risk groups. Univariate and multivariate analyses were used to determine whether risk score was an independent predictor of prognosis in patients with colon cancer. Chi-square test was performed to analyze the relationship between the signature and clinical variables and the Wilcoxon signed-rank test was used to show the risk score differences between different groups for these clinical characteristics. A nomogram model was developed based on three independent prognosis factors that were significant in both the univariate and multivariate analyses ( $p < 0.05$ ). Calibration graphs were constructed to show the differences between nomogram-predicted and actual survival rates of patients with colon cancer.

## Immune Cell Infiltration Analysis

To better understand the relationship between the calculated risk score and tumor immune cell infiltration status, datasets

including XCELL, TIMER, QUANTISEQ, EPIC, CIBERSORT-ABS, and CIBERSORT were used to analyze the immune cell infiltration status. The lollipop diagram was drawn to show the correlation between risk score and immune infiltrated cells *via* Spearman correlation method. The differences of immune cell content in high-risk and low-risk groups were shown as boxplots using Wilcoxon signed-rank test.

## Expression of Immune Checkpoint Genes in High-Risk and Low-Risk Groups

To understand the differences in the expression levels of immune checkpoint genes in the high-risk and low-risk groups, six immune checkpoint genes were selected, including CTLA4, HAVCR2, IDO1, lymphocyte-activation gene 3 (LAG3), PD-1, and PD-L1. Violin plots were drawn to show the results using *ggpubr* R package.

## Evaluating the Differences in Chemosensitivity Between COAD Patients in High-Risk and Low-Risk Groups

IC50, half of the maximum inhibitory concentration, represents the concentration of drug required for 50% inhibition of cancer cells. IC50 was calculated to evaluate the significance of lncRNA-based signature in six types of chemotherapeutic drugs, including camptothecin, doxorubicin, erlotinib, gemcitabine, paclitaxel, and rapamycin, which have been used in the treatment of patients with colon cancer. Wilcoxon signed-rank test was performed to analyze the differences in IC50 in high-risk and low-risk groups. The results are shown as boxplots using *ggpubr*, *pRRophetic*, and *ggplot2* R packages.

## The Verification of LINC02195 and SCARNA9 by Quantitative Real-Time Polymerase Chain Reaction

Ten pairs of colon cancer tissues and adjacent non-cancer tissues were collected from The First Affiliated Hospital of Anhui Medical University, and approved by the Ethics Committee. All participants signed an informed consent form. These samples were collected after surgical resection from colon cancer patients who had never received preoperative chemotherapy or radiotherapy. The HiPure Universal RNA Kit (Shanghai, Magen) was used to extract total RNA from the colon cancer and adjacent non-cancer tissues stored in liquid nitrogen following the manufacturer's instructions. The concentration and purity of RNA samples was measured using NanoDrop 2000 (Thermo Fisher Scientific, United States). Extracted RNA was reverse transcribed into cDNA using the PrimeScript RT kit (Vazyme, Nanjing, China) according to the protocol. Finally, the concentration of cDNA was measured using TB Green Premix Ex Taq II (GenStar, China) under the LightCycler480 System (Applied Biosystems, Waltham, MA, United States) according to the manufacturer's instruction. The primer sequences for PCR amplification were as follows: LINC02195, forward: 5'-GTCA

CACAGCAAGCCTAAAGAAACG-3', reverse: 5'-TCAGCCA TAGAGGAGACAGCAAGG-3'; SCARNA9, forward: 5'-AAGG GCATATGTCTGGTGTGTGTG-3', reverse: 5'-CCCCACCCTC AATCTCATTTCATTC-3'; GAPDH, forward: 5'-GGGAAGG TGAAGGTCGGAGT-3', reverse: 5'-GGGGTCATTGATGGCA ACA-3'. GAPDH was used as an internal control, and each sample was repeated three times. The relative expression levels of LINC02195 and SCARNA9 were calculated using the  $2^{-\Delta\Delta Ct}$  method. The differences in LINC02195 and SCARNA9 expression between colon cancer tissues and adjacent non-cancer tissues were tested by *t*-tests. The graphs were drawn using GraphPad Prism software (version 9.2.0).

## RESULTS

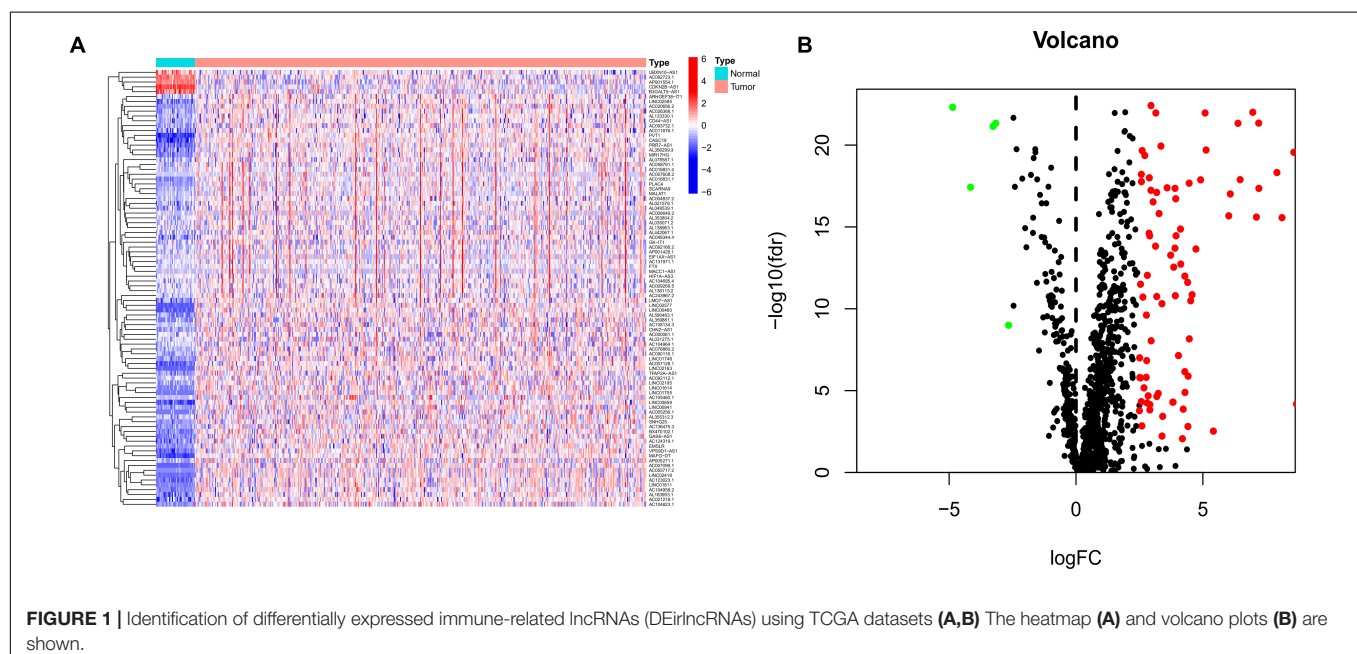
### Differentially Expressed Immune-Related Long Noncoding Ribonucleic Acids

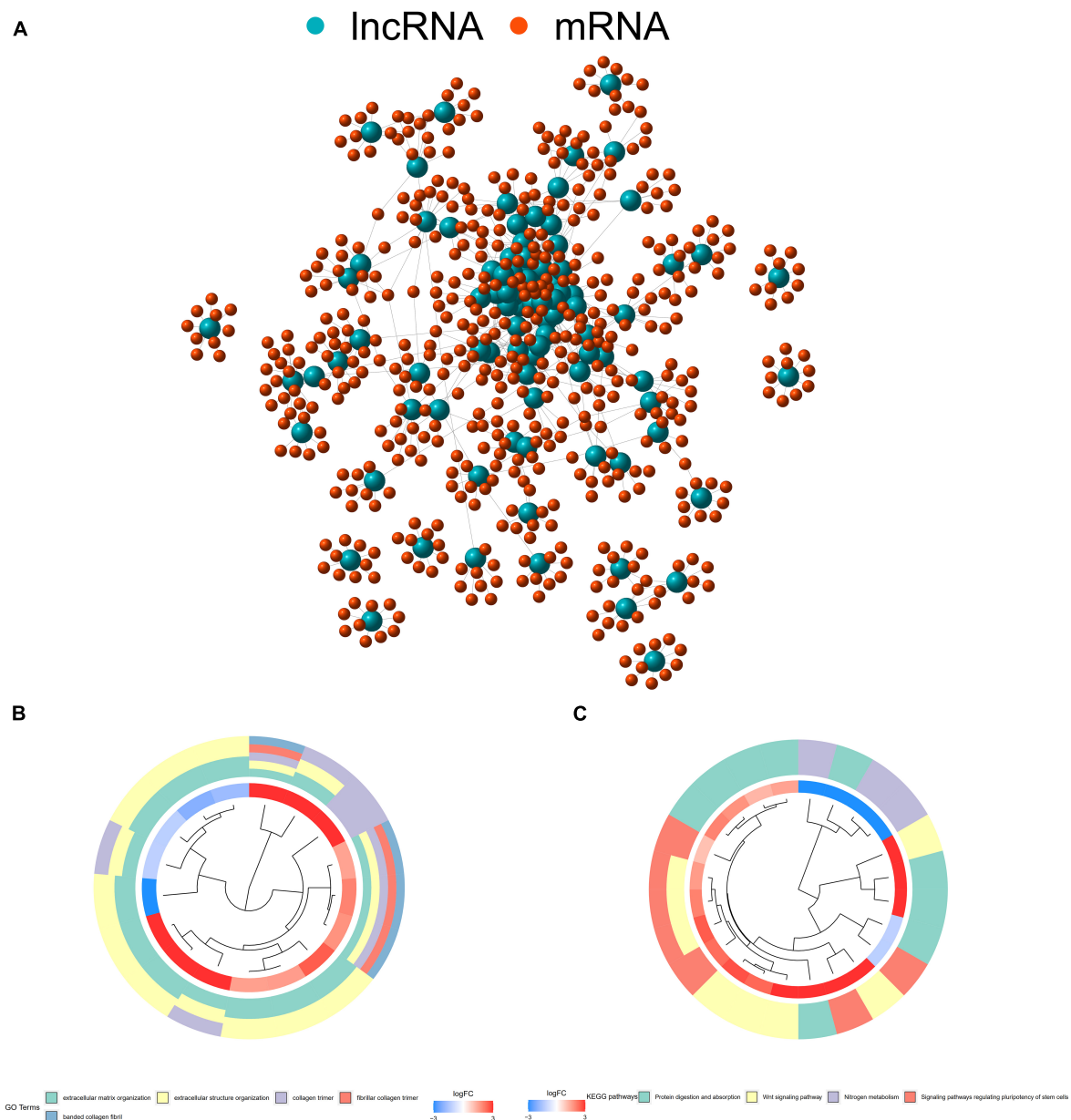
Expression patterns and clinical information from 457 COAD patients were downloaded from TCGA database. A total of 393 patients with complete clinical information and follow-up time >0 days were included in the study. Detailed clinical characteristics of the 393 cases are shown in **Supplementary Table 1**. Human immune-related genes were identified using the ImmPort database. Co-expression analysis was performed to identify irlncRNAs based on immune-related genes. As shown in **Figure 1A**, there were 90 irlncRNAs with  $\log |FC| > 2.5$  and *p* value < 0.01, among which 85 lncRNAs were upregulated while five were downregulated (**Figure 1B**). To understand the biological functions and pathways involved in the 90 DEirlncRNAs, the expression correlation between the DEirlncRNAs and mRNAs were shown as a lncRNA-mRNA co-expression network, and lncRNAs and associated

mRNAs were linked together with lines (**Figure 2A**). As seen in **Figure 2B**, the 90 lncRNAs were primarily related to the biological functions of extracellular matrix organization, extracellular structure organization, collagen trimer, fibrillar collagen trimer, and banded collagen fibril (**Figure 2B**). These DEirlncRNAs also participated in the pathways of protein digestion and absorption, WNT signaling, nitrogen metabolism, and regulating pluripotency of stem cells (**Figure 2C**).

### Construction of Differentially Expressed Immune-Related Long Non-coding Ribonucleic Acid Pairs and Prognosis Signature

A total of 2,720 valid lncRNA pairs were constructed based on the 90 DElncRNAs and 22 were screened out using LASSO regression analysis (**Figures 3A,B**). As shown in **Figure 3C**, all the 22 lncRNA pairs were significant in univariate Cox regression analysis (*p* < 0.05). The risk score was defined as  $\sum_{i=1}^n \beta_i^* \lambda_i$ . Then, 5-year AUC for ROC curves of the 22 lncRNA pairs were calculated. The maximum AUC value of the signature to predict 5-year survival was 0.951; the AIC value was also calculated to identify the ideal cut-off point to divide patients into high-risk and low-risk groups (**Figure 4A**). As shown in **Figure 4B**, the AUC value of the signature obtained in this study was much higher than that of three other lncRNA-based signatures of patients with colon cancer from three other studies, including LiLncSig (Li Z. et al., 2020) (AUC = 0.721), LinLncSig (Lin et al., 2020) (AUC = 0.796), and XingLncSig (Xing et al., 2018) (AUC = 0.665). The AUC for 1-, 3-, and 5-year survival were 0.851, 0.893, and 0.951, respectively (**Figure 4C**). AUC of other clinical-pathological features were also presented. In the constructed signature, risk score had a higher efficiency for predicting 1-, 3-, and 5-year survival than other variables

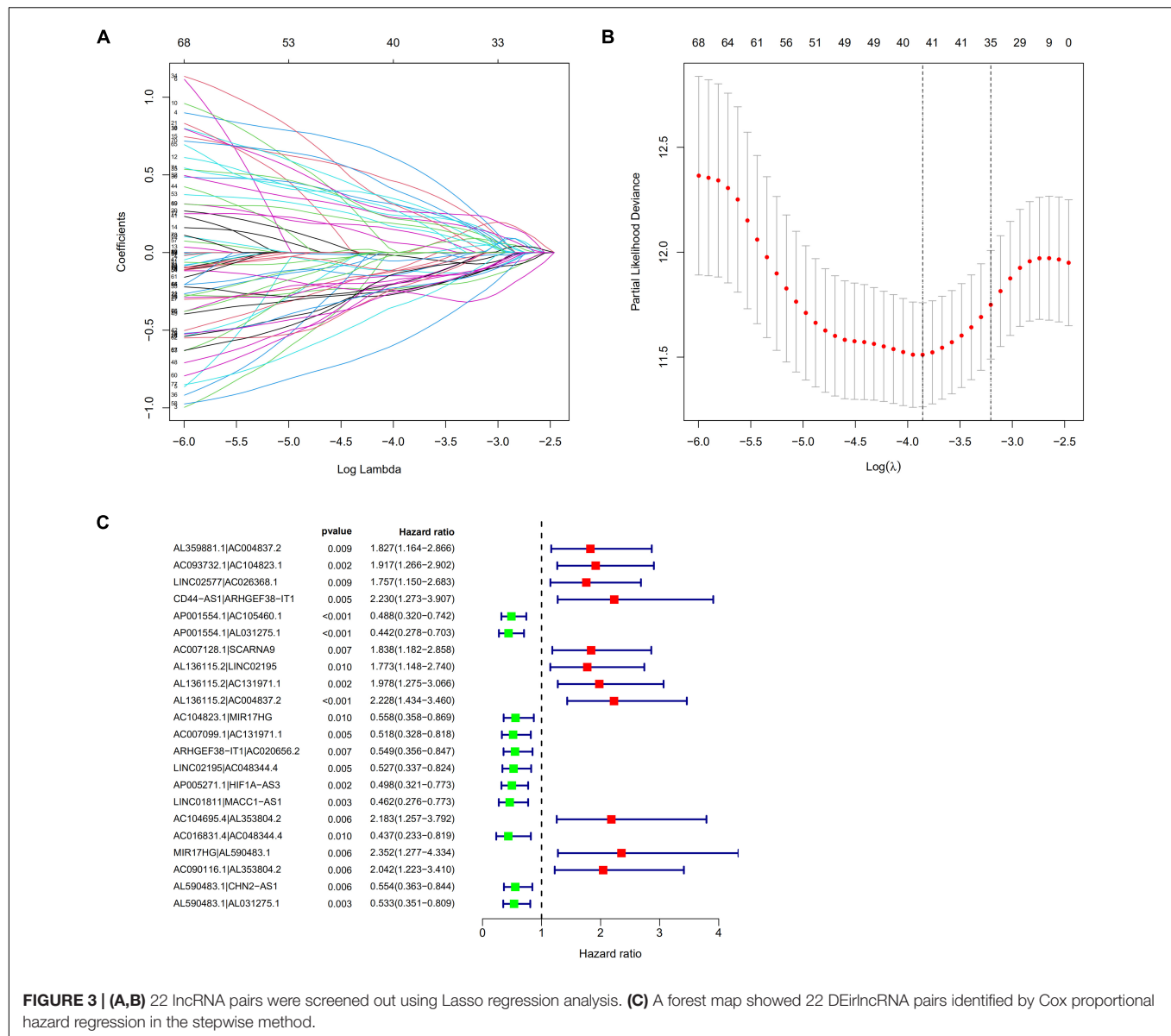




**FIGURE 2 | (A)** The expression correlation between DElncRNAs and mRNAs were shown as an Long non-coding ribonucleic acids (lncRNAs)–mRNA co-expression network, wherein lncRNAs and associated mRNAs were linked together with lines. **(B)** DElncRNAs were primarily related to the biological functions of extracellular matrix organization, extracellular structure organization, collagen trimers, fibrillar collagen trimers, and banded collagen fibrils. **(C)** These DElncRNAs also participated in the pathways of protein digestion and absorption, WNT signaling, nitrogen metabolism, and signaling pathways regulating the pluripotency of stem cells.

in COAD patients (Figures 4D–F). Based on the risk score, patients were divided into high-risk and low-risk groups using the calculated cut-off point (Figure 5A), and patients with higher risk scores had a higher risk of mortality (Figure 5B). As seen in Figure 5C, survival curve was plotted to show the survival differences of COAD patients in the two groups. Patients in the high-risk group had a significantly lower probability of survival than those in the low-risk group ( $p < 0.001$ ). Chi-square tests were performed to investigate the relationship between risk score

and other clinical-pathological features. A heatmap was plotted, showing that age, clinical stage, T stage, N stage, and M stage were significantly related to the risk score (Figure 6A). Univariate and multivariate Cox regression analyses were performed to identify prognosis-related factors in COAD patients (Figures 6B,C). Factors with  $p$  value  $< 0.05$  in the univariate analysis were included in the multivariate analysis. Forest maps showed that age ( $p < 0.001$ , HR = 1.054, 95% CI [1.031–1.078]), stage ( $p = 0.035$ , HR = 2.344, 95% CI [1.060–5.182]), and the risk



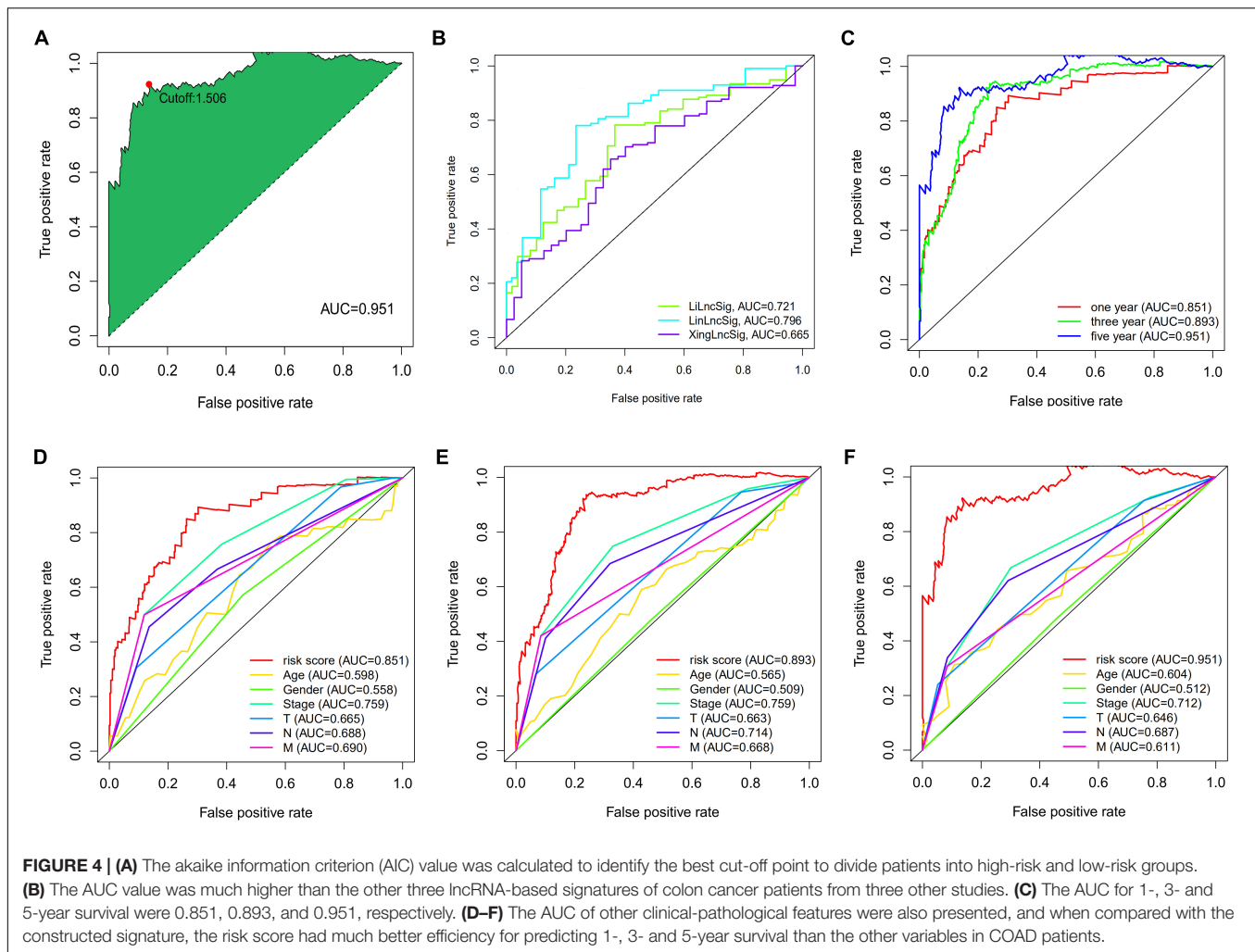
score ( $p < 0.001$ , HR = 1.042, 95% CI [1.029–1.056]) were still significant after multivariate analysis. Therefore, the risk score was independently associated with the prognosis of COAD patients. Wilcoxon signed-rank test showed that clinical stage (Figure 6D), T stage (Figure 6E), N stage (Figure 6F), and M stage (Figure 6G) were significantly related to the calculated risk score. To better predict 1-, 3-, and 5-year survival rates of COAD cases, a nomogram model was constructed based on the results of univariate and multivariate Cox regression analyses (Figure 7A). Age, clinical stage, and risk score were included in the nomogram model. Accordingly, a 60-year-old patient with stage IV colon cancer and risk score of 45 has an estimated 5-year survival rate of less than 10%. Moreover, calibration plots depicting the differences between nomogram-predicted and actual survival probabilities of COAD patients showed that the predicted 1-, 3-, and 5-year survival probabilities were close to the actual survival

probabilities (Figures 7B–D), indicating that this nomogram model accurately predicted survival of COAD patients.

## Evaluation of the Relationship Between Risk Score and Immune Cell Infiltration Status

To better understand the correlation between risk score and tumor immune microenvironment, the Spearman correlation and Wilcoxon signed-rank tests were performed. As Figure 8A shows, the risk score correlated with many types of immune cells, including granulocyte-monocyte progenitors, neutrophils, CD4+ T cells, myeloid dendritic cells, cancer associated fibroblasts, and activated natural killer (NK) cells. High risk score was positively associated with tumor infiltrating immune cells including CD4+ T cells (Figures 8C,F), neutrophils (Figure 8D),





and activated NK cells (Figures 8H,I), and negatively associated with hematopoietic stem cells (Figure 8B), myeloid dendritic cells (Figure 8E), and uncharacterized cells (Figure 8G).

## Expression of Immune Checkpoint Genes in High-Risk and Low-Risk Groups

To use the risk score to predict potential checkpoint blockade therapy, violin plots were drawn to show the differences of immune checkpoint gene expression in high-risk and low-risk groups. LAG3 (Figure 9D,  $p < 0.05$ ) and PD-1 (Figure 9E,  $p < 0.01$ ) expressions were significantly different in the two groups, whereas CTLA4 (Figure 9A,  $p > 0.05$ ), HAVCR2 (Figure 9B,  $p > 0.05$ ), IDO1 (Figure 9C,  $p > 0.05$ ), and PD-L1 (Figure 9F,  $p > 0.05$ ) expressions showed no significant difference between the groups.

## Using the Risk Score to Predict Chemosensitivity of COAD Patients

The differences in chemosensitivity evaluated using IC50 values in high-risk and low-risk groups were analyzed using

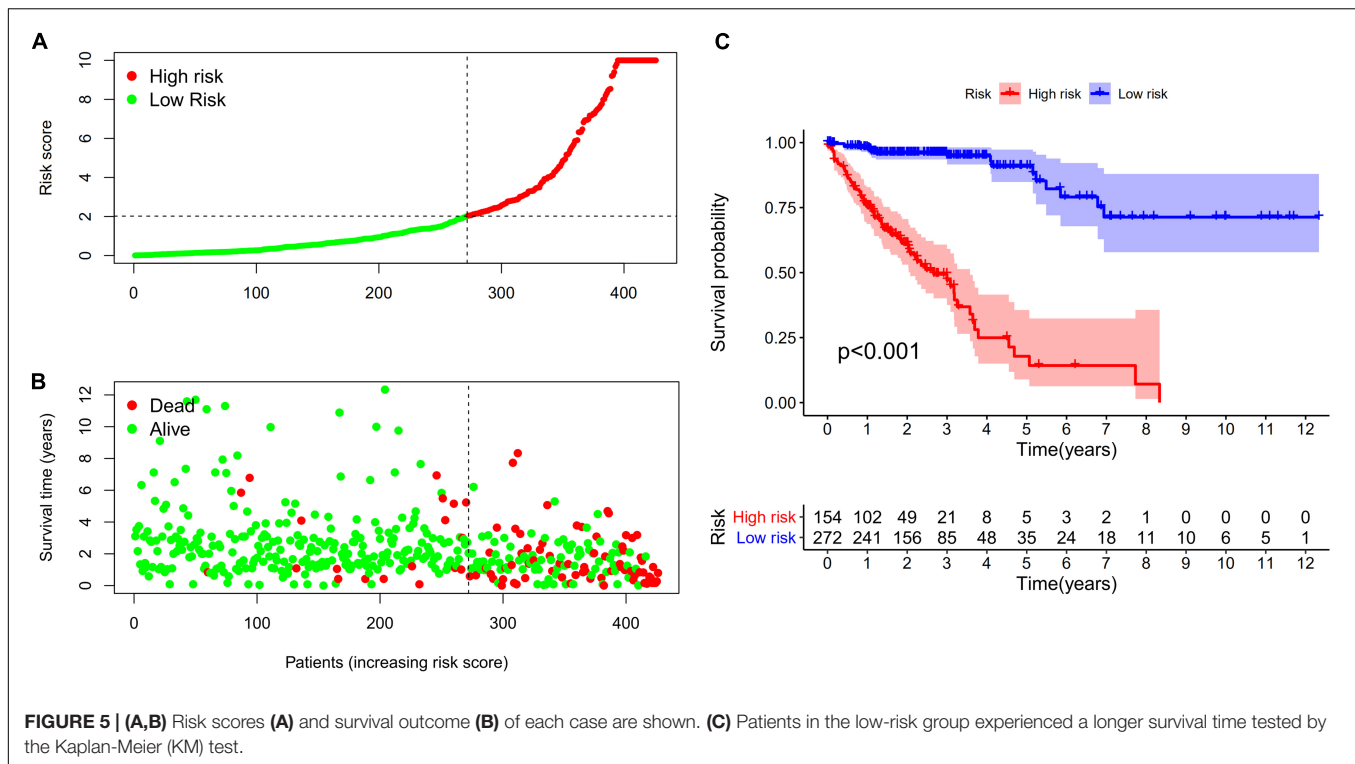
Wilcoxon signed-rank test. The results indicated that lower IC50 values of camptothecin, doxorubicin, erlotinib, gemcitabine, paclitaxel, and rapamycin were associated with higher risk scores (Figure 10). These results might provide reference for clinical treatment of COAD.

## Validating Expression Levels of LINC02195 and SCARNA9 via Quantitative Real-Time Polymerase Chain Reaction

To explore the expression levels of LINC02195 and SCARNA9, these lncRNAs were tested in colon cancer and adjacent non-cancer tissues using qRT-PCR method. As Figure 11 shows, the expression levels of LINC02195 and SCARNA9 in colon cancer were significantly higher than that in adjacent non-cancer tissues.

## DISCUSSION

Recent studies have presented signatures for cancer diagnosis and survival prediction based on the specific expression



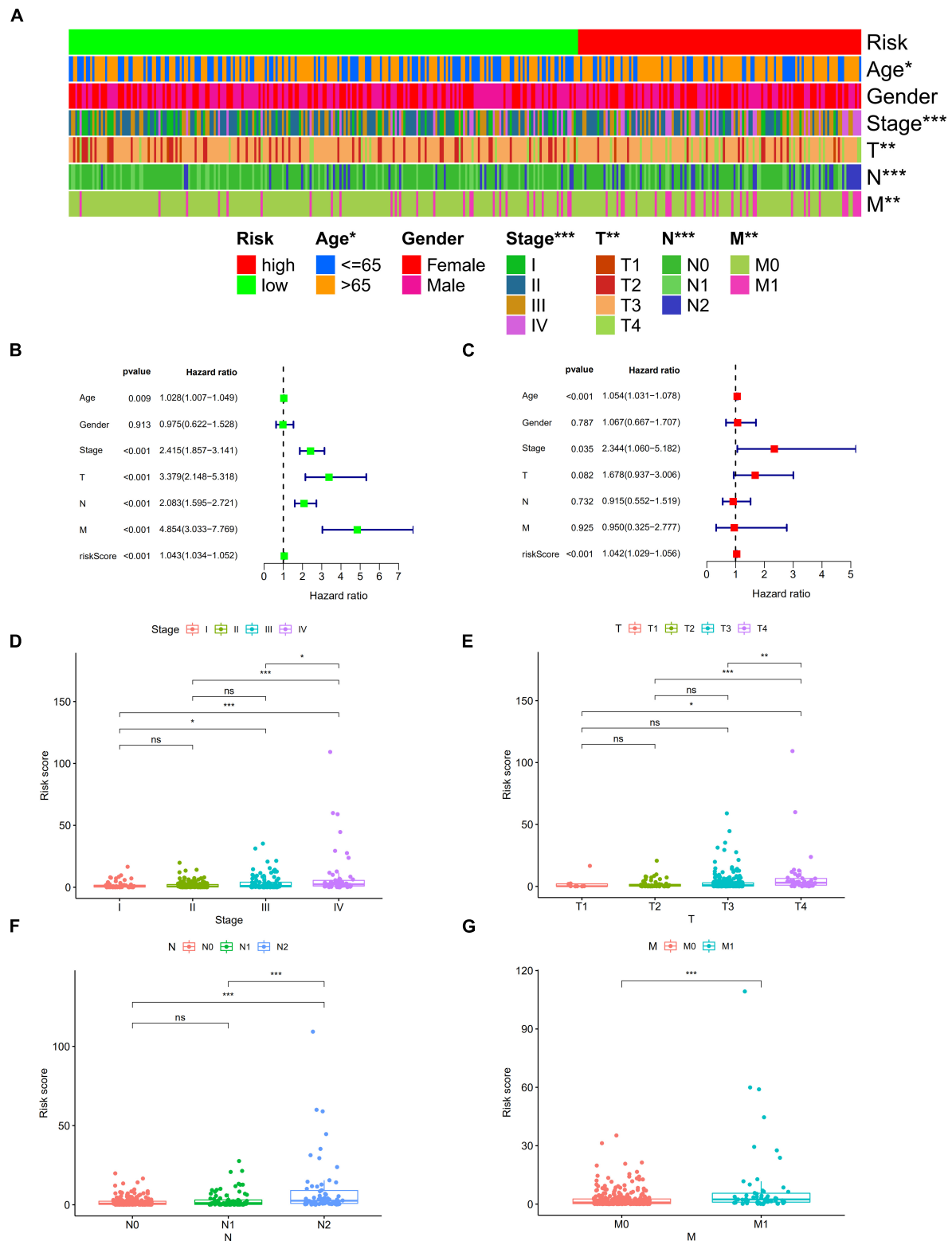
levels of coding genes or noncoding RNAs (Liu et al., 2021; Wang R. et al., 2021; Wang X. et al., 2021; Wu et al., 2021), which required certain testing methods. In this study, lrlncRNA pairs were constructed and used for the development of prognosis signature. The combination of two lncRNAs did not require the exact expression quantities to be measured by certain methods, making it much more feasible and convenient in clinical use.

First, the expression and clinical data were retrieved from TCGA database. Differential co-expression analysis was performed to identify DElrlncRNAs and GO and KEGG analyses were performed to explore relevant pathways and molecular biological functions. The results suggested that DElrlncRNAs were related to the biological functions of the organization of cellular and noncellular components. Malignant tumors are surrounded by extracellular matrix and stromal cells and these cellular and non-cellular components build up the tumor microenvironment (Wang et al., 2017). The interactions between tumor microenvironment and cancer cells have great importance in cancer progression and metastasis (Joyce and Pollard, 2009; Quail and Joyce, 2013). Results showed that DElrlncRNAs participated in the pathways of protein digestion and absorption, WNT signaling, nitrogen metabolism, and regulating pluripotency of stem cells. The WNT signaling pathway is related to several cancer types, especially colorectal cancer (CRC; Cancer Genome Atlas Network, 2012). Wnt- $\beta$ -catenin signal activation leads to the accumulation of  $\beta$ -catenin in the nucleus, which has been detected in over 80% of CRC tumor tissues (Wanitsuwan et al., 2008). In addition, high levels of nuclear  $\beta$ -catenin are associated with poor prognosis in patients with CRC (Baldus et al., 2004). A recent study indicated

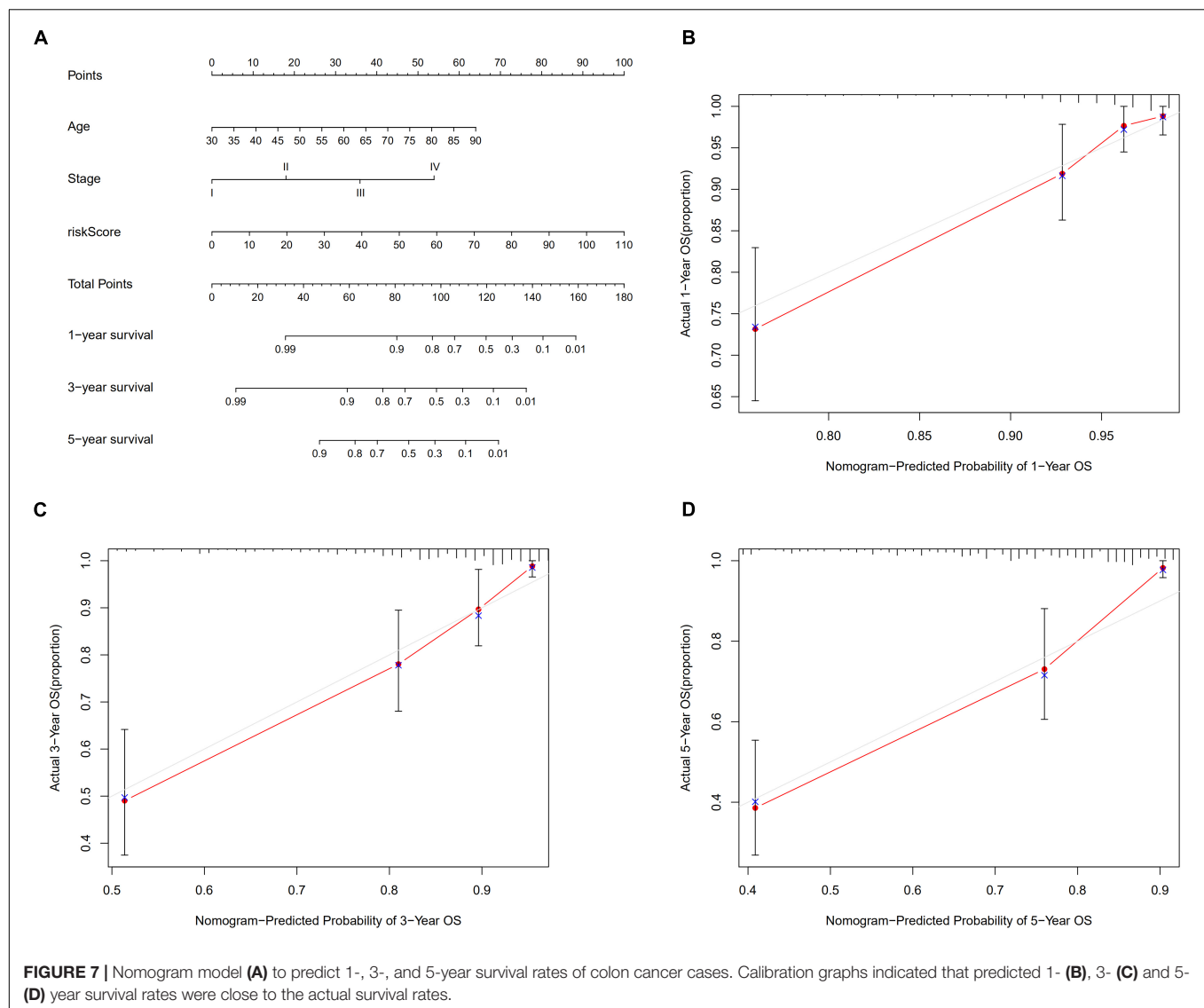
that nitrogen metabolism in was changed in various types of cancer, which was detectable in body fluids and might cause new mutations in cancer tissues (Lee et al., 2018). Crespo et al. (2018) suggested that colonic organoids from human-induced pluripotent stem cells can be used for modeling CRC.

A total of 2,720 valid lncRNA pairs were constructed using 90 DElncRNAs. To explore the impact of DElncRNA pairs on prognosis in colon cancer patients, 22 prognosis-related DElncRNA pairs were identified using LASSO regression analysis and Cox regression analysis. Some of the DElrlncRNAs used for modeling have already been demonstrated to play an important role in CRC and other types of malignant tumors. Anirban et al. (Li H. et al., 2020) demonstrated that LINC02195 is a regulator of histocompatibility complex class I molecules and a prognosis biomarker for head and neck squamous cell carcinoma. Wang et al. (2020) showed that SCARNA9 is associated with the prognosis of patients with endometrial cancer. Xu et al. (2019) suggested that MIR17HG is an immune-related lncRNA, as it was upregulated in CRC tissues compared with normal tissues. Moreover, MIR17HG also contributed to tumorigenesis and metastasis in CRC cells both *in vitro* and *in vivo*. Cao et al. (2019) also showed that MIR17HG is upregulated in glioma tissues and cell lines and that downregulation of MIR17HG is related to inhibition of glioma cell progression. Therefore, the constructed signature can identify new biomarkers for further studies.

Subsequently, every AUC value was calculated to construct a signature with the maximum AUC value. The 1-, 3- and 5-year AUCs of the prognosis signature were also compared with other clinical variables. AIC value was calculated to find the



**FIGURE 6 |** Strip chart (A) showed that age, clinical stage, tumor infiltration depth, lymph node metastasis, and distant metastasis were significantly associated with the risk score. Forest plots of univariate (B) and multivariate (C) Cox regression analyses in colon cancer. Scatters diagram also showed that (D) clinical stage, (E) tumor infiltration depth, (F) lymph node metastasis, and (G) distant metastasis status significantly correlated with the risk score. \* $p < 0.05$ ; \*\* $p < 0.01$ ; and \*\*\* $p < 0.001$ .

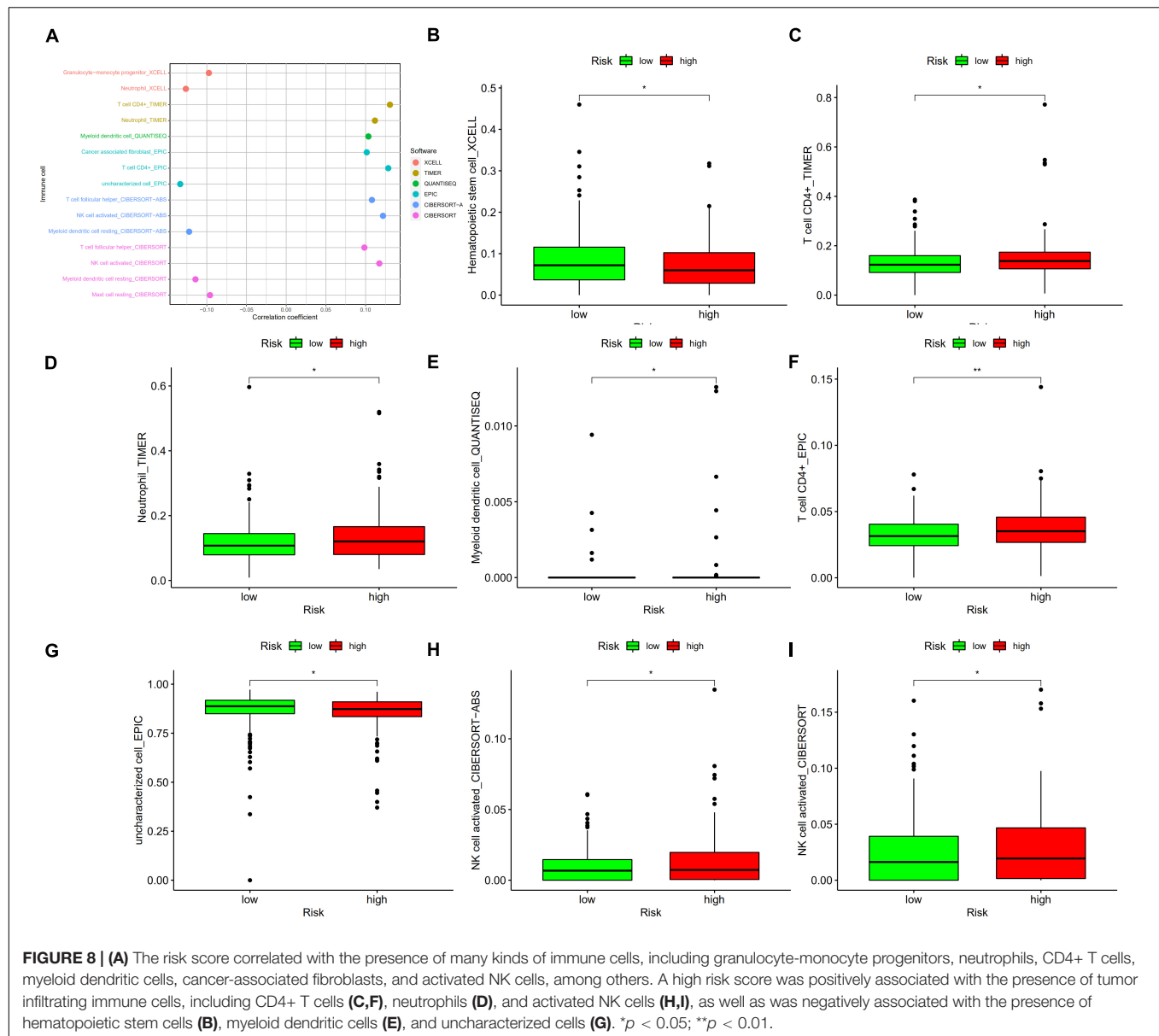


ideal cut-off point to divide patients into high- and low-risk groups. KM curve showed that survival rates of low-risk patients were much higher than those of high-risk patients. To better understand the utility of clinical variables and risk score on predicting patient outcomes, univariate and multivariate Cox analyses were performed. These results showed that the risk score remained significant after these analyses, indicating that the calculated risk score was an independent predictor of patient prognosis. The relationship between the risk score and other clinical features was also determined. The results suggested that the risk score correlated with tumor stage, tumor infiltration depth, lymph node metastasis, and distant metastasis, indicating that the risk score might be related to the development and migration of colon cancer. Nomogram is a prediction tool in oncology, especially for cancer prognosis (Iasonos et al., 2008; Balachandran et al., 2015). A nomogram model was established to visualize the effects of clinical features and risk score on 1-, 3- and 5-year survival probabilities of patients. Calibration graphs

showed that the nomogram-predicted survival rates were close to actual survival rates, indicating that the nomogram model had high prediction efficiency.

Tumor-infiltrating immune cells affect the response to tumor anti-checkpoint blockades. Tumor-infiltrating CD4<sup>+</sup> T cells upregulated PD-1, cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), T cell immunoglobulin and mucin domain-3 (TIM-3) and LAG-3 (Toor et al., 2019). Six common methods were used to evaluate the relationship between tumor infiltration immune cells and risk score, including XCELL (Aran et al., 2017), TIMER (Li et al., 2017), QUANTISEQ (Finotello et al., 2019), EPIC (Van Veldhoven et al., 2011), CIBERSORT-A (Tamminga et al., 2020), and CIBERSORT (Newman et al., 2015). The results revealed that the risk score was positively related to CD4<sup>+</sup> T cells, neutrophils, and activated NK cells, and negatively related to hematopoietic stem cells and myeloid dendritic cells. A previous study showed that immune scores based on immune genomic analysis can indicate the therapeutic benefits

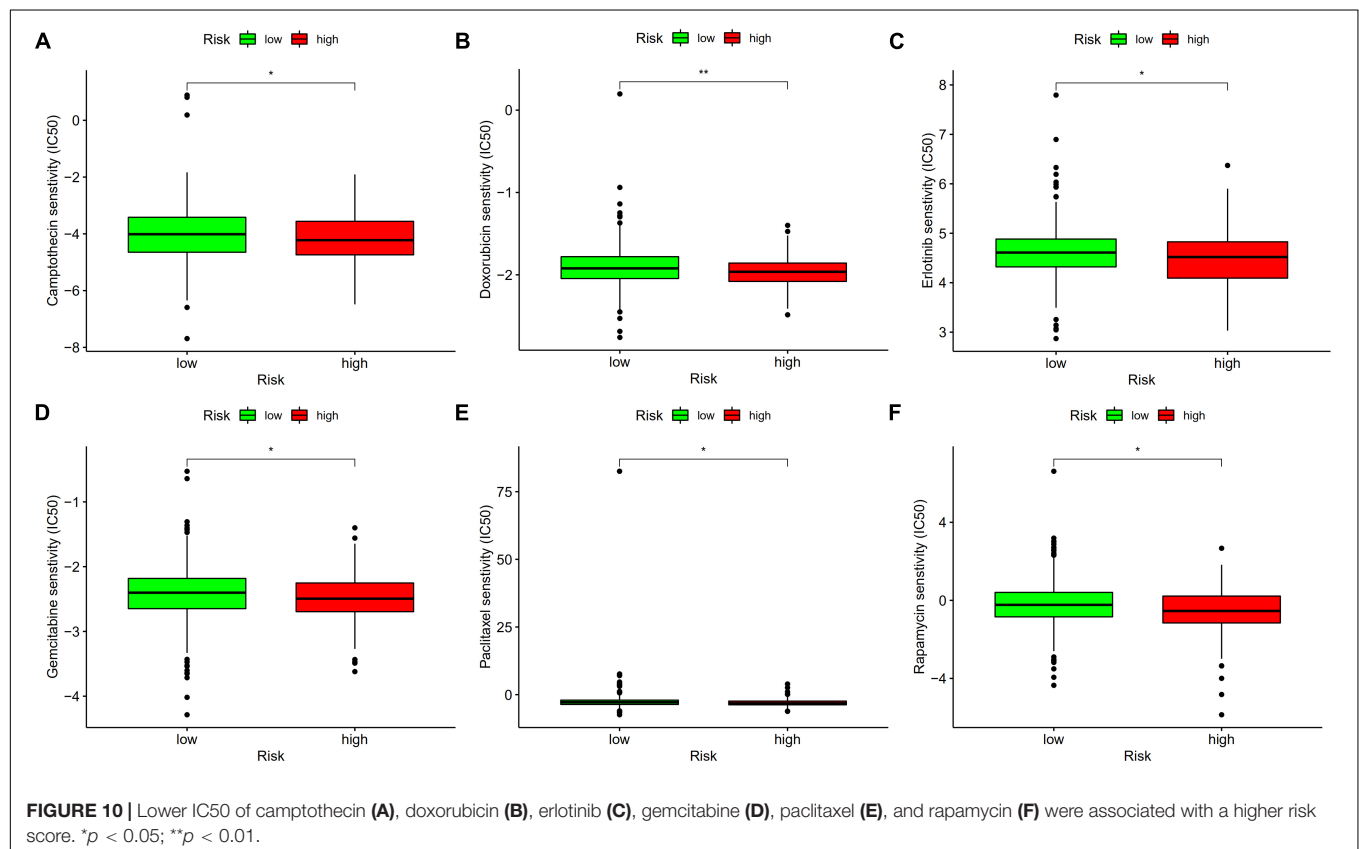
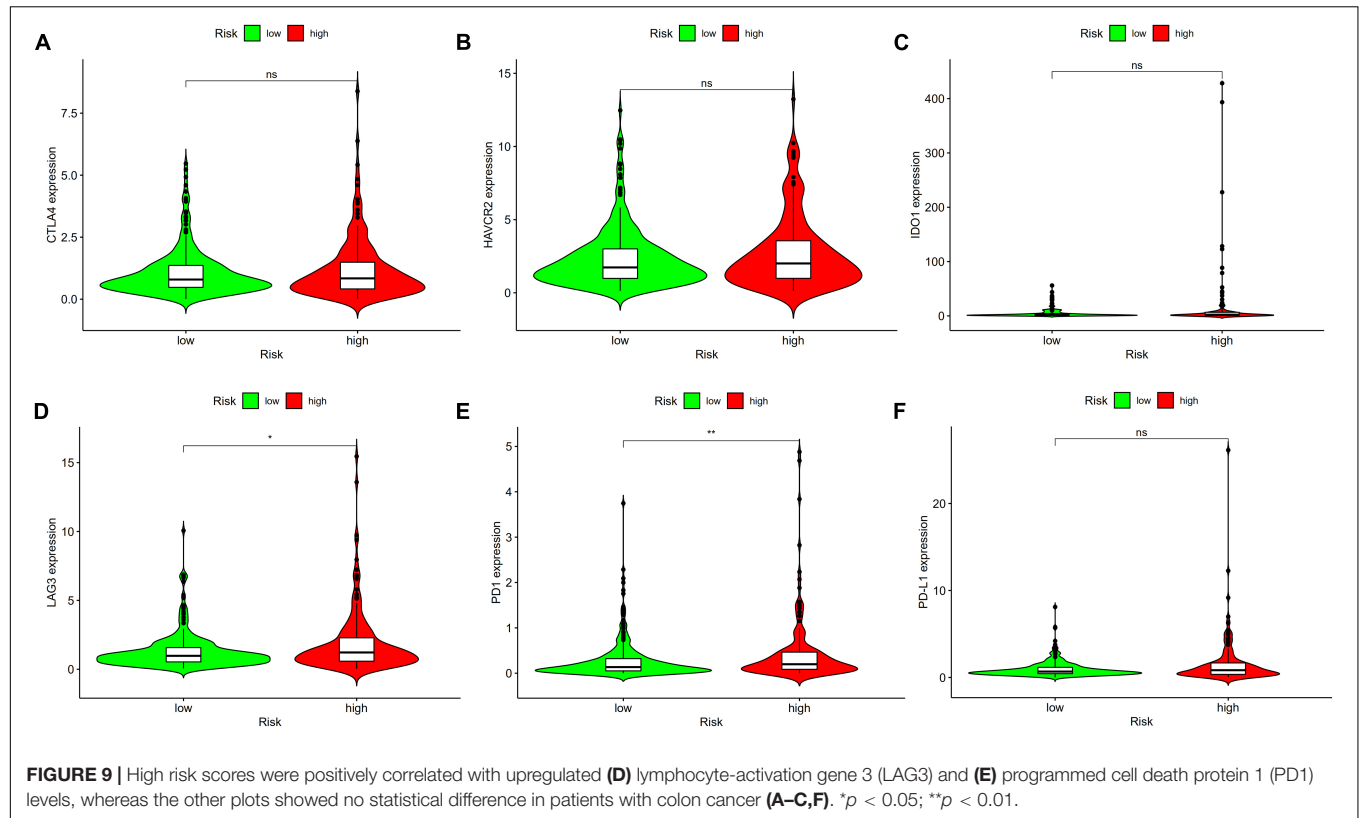


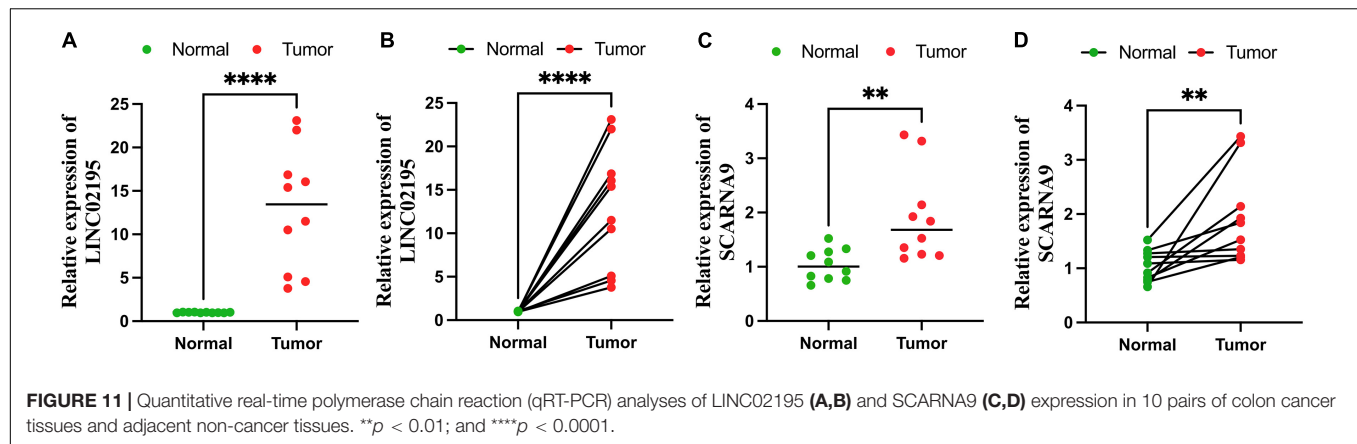


of immunotherapy and chemotherapy (Dai et al., 2020). To predict potential checkpoint blockade therapy, the relationship between risk score and immune checkpoint gene expression was explored and the results showed that PD-1 and LAG-3 expressions were significantly different in high- and low-risk groups. Anti-PD-1 inhibitor is effective in treating patients with dMMR/MSI-H mCRC (He et al., 2020). Xiao et al. (Xiao and Freeman, 2015) suggested that LAG-3 creates an immunosuppressive microenvironment in MSI-H CRC, possibly helping MSI-H tumors escape immune destruction by infiltrating immune cells. Our study also revealed that the risk score was associated with sensitivity to chemotherapeutics such as camptothecin, doxorubicin, erlotinib, gemcitabine, paclitaxel, and rapamycin. The findings our study can be applied for guiding clinical immunotherapy and chemotherapy in

patients with colon cancer. Among lncRNAs that were used to construct the signature, LINC02195 and SCARNA9 were found to be associated with neck squamous cell carcinoma and endometrial cancer in previous researches (Li H. et al., 2020; Wang et al., 2020), however, the effects of these two lrlncRNAs on colon cancer remains unknown. We further validated the expression levels of LINC02195 and SCARNA9 using qRT-PCR method, the results showed that LINC02195 and SCARNA9 were significantly upregulated in colon cancer compared with adjacent non-cancer tissues, indicating that the two lrlncRNAs may be potential biomarkers for diagnosis and therapy in colon cancer.

However, the study has some shortcomings and limitations. All data in our study were downloaded from TCGA database because we could not find other datasets that simultaneously





included lncRNA expression levels, clinicopathological characteristics, and survival outcomes for patients with colon cancer. The individual data source might result in unreliable results. Subsequent molecular biological experiments are needed to further examine the function of DElncRNAs in colon cancer development and to better understand carcinogenic mechanisms. In addition, clinical cases are required in further studies to increase stability and the predictive ability of our established signature.

## CONCLUSION

In conclusion, our analysis of lncRNA expression profiles and clinical features identified DElncRNAs in colon cancer. lncRNA pairs were constructed and used for the development of prognosis signature, which did not require the exact expression quantities tested by certain methods. The constructed signature could effectively evaluate the prognosis of patients with colon cancer and guide clinical therapy. Additional studies are needed to validate the findings of this study and provide a basis for individualized treatment of patients with colon cancer.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee on Medical Ethics of The First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

XW and KC are responsible for writing and submitting the manuscript. ZW, BC, and YX are responsible for data collection and analysis. LD and TB are responsible for the production of pictures. WY and WC are responsible for the ideas and guidance. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.750709/full#supplementary-material>

**Supplementary Table 1 |** Clinicopathologic characteristics of TCGA colon cancer patients.

**Supplementary Table 2 |** List of 90 DElncRNAs.

**Supplementary Table 3 |** Univariate Cox regression analysis of the 22 identified lncRNA pairs.

**Supplementary Table 4 |** Univariate Cox regression analysis of risk score and other clinical features.

**Supplementary Table 5 |** Multivariate Cox regression analysis of risk score and other clinical features.

**Supplementary Table 6 |** The correlation between risk score and immune infiltration of cells via the Spearman correlation method.

**Supplementary Table 7 |** Raw data of qRT-PCR.

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# MCM3AP-AS1: An Indispensable Cancer-Related LncRNA

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Long non-coding RNAs (lncRNAs) are a class of RNA molecules with transcripts longer than 200 nucleotides that have no protein-coding ability. MCM3AP-AS1, a novel lncRNA, is aberrantly expressed in human cancers. It is significantly associated with many clinical characteristics, such as tumor size, tumor-node-metastasis (TNM) stage, and pathological grade. Additionally, it considerably promotes or suppresses tumor progression by controlling the biological functions of cells. MCM3AP-AS1 is a promising biomarker for cancer diagnosis, prognosis evaluation, and treatment. In this review, we briefly summarized the published studies on the expression, biological function, and regulatory mechanisms of MCM3AP-AS1. We also discussed the clinical applications of MCM3AP-AS1 as a biomarker.

**Keywords:** long non-coding RNA, MCM3AP-AS1, biological function, regulatory mechanism, biomarker

## INTRODUCTION

Cancer is a fatal disease that is often caused by somatic mutations (Kennedy et al., 2019; Andrei et al., 2020; Costa et al., 2020). Genomic alterations can lead to a series of malignant features, including cell migration, invasion, and metastasis. Conventional cancer therapies, including surgery, radiotherapy, and chemotherapy, have limitations (Liang et al., 2021). Molecular targeted therapy, a new therapeutic approach, overcomes these limitations and has advantages in cancer treatment (Ethier et al., 2021).

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with transcript lengths longer than 200 nucleotides that have no protein-coding ability (Thum, 2014; Lorenzen and Thum, 2016; Liu et al., 2017; Xu et al., 2020; Jin et al., 2021). The function of lncRNAs is related to their special subcellular localization. The lncRNAs located in the nucleus participate in gene regulation at the epigenetic and transcription levels. Moreover, lncRNAs in the cytoplasm are involved in interactions with proteins in the cytoplasm and the regulation of the metabolism of mRNAs, such as endogenous competitive RNAs (ceRNAs), which interact with microRNAs. Increasing evidence indicates that lncRNAs are important modulators of different biological functions (Zehendner et al., 2020). The overexpression of lncRNA OTUD6B-AS1 inhibits cell proliferation, migration, and invasion in clear cell renal cell carcinoma (Wang et al., 2019a). Elevated levels of lncRNA H19 decreased sensitivity to tamoxifen in breast cancer (Wang et al., 2019b). Moreover, lncRNAs have been reported to function via multiple signaling pathways in cancer progression. LNRIL6 promotes cancer progression by activating the IL-6/STAT3 pathway

in colorectal cancer (CRC) (Wang et al., 2019c). Long non-coding RNA EPB41L4A-AS2 inhibits cell proliferation and migration by downregulating miR-301a-5p expression and upregulating FOXL1 expression in hepatocellular carcinoma (HCC) (Wang et al., 2019f).

MCM3AP-AS1 is located in 46,228,977-46,259,390 of chromosome 21, and the subcellular localization of MCM3AP-AS1 is chromatin and nucleoplasm (**Figure 1A**). MCM3AP-AS1 was found to be dysregulated in a variety of cancers, including breast cancer, CRC, gastric cancer, HCC, and prostate cancer (PCa). MCM3AP-AS1 has great potential for use in cancer diagnosis, prognosis evaluation, and treatment. In this review, we first summarized the expression profile of MCM3AP-AS1 and the cellular processes in which MCM3AP-AS1 is involved. Then, we clarified the mechanism of MCM3AP-AS1 in two parts: *in vitro* cell experiments and *in vivo* experiments. The former part enabled superficial function verification, while the latter enabled further confirmation of the potential regulatory mechanism of MCM3AP-AS1. Briefly, we outline the role of lncRNA MCM3AP-AS1 in tumorigenesis by integrating recent research findings.

## EVIDENCE ACQUISITION

We carried out exhaustive research employing PubMed and the Web of Science database to seek articles up to August 2021 using the keywords MCM3AP-AS1, MCM3AP-AS, MCM3APAS, tumor, cancer, and carcinoma. We assessed all results according to the titles and abstracts and selected articles related to our theme. All unrelated articles, letters, meeting proceedings, correction articles, and retracted articles were excluded. After this, the full text of any selected article was reviewed independently by two authors. **Figure 1B** shows a flow diagram of the study selection process.

## EXPRESSION AND BIOLOGICAL FUNCTIONS OF MCM3AP-AS1 IN HUMAN CANCER

The expression levels of MCM3AP-AS1 are significantly dysregulated in human cancers (**Table 1**). MCM3AP-AS1 plays a vital role in the occurrence and development of various cancers. Its expression is significantly associated with several clinical characteristics. Moreover, *in vitro* assays have shown that it markedly promotes or suppresses tumor progression by controlling cell biological functions. In this section, we discussed the emerging roles of MCM3AP-AS1 in different cancers (**Table 2**).

### Breast Cancer

Breast cancer is one of the most common types of malignancy in women worldwide (Liang et al., 2020; Carreira et al., 2021; Han et al., 2021). The levels of lncRNA MCM3AP-AS1 are significantly upregulated in breast cancer tissues and cell lines (Chen et al., 2020; Riahi et al., 2021). The level of

MCM3AP-AS1 was positively associated with estrogen receptor (ER) and progesterone receptor (PR) expression, whereas no significant differences were observed between MCM3AP-AS1 and HER2 expression profiles in breast cancer patients. Functionally, MCM3AP-AS1 affected cell biological functions to control breast cancer progression by regulating specific pathways. MCM3AP-AS1 knockout inhibited the proliferation, invasion, and migration of breast cancer cell lines. These findings may facilitate the development of novel therapeutics for breast cancer.

### Colorectal Cancer

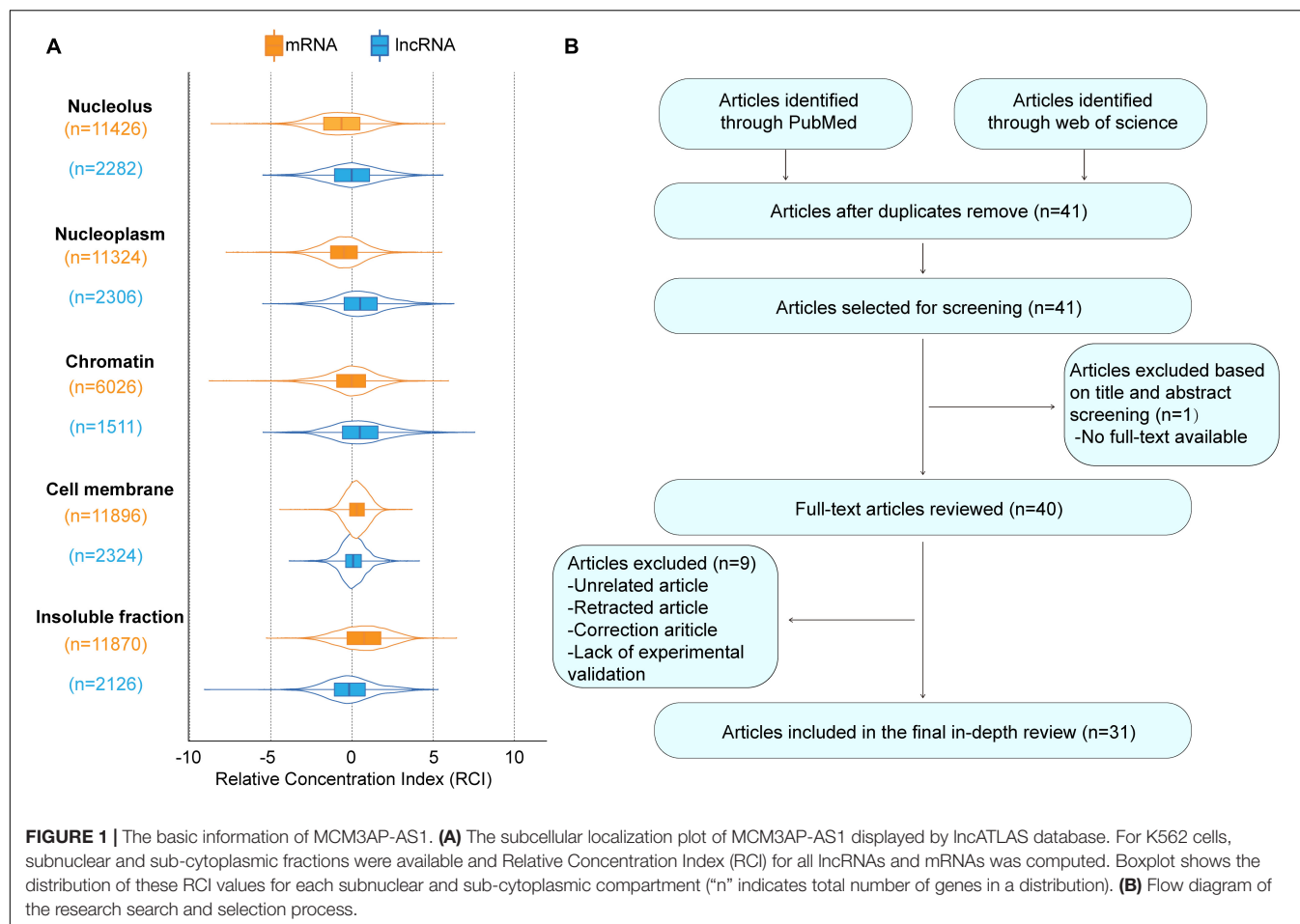
Colorectal cancer (CRC) is one of the most prevalent cancers and a leading cause of cancer-related death worldwide (Oki et al., 2016; Wang et al., 2019d; Yarla et al., 2019; Zhao et al., 2021). Some studies have revealed that MCM3AP-AS1 expression is markedly upregulated in CRC tissues compared to corresponding normal tissues (Ma et al., 2020; Zhou et al., 2021). In terms of prognosis, MCM3AP-AS1 levels are negatively associated with overall survival (OS). Functionally, elevated MCM3AP-AS1 expression promotes cell proliferation, colony formation, migration, and invasion and arrests the cell cycle at the G1 phase in CRC cell lines (Ma et al., 2020; Zhou et al., 2021) (**Figure 2**). In contrast, Dai et al. found that MCM3AP-AS1 expression was decreased in CRC tissues (Dai et al., 2021). The expression of MCM3AP-AS1 was positively correlated with OS in CRC patients. Moreover, the levels of MCM3AP-AS1 were negatively associated with tumor-node-metastasis (TNM) stage, tumor size, and carcinoembryonic antigen (CEA) levels in CRC. Functionally, MCM3AP-AS1 significantly reduced the proliferation and migration of CRC cells (**Figure 2**). The results were validated by siRNA knockdown experiments. However, MCM3AP-AS1 expression needs to be further studied in CRC. Further evidence-based basic and clinical studies are needed to increase the evidence base.

### Gastric Cancer

Gastric cancer is the fifth most common malignancy and the second leading cause of cancer-related mortality worldwide (Liu et al., 2016; Seidlitz et al., 2019; Kang et al., 2020; Harada et al., 2021; Li et al., 2021). Cisplatin (CDDP) is a well-known chemotherapeutic agent used to treat gastric cancer (Germann et al., 2002; Ivanova et al., 2013; Huang et al., 2019). Cisplatin resistance is the main reason for the poor therapeutic effects in gastric cancer (Wang et al., 2018; Zhang Q. et al., 2020). The expression levels of MCM3AP-AS1 were evidently upregulated in MGC-803, SGC-7901, NCI-N87 (NCI-N87/CDDP), and AGS cells (AGS/CDDP) (Wang et al., 2020; Sun et al., 2021). *In vitro* evidence suggested that the levels of MCM3AP-AS1 are positively associated with the half-maximal inhibitory concentration (IC<sub>50</sub>) of CDDP in gastric cancer. MCM3AP-AS1 was also found to facilitate cell proliferation, migration, and invasion and decrease CDDP sensitivity in gastric cancer cell lines (Sun et al., 2021).

### Liver Cancer

Hepatocellular carcinoma (HCC) is a common primary malignancy of the liver and typically occurs in patients with underlying chronic liver disease (Degaspero et al., 2020;



Goh et al., 2020; Yip et al., 2020; Zhou et al., 2020; Xia et al., 2021). MCM3AP-AS1 expression is upregulated in HCC tissues and cell lines (Wang et al., 2019e). The levels of MCM3AP-AS1 are positively associated with tumor size, stage, and grade in HCC patients. Increased MCM3AP-AS1 expression was associated with a worse prognosis in HCC (Wang et al., 2019e; Zhang et al., 2019). *In vitro* cell experiments suggested that silencing MCM3AP-AS1 inhibited the formation of human lymphatic endothelial cells in HCC. Moreover, MCM3AP-AS1 significantly promotes the proliferation, colony formation, cell cycle progression, and metastasis of HCC cells (Wang et al., 2019e; Zhang et al., 2019).

## Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC), a head and neck cancer, seriously affects the quality of life of affected patients, both psychologically and physically (Ng et al., 2017; Bray et al., 2018; Almangush et al., 2020). Hou et al. revealed that MCM3AP-AS1 expression was elevated in both OSCC cells and tissues and that a high expression level of MCM3AP-AS1 was correlated with a poor prognosis in OSCC patients. Moreover, overexpression of MCM3AP-AS1 could enhance the proliferation, migration, and invasion of OSCC cells. Inhibiting MCM3AP-AS1 had the opposite effect on the above cell events

(Hou et al., 2020). Li et al. found similar phenomena in OSCC cells and found different molecular mechanisms than Hou, which would be elaborated in a later section (Li and Jiang, 2020). Therefore, we have adequate evidence to indicate that MCM3AP-AS1 has the potential to act as a biomarker for OSCC patients.

## Prostate Cancer

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related death in Americans (Amarasekera et al., 2019; Berger et al., 2019; Ge et al., 2020). The expression levels of MCM3AP-AS1 were markedly increased in PCa tissues and cell lines (Jia et al., 2020; Li et al., 2020a; Wu et al., 2020). MCM3AP-AS1 levels were negatively correlated with OS in PCa patients (Jia et al., 2020; Chen et al., 2021).

A negative association has also been reported between MCM3AP-AS1 and disease-free survival in PCa (Wu et al., 2020). MCM3AP-AS1 expression correlates with the Gleason score, pathological stage, and androgen receptor expression in PCa (Li et al., 2020a). MCM3AP-AS1 knockdown inhibited cell proliferation, migration, and invasion and promoted the apoptosis of PCa cell lines (Jia et al., 2020; Li et al., 2020a; Wu et al., 2020). In addition, MCM3AP-AS1 expression



**TABLE 1** | Expression and associated clinical features of the lncRNA MCM3AP-AS1 in cancer.

Type	Expression	Feature	References
Breast cancer	upregulated	tumor estrogen receptor expression, and tumor progesterone receptor expression	Riahi et al., 2021
Burkitt Lymphoma	upregulated	tumor size, tumor stage, and poor prognosis	Guo et al., 2020
Cervical squamous cell carcinoma	downregulated	poor survival	Lan et al., 2020
Clear cell renal cell carcinoma	upregulated	/	Wang et al., 2019a
Colorectal cancer	upregulated	poor survival	Ma et al., 2020
Colorectal cancer	downregulated	poor prognosis, higher TNM stage, tumor size, and CEA level	Dai et al., 2021
Colorectal cancer	upregulated	poor survival	Zhou et al., 2021
Endometrioid carcinoma	upregulated	/	Yu J. et al., 2021
Hepatocellular carcinoma	upregulated	large tumor size, high tumor grade, advanced tumor stage, and poor prognosis	Wang et al., 2019e
Hepatocellular carcinoma	/	overall survival	Zhang et al., 2019
Nasopharyngeal carcinoma	upregulated	poor survival	Sun et al., 2020
Lung cancer	upregulated	/	Li et al., 2020b
Small cell lung cancer	upregulated	survival rate	Luo et al., 2021
Non-small cell lung cancer	upregulated	/	Shen et al., 2021
Oral squamous cell carcinoma	upregulated	poor prognosis	Hou et al., 2020
Oral squamous cell carcinoma	upregulated	/	Li and Jiang, 2020
Pancreatic cancer	upregulated	survival rates	Yang et al., 2019
Prostate cancer	upregulated	overall survival	Jia et al., 2020
Prostate cancer	upregulated	pathological stage, Gleason score, and AR expression	Li et al., 2020a
Prostate cancer	/	overall survival	Chen et al., 2021
Prostate cancer	upregulated	disease-free survival	Wu et al., 2020

was significantly associated with bone metastases in PCA (Chen et al., 2021).

## Lung Cancer

Lung cancer (LC) continues to be one of the most frequent cancers worldwide. The number of patients and deaths related to LC has continued to increase in recent years (Schwartz and Cote, 2016; Jones and Baldwin, 2018; Bade and Dela Cruz, 2020). MCM3AP-AS1 was elevated in small cell lung cancer (SCLC), and a high expression level of MCM3AP-AS1 was accompanied by a low survival rate. MCM3AP-AS1 overexpression could facilitate the migration and invasion of SCLC cells (Luo et al., 2021). In non-small-cell lung cancer (NSCLC), MCM3AP-AS1 was also obviously upregulated. MCM3AP-AS1 also could promote the proliferation, invasion, and migration of NSCLC cells. Li et al. generally found that MCM3AP-AS1 could accelerate angiogenesis, in addition to cell proliferation and migration, in LC. In conclusion, MCM3AP-AS1 had the potential to act as a biomarker in LC (Li et al., 2020b).

## Reproductive System Cancers

For females, the incidence and motility of reproductive system cancers are the highest among cancers worldwide, indicating that they seriously threaten women's health (Torre et al., 2017; Hernandez-Silva et al., 2020). Interestingly, MCM3AP-AS1 participates in the progression of most reproductive system cancers, including cervical cancer and endometrial cancer. For endometrioid carcinoma (EC), MCM3AP-AS1 expression was upregulated in cancer tissues compared with adjacent

normal tissues. Overexpression of MCM3AP-AS1 increased the migration and invasion rate of EC cells. Both migration and invasion were inhibited when MCM3AP-AS1 was knocked down (Yu J. et al., 2021). However, Lan et al. demonstrated that MCM3AP-AS1 expression was reduced in cervical squamous cell carcinoma (CSCC) and that MCM3AP-AS1 overexpression significantly inhibit the proliferation of CSCC cells (Lan et al., 2020). These results suggest that MCM3AP-AS1 plays dual roles in reproductive tumors, and the underlying mechanism is probably worth studying.

## Other Cancers

MCM3AP-AS1 was also found to be upregulated in Burkitt lymphoma, glioblastoma, LC, nasopharyngeal carcinoma, clear cell renal cell carcinoma, pancreatic cancer, and papillary thyroid cancer tissues compared to the corresponding normal tissues (Yang et al., 2017, 2019; Liang et al., 2019; Guo et al., 2020; Li et al., 2020b; Qiu et al., 2020; Sun et al., 2020). The MCM3AP-AS1 expression profile was positively associated with tumor size and stage in Burkitt lymphoma. Higher levels of MCM3AP-AS1 indicate a worse prognosis in Burkitt lymphoma, nasopharyngeal carcinoma, and pancreatic cancer. Further *in vitro* experiments confirmed that MCM3AP-AS1 expression was upregulated in pancreatic cancer cell lines (PANC-1, BxPC-3, MIA PaCa-2, Capan-2, and AsPC-1). MCM3AP-AS1 inhibited cell proliferation and migration in LC, pancreatic cancer, and papillary thyroid cancer. It also promoted tumor angiogenesis in glioblastoma and LC and increased cell viability in Burkitt lymphoma and glioblastoma.

**TABLE 2 |** The biological functions and molecular mechanisms of MCM3AP-AS1.

Type	Expression	Function	Related genes	References
Breast cancer	upregulated	cell proliferation, migration, and invasion	miR-28-5p, and CENPF	Chen et al., 2020
Breast cancer	upregulated	cell proliferation	miR-708-5p	Riahi et al., 2021
Burkitt lymphoma	upregulated	cell viability, apoptosis, and chemoresistance	miR-15a, and EIF4E	Guo et al., 2020
Cervical squamous cell carcinoma	downregulated	cell proliferation	miR-93	Lan et al., 2020
Colorectal cancer	upregulated	cell cycle	miR-545, and CDK4	Ma et al., 2020
Clear cell renal cell carcinoma	upregulated	proliferation, inflammation, Pro-angiogenesis	E2F1, DPP4	Wang et al., 2019a
Colorectal cancer	downregulated	cell proliferation, and migration	miR-19a-3p, and FOXF2	Dai et al., 2021
Colorectal cancer	upregulated	cell proliferation, colony formation, migratory, and invasive ability	miR-193a-5p, and SENP1	Zhou et al., 2021
Colorectal cancer	/	/	miR-599, and ARPP19	Yu Y. et al., 2021
Endometrioid carcinoma	upregulated	invasion, and migration	miR-708-5p	Yu J. et al., 2021
Gastric cancer	upregulated (CDDP resistance)	CDDP resistance	miR-138, and FOXC1	Sun et al., 2021
Gastric cancer	upregulated	cell proliferation, and apoptosis	miR-708-5p	H Wang et al., 2020
Glioblastoma	upregulated	cell viability, migration, tube formation of GECs, and angiogenesis	miR-211, KLF5, and AGGF1	Yang et al., 2017
Hepatocellular carcinoma	upregulated	cell proliferation, colony formation, cell cycle progression, and induced apoptosis	miR-194-5p, FOXA1, and FOXA1 restoration	Wang et al., 2019e
Hepatocellular carcinoma	/	invasion, and HDLECs	miR-455, and EGFR	Zhang et al., 2019
Lung cancer	upregulated	cell proliferation, migration, and angiogenesis	YY1, miR-340-5p, and KPNA4	Li et al., 2020b
Small cell lung cancer	upregulated	invasion, and migration	miR-148a, and ROCK1	Luo et al., 2021
Non-small cell lung cancer	upregulated	proliferation, migration and invasion	miR-195-5p, and E2F	Shen et al., 2021
Nasopharyngeal carcinoma	upregulated	cell proliferation, and apoptosis	miR-34a	Sun et al., 2020
Oral squamous cell carcinoma	upregulated	proliferation, migration and invasion	miR-363-5p	Hou et al., 2020
Oral squamous cell carcinoma	upregulated	proliferation, migration and invasion	miR-204-5p, and FOXC1	Li and Jiang, 2020
Pancreatic cancer	upregulated	proliferation, migration, and invasion	miR-138-5p, and FOXK1	Yang et al., 2019
Papillary thyroid cancer	upregulated	proliferation, migration, and invasion	miR-211-5p, and SPARC	Liang et al., 2019
Prostate cancer	upregulated	proliferation, and invasion	miR-543-3p, SLC39A10, and PTEN	Jia et al., 2020
Prostate cancer	upregulated	proliferation, invasion, migration, and apoptosis	DNMT1, DNMT3, NPY1R, and MAPK	Li et al., 2020a
Prostate cancer	/	bone metastasis	/	Chen et al., 2021
Prostate cancer	upregulated	proliferative ability, and apoptosis	miR-876-5p, WNT5A, and WNT5A	Wu et al., 2020

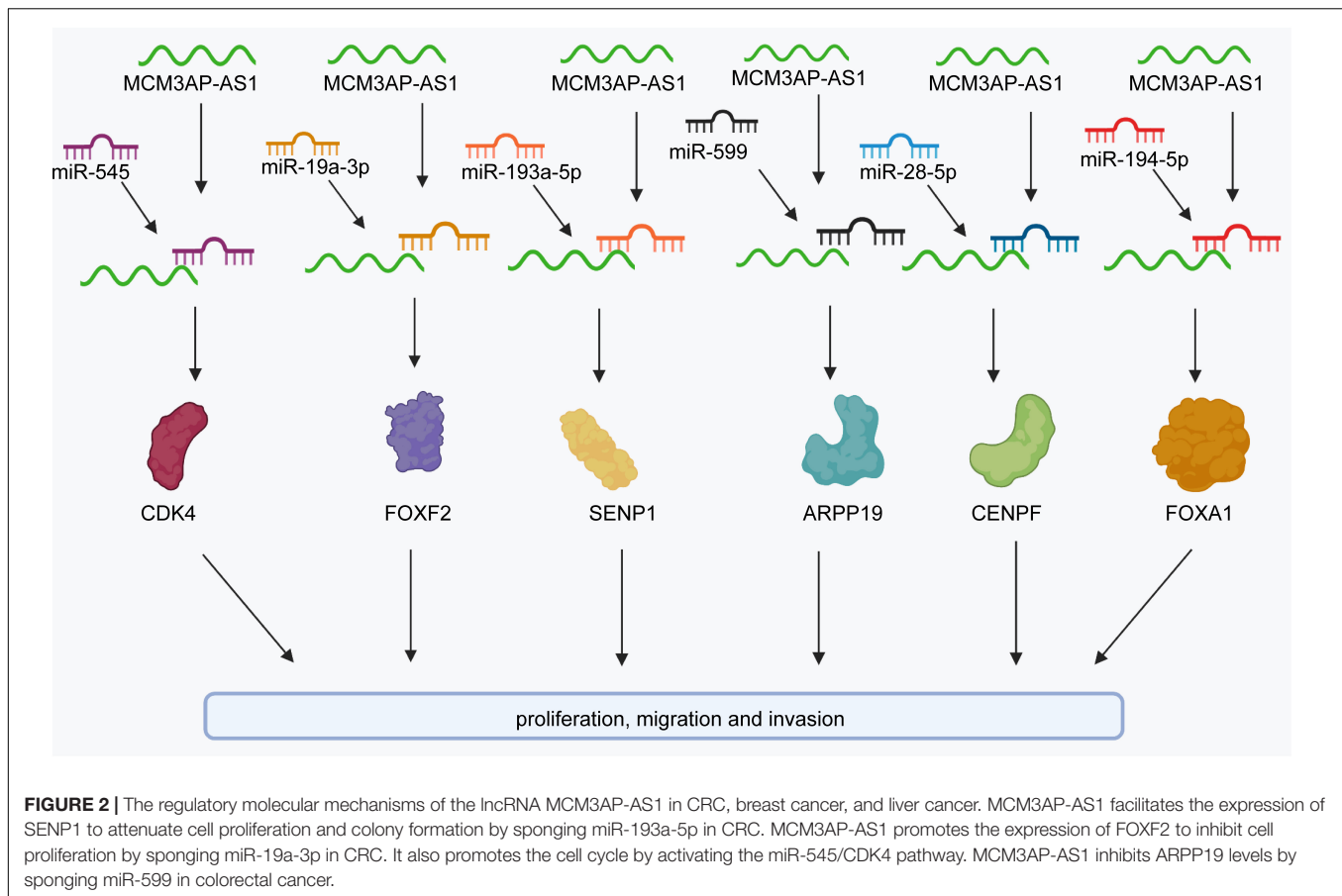
In contrast to the above findings, MCM3AP-AS1 expression is markedly downregulated in CSCC tissue samples and predicts a poor outcome. Overexpression of MCM3AP-AS1 reduces CSCC cell proliferation, and MCM3AP-AS1 acts as a tumor suppressor during CSCC development and progression.

In conclusion, MCM3AP-AS1 expression was markedly elevated in breast cancer (Chen et al., 2020; Riahi et al., 2021), Burkitt lymphoma (Guo et al., 2020), gastric cancer (Wang et al., 2020; Sun et al., 2021), glioblastoma (Yang et al., 2017), HCC (Wang et al., 2019e), LC (Li et al., 2020b), nasopharyngeal carcinoma (Sun et al., 2020), pancreatic cancer (Yang et al., 2019), papillary thyroid cancer (Liang et al., 2019), and PCA (Li et al., 2020a; Wu et al., 2020). However, MCM3AP-AS1

expression was downregulated in CSCC patients (Lan et al., 2020) (Table 1). Collectively, MCM3AP-AS1 has the potential to act as a prognostic biomarker for many cancers.

## REGULATORY MOLECULAR MECHANISMS OF MCM3AP-AS1 IN HUMAN CANCER

From the aforementioned studies, we can conclude that MCM3AP-AS1 plays an important role in the regulation of various biological functions, such as cell growth, motility, cell cycle, drug resistance, and angiogenesis. In this section,



we summarized the regulatory molecular mechanisms of MCM3AP-AS1 in cancer, both *in vivo* and *in vitro*.

## In vitro Cell Experiment

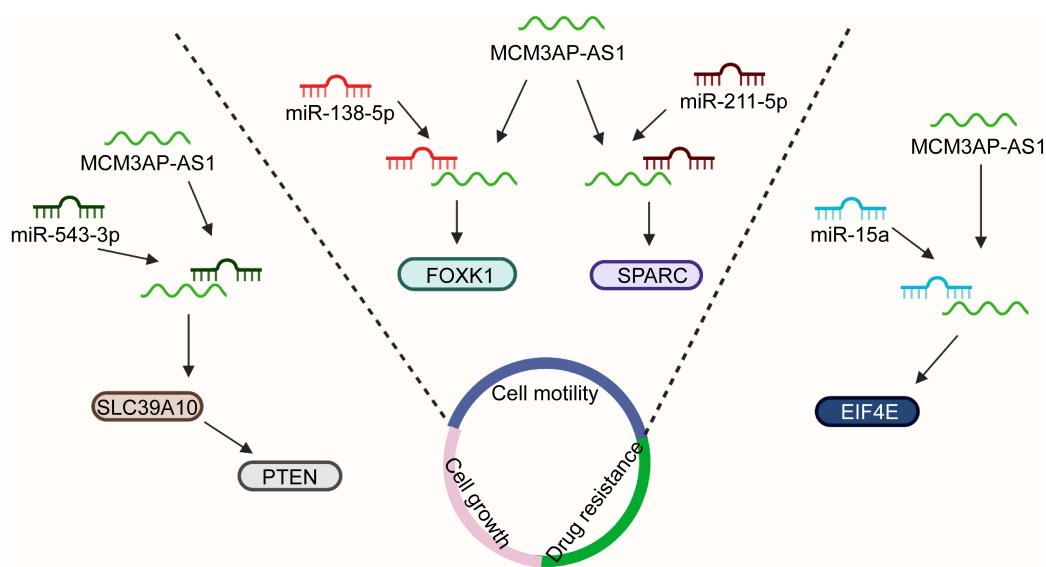
### Cell Growth

MCM3AP-AS1 facilitates cell proliferation by reducing miR-708-5p levels in breast cancer cells (Riahi et al., 2021). MCM3AP-AS1 facilitates the expression of SENP1 to attenuate cell proliferation and colony formation by sponging miR-193a-5p in CRC cells (Zhou et al., 2021). It also inhibited gastric cancer cell proliferation and promoted apoptosis by downregulating miR-708-5p levels (Wang et al., 2020). In HCC, MCM3AP-AS1 regulates cellular processes, such as cell proliferation, cell cycle progression, and cell apoptosis by activating the miR-194-5p/FOXA1 pathway (Wang et al., 2019e). MCM3AP-AS1 enhances the proliferation of PCa cells by the miR-543-3p/SLC39A10/PTEN (Figure 3) and miR-876-5p/WNT5A pathways in PCa (Jia et al., 2020; Li et al., 2020a). Additionally, it activates the MAPK pathway to induce cell proliferation by promoting methylation of the NPY1R promoter in PCa (Wu et al., 2020). Silencing of MCM3AP-AS1 suppresses KPNA4 expression to impair cell proliferation by acting as a sponge of miR-340-5p in LC (Li et al., 2020b). MCM3AP-AS1 does not affect the levels of miR-34a, whereas elevated miR-34a expression suppresses cell proliferation by downregulating MCM3AP-AS1

expression in nasopharyngeal carcinoma (Sun et al., 2020). Silencing MCM3AP-AS1 expression inhibits cell proliferation and colony formation by regulating the miR-138-5p/FOXK1 axis in pancreatic cancer (Yang et al., 2019) and plays the same role by controlling the miR-211-5p/SPARC pathway in papillary thyroid cancer (Liang et al., 2019). In contrast, MCM3AP-AS1 markedly downregulates the expression of miR-93 and inhibited cell proliferation in CSCC (Lan et al., 2020). Some researchers have found that MCM3AP-AS1 promotes the expression of FOXF2 to enhance cell proliferation by sponging miR-19a-3p in CRC (Dai et al., 2021).

### Cell Motility

Cell motility is a physiological process that is required for embryonic development, wound healing, immune surveillance, and cancer metastasis (Tojkander et al., 2015; Swaminathan et al., 2016; Cummins et al., 2018). MCM3AP-AS1 functions as a sponge of miR-193a-5p to upregulate SENP1 expression and facilitate cell migration and invasion in CRC (Zhou et al., 2021). Overexpression of MCM3AP-AS1 enhances cell migration and invasion by regulating DNMT1/DNMT3 (A/B) methylation-mediated overexpression of NPY1R and the miR-543-3p/SLC39A10/PTEN pathway in PCa (Jia et al., 2020; Li et al., 2020a). MCM3AP-AS1 expression is mediated by YY1 and promotes the upregulation of KPNA4, which facilitated the migration of LC cells by sponging miR-340-5p (Li et al., 2020b).



**FIGURE 3 |** MCM3AP-AS1 functions by interacting with specific molecular pathways. MCM3AP-AS1 enhances cell proliferation via the miR-543-3p/SLC39A10/PTEN axis in PCa. MCM3AP-AS1 reduces the levels of miR-138-5p and increases the expression of FOXK1 to promote cell migration in pancreatic cancer. Cell invasion is enhanced by the activation of the miR-211-5p/SPARC pathway in papillary thyroid cancer. MCM3AP-AS1 regulates the sensitivity of lymphoma cells to doxorubicin by regulating the miR-15a/EIF4E pathway.

LncRNA MCM3AP-AS1 reduces the levels of miR-138-5p and increased the expression of FOXK1, promoting cell migration in pancreatic cancer (Yang et al., 2019) (**Figure 3**). Cell proliferation and invasion are enhanced by the activation of the miR-211-5p/SPARC pathway in papillary thyroid cancer (Liang et al., 2019).

### Angiogenesis and Drug Resistance

Angiogenesis, the formation of new blood vessels from existing vessels, plays a critical role in physiological and pathological conditions (Ramjiawan et al., 2017; Li et al., 2019). Aberrant angiogenesis can support the metabolism of tumors and contribute to tumor progression (Cebulla et al., 2014; Wu et al., 2014; Mao et al., 2015). The lncRNA MCM3AP-AS1 accelerated tumor angiogenesis by targeting the miR-211/KLF5/AGGF1 pathway in glioblastoma (Yang et al., 2017). The elevated expression of MCM3AP-AS1 facilitates angiogenesis by regulating the miR-340-5p/KPNA4 axis in LC (Li et al., 2020b). Resistance to chemotherapy is the main cause of chemotherapy failure in cancers (Si et al., 2019; Gao et al., 2020; Jena and Mandal, 2021). *In vitro* evidence suggests that increased MCM3AP-AS1 controls the sensitivity of lymphoma cells to doxorubicin by regulating the miR-15a/EIF4E pathway (Guo et al., 2020) (**Figure 3**). MCM3AP-AS1 reduces gastric cancer cell sensitivity to cisplatin by regulating the miR-138/FOXK1 pathway (Sun et al., 2021). The results may provide novel ideas for targeted therapy of lymphoma and gastric cancer.

### Experiments *in vivo*

The results of the *in vitro* experiments were further confirmed by experiments using animal models *in vivo*. In a nude mouse model of CRC, MCM3AP-AS1 expression was positively associated with

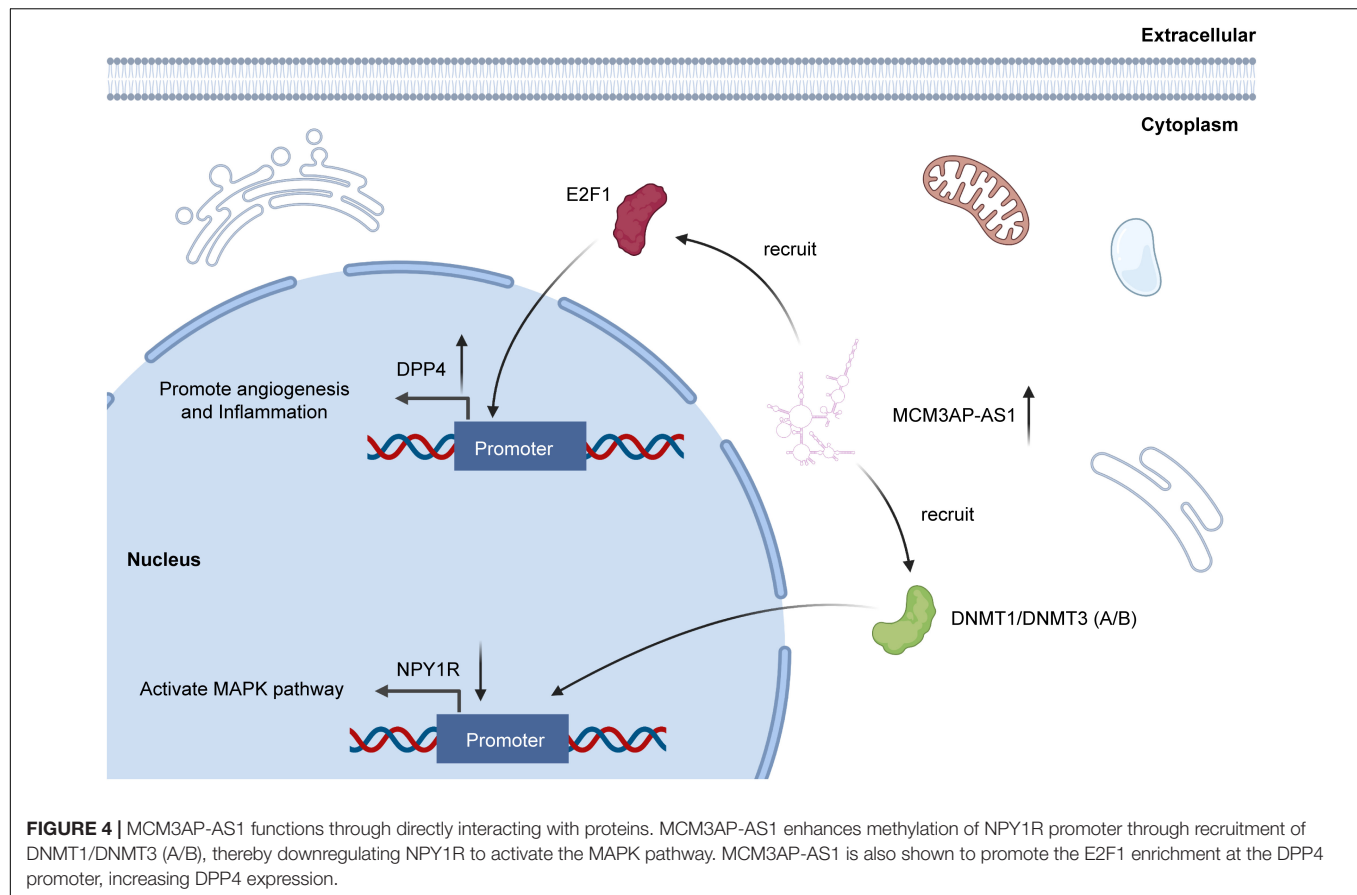
tumor growth, tumor weight, and the number of lung metastatic tumor nodules (Zhou et al., 2021). MCM3AP-AS1 promotes tumor growth by activating the miR-28-5p/CENPF pathway in breast cancer *in vivo* (Chen et al., 2020). Furthermore, MCM3AP-AS1 enhanced the levels of FOXA1 to suppress tumorigenesis by sponging miR-194-5p in an HCC xenograft model (Wang et al., 2019e). Moreover, MCM3AP-AS1 silencing decreased the volume of PCa and inhibited the expression of SLC39A10 in BALB/c mice (Jia et al., 2020). MCM3AP-AS1 also contributed to PCa progression via regulation of the MAPK/NPY1R axis *in vivo* (Li et al., 2020a). Animal experiments have demonstrated that downregulation of MCM3AP-AS1 contributes to the expression of miR-15a and PARP, whereas it inhibits the expression of Mcl-1 and EIF4E in lymphoma (Guo et al., 2020). MCM3AP-AS1 significantly promotes tumor growth by activating the miR-211-5p/SPARC pathway in papillary thyroid cancer (Liang et al., 2019).

*In vivo* and *in vitro*, MCM3AP-AS1 has various biological functions, including cell proliferation, colony formation, migration, invasion, and chemoresistance. Interestingly, the mechanisms related to MCM3AP-AS1 are all similar and involve the ceRNA network. For researchers in related fields, this idea is worthy of reference.

### MCM3AP-AS1 AS A BIOMARKER AND TREATMENT TARGET IN CANCER

Cancer prognosis monitoring is critical for reducing cancer-related deaths. Dysregulated expression patterns of MCM3AP-AS1 have great value for the diagnosis and prognosis of cancer. The levels of lncRNA MCM3AP-AS1 are negatively





associated with OS of CRC patients (Zhou et al., 2021). MCM3AP-AS1 levels are also negatively correlated with OS in PCa, papillary thyroid cancer, and nasopharyngeal carcinoma (Liang et al., 2019; Jia et al., 2020; Sun et al., 2020; Chen et al., 2021). In addition, receiver operating characteristic (ROC) analyses showed that the specificity and sensitivity values of MCM3AP-AS1 were 0.58 and 0.76, respectively, in breast cancer patients (Riahi et al., 2021). Therefore, MCM3AP-AS1 can be regarded as a potential diagnosis and prognosis biomarker in multiple cancers.

Treatment of cancer using molecular-targeted therapy is a promising strategy. MCM3AP-AS1 is a novel molecular target for cancer therapy. MCM3AP-AS1 regulates cancer progression through a series of pathways, such as miR-194-5p/FOXA1 (Wang et al., 2019e), miR-138-5p/FOXK1 (Yang et al., 2019), miR-211-5p/SPARC (Liang et al., 2019), and miR-15a/EIF4E (Guo et al., 2020). And the miR-194-5p/FOXA1 axis is further confirmed *in vivo* experiments through constructing HCC xenograft model. MCM3AP-AS1 knockdown inhibits cell proliferation and colony formation in CRC (Yu Y. et al., 2021). Knockdown of MCM3AP-AS1 suppresses cell proliferation, migration, and invasion and promotes apoptosis in PCa cells and decreases the volume of PCa in BALB/c mice (Jia et al., 2020; Li et al., 2020a; Wu et al., 2020). MCM3AP-AS1 knockdown increases gastric cancer cell sensitivity to cisplatin (Sun et al., 2021). Silencing miR-708-5p attenuates the inhibition of cell

proliferation caused by MCM3AP-AS1 in gastric cancer (Wang et al., 2020). The upregulation of NPY1R inhibits the function of MCM3AP-AS1 by inactivating the MAPK pathway in PCa (Li et al., 2020a).

## CONCLUSION AND FUTURE PERSPECTIVES

MCM3AP-AS1 is aberrantly expressed in human cancers, such as breast cancer, CRC, gastric cancer, HCC, and PCa. Its expression is significantly associated with several clinical characteristics. The levels of MCM3AP-AS1 are significantly associated with tumor size, TNM stage, pathological grade, and prognosis in different cancers. Additionally, it markedly promotes or suppresses tumor progression by controlling the biological functions of cells. For example, MCM3AP-AS1 upregulation promotes cell proliferation, colony formation, migration, and invasion and arrests the cell cycle at the G1 phase in CRC. MCM3AP-AS1 also plays an important role by interacting with specific molecules through a ceRNA mechanism. MCM3AP-AS1 facilitates proliferation by regulating the miR-193a-5p/SENPI, miR-543-3p/SLC39A10/PTEN, and miR-876-5p/WNT5A pathways.

Beyond participating in ceRNA network, lncRNA can also interact with protein directly (Ferre et al., 2016). And RNA

binding protein immunoprecipitation (RIP) and RNA pull-down technology could verify the interactions between lncRNAs and protein (Bierhoff, 2018). As for MCM3AP-AS1, recent researches have revealed that MCM3AP-AS1 can directly interact with special proteins and further effect the biological functions of several cancers. Qiu et al. demonstrated that MCM3AP-AS1 could interact with E2F1 and enhance the enrichment of E2F1 at the DPP4 promoter, increasing the expression of DPP4. As a result, MCM3AP-AS1 promoted angiogenesis and inflammation in clear cell renal cell carcinoma (Qiu et al., 2020). Another study also revealed that MCM3AP-AS1 can recruit DNMT1/DNMT3 (A/B) to induce methylation of NPY1R promoter. In this way, MCM3AP-AS1 decreased NPY1R expression (Li et al., 2020a) (**Figure 4**). The interaction between lncRNA and protein is recently proposed molecular mechanism of lncRNA. However, for MCM3AP-AS1, researches related with this mechanism are still limited. Therefore, to explore the interaction between MCM3AP-AS1 and protein is an indispensable idea for future researchers.

Exosome is a kind of extracellular vesicles secreted by cells. Exosome exerts biological functions through transporting DNA, RNA, protein, and liquid among cells (Zhang Y. et al., 2020). And these components of exosomes could play a role in receptor cells to accomplish intercellular communication (Sun et al., 2018). Recent studies revealed that lncRNA could act as component of exosome and participate in the initiation and progression of cancers. Lang et al. demonstrated that gliomas could secrete exosomes containing lncRNA POU3F3 to promote the angiogenesis (Lang et al., 2017). Similar phenomenon was observed in breast cancer. lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was upregulated in BC, and MALAT1 was transported by exosomes to accelerate BC cell proliferation (Zhang et al., 2018). More importantly, as long as released from cells, exosomes could enter circulatory system and be isolated from available body fluid for detection (Dong et al., 2019). Thus, it is probably a crucial research direction to further explore the roles of exosome lncRNA in human tumors.

Moreover, MCM3AP-AS1 is a promising biomarker for cancer diagnosis, prognosis evaluation, and treatment. However, there is a need for additional basic and clinical experimental results before these findings can be applied in the clinic. The process of drug development is difficult and challenging. At present, RNA drugs for some diseases have been successfully listed (Crooke et al., 2018). The types of RNA drugs considered feasible include oligonucleotides, mRNA, and RNA related small molecules. Considering the drug targeting and toxicity, oligonucleotide is a promising strategy and avenue for implementing gene therapy. The advantages of oligonucleotide lie in the convenience and efficient design. Delivering oligonucleotides directly in saline solution may maintain toxicity at a low level. Chemical modifications are also feasible methods to control toxicity and reduce off-target effects. Thus, oligonucleotides are a potential strategy for drug research and development (Roberts et al., 2020). Further drug research and development can refer to our point of view.

## AUTHOR CONTRIBUTIONS

WG, YH, and SZ designed the review. XY, QZe, and QZa wrote this manuscript. XY searched the articles and made figures. All authors worked collaboratively on the work presented here, read, and approved the final manuscript.

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# CircITCH: A Circular RNA With Eminent Roles in the Carcinogenesis

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Circular RNAs (circRNAs) are a group of long non-coding RNAs with enclosed structure generated by back-splicing events. Numerous members of these transcripts have been shown to affect carcinogenesis. Circular RNA itchy E3 ubiquitin protein ligase (circITCH) is a circRNA created from back splicing events in *ITCH* gene, a protein coding gene on 20q11.22 region. *ITCH* has a role as a catalyzer for ubiquitination through both proteolytic and non-proteolytic routes. CircITCH is involved in the pathetiology of cancers through regulation of the linear isoform as well as serving as sponge for several microRNAs, namely miR-17, miR-224, miR-214, miR-93-5p, miR-22, miR-7, miR-106a, miR-10a, miR-145, miR-421, miR-224-5p, miR-197 and miR-199a-5p. CircITCH is also involved in the modulation of Wnt/ $\beta$ -catenin and PTEN/PI3K/AKT pathways. Except from a single study in osteosarcoma, circITCH has been found to exert tumor suppressor role in diverse cancers. In the present manuscript, we provided a comprehensive review of investigations that reported function of circITCH in the carcinogenesis.

**Keywords:** circular RNA, circITCH, cancer, expression, ncRNAs

## INTRODUCTION

Circular RNAs (circRNAs) are a group of long non-coding RNAs with enclosed structure. This structure is created through establishment of a covalent bond between 5' and 3' termini through a back-splicing event in exons of a certain pre-mRNA (1). Several studies have indicated broad expression of circRNAs in mammalian cells in a cell type- or tissue-specific manner (1). CircRNAs have been shown to affect different cellular and biological processes, namely cell proliferation (1), differentiation, pluripotency (2) and epithelial-mesenchymal transition (EMT) (3). Moreover, they can participate in the remodeling of endoplasmic reticulum stress, autophagy and phagocytosis, DNA repair mechanisms as well as drug efflux (4). Different mechanisms have been suggested for circRNAs effects in these processes with the most appreciated one being their function as decoys for microRNAs (miRNAs) or RNA-binding proteins. Through this mechanism, circRNAs can influence expression of genes or translation of proteins with regulatory functions (1). CircRNA have the ability to base pair with other types of RNAs as well (5). Moreover, circRNAs can suppress activity of certain proteins, particularly cell cycle proteins through interacting with them (6). While circRNAs are mainly considered as non-coding RNAs, they might be served as a template for production of proteins under some conditions (5). Cumulatively, circRNAs can influence

expression of cellular proteins, interfere with RNA-binding proteins to affect transcription of genes, regulate gene transcription *in cis*, and modulate splicing events (5). Yet, the competing endogenous function of circRNAs is the chief way through which they exert their biological effects (5). Several studies have emphasized on the role of circRNAs in cancer development and induction of chemo/radioresistance (4, 5).

Circular RNA *ITCH* E3 ubiquitin protein ligase (circITCH) is an example of cancer-related circRNAs which can be used as target for therapeutic interventions. It is created from back splicing events in *ITCH* gene, a protein coding gene on 20q11.22 region. *ITCH* has a role as a catalyzer for ubiquitination through both proteolytic and non-proteolytic routes (7). It has been shown to affect tumorigenesis in a context-dependent manner (7). Recent studies have shown involvement of the circRNA from this locus in the carcinogenesis process. In the present manuscript, we provided a comprehensive review of investigations that reported function of circITCH in this process. The evidence regarding the role of circITCH in cancers is classified based on the samples/models used in the original papers to *in vitro*, *in vivo* and clinical studies.

## CELL LINE STUDIES

### Bladder Cancer

CircITCH has been found to be down-regulated in bladder cancer cell lines. Forced over-expression of circITCH could inhibit proliferation, migratory potential, invasive properties and metastatic ability of bladder cancer cells. Functionally, circITCH acts as a sponge for miR-17 and miR-224 to up-regulate expression of their target genes p21 and PTEN. Cumulatively, circITCH functions as a tumor suppressor circRNA in bladder cancer (8).

### Breast Cancer

Expression of circITCH has also been shown to be decreased in triple negative breast cancer cell lines. Stable transfection of MDA-MB-231 and BT-549 cells with circITCH-expressing vectors has resulted in inhibition of proliferation, invasiveness and metastatic ability of these cells. Mechanistically, circITCH serves as a molecular sponge for miR-214 and miR-17 leading to enhancement of expression of the linear form of *ITCH*. This circRNA functionally inactivates Wnt/ $\beta$ -catenin signaling (9).

### Cervical Cancer

Expression of circITCH has also been shown to be down-regulated in cervical cancer cell lines. Up-regulation of circITCH in cervical cancer cells has inhibited their proliferation, migration, and invasiveness. Mechanistically, circITCH acts a sponge for miR-93-5p to regulate expression of FOXK2 (10).

### Osteosarcoma

Down-regulation of circITCH has also been verified in osteosarcoma cells. Overexpression of circITCH has

induced cell apoptosis and decreased cell viability, proliferation, migratory potential and invasive properties of MG63 and Saos-2 osteosarcoma cells. This circRNA could decrease expression of miR-22 in osteosarcoma cells, thus suppressing PTEN/PI3K/AKT and SP-1 signals (11). On the other hand, another study in the hFOB1.19 osteoblast cell line and multiple osteosarcoma cell lines has shown up-regulation of circITCH in neoplastic cells compared with the osteoblast cells. Functionally, circITCH enhanced migration, invasive properties, and growth of these neoplastic cells through sponging miR-7 and increasing expression of EGFR (12).

**Figure 1** shows the tumor suppressor role of circITCH in bladder, breast and cervical cancers as well as dual role of this circRNA in osteosarcoma.

### Thyroid Cancer

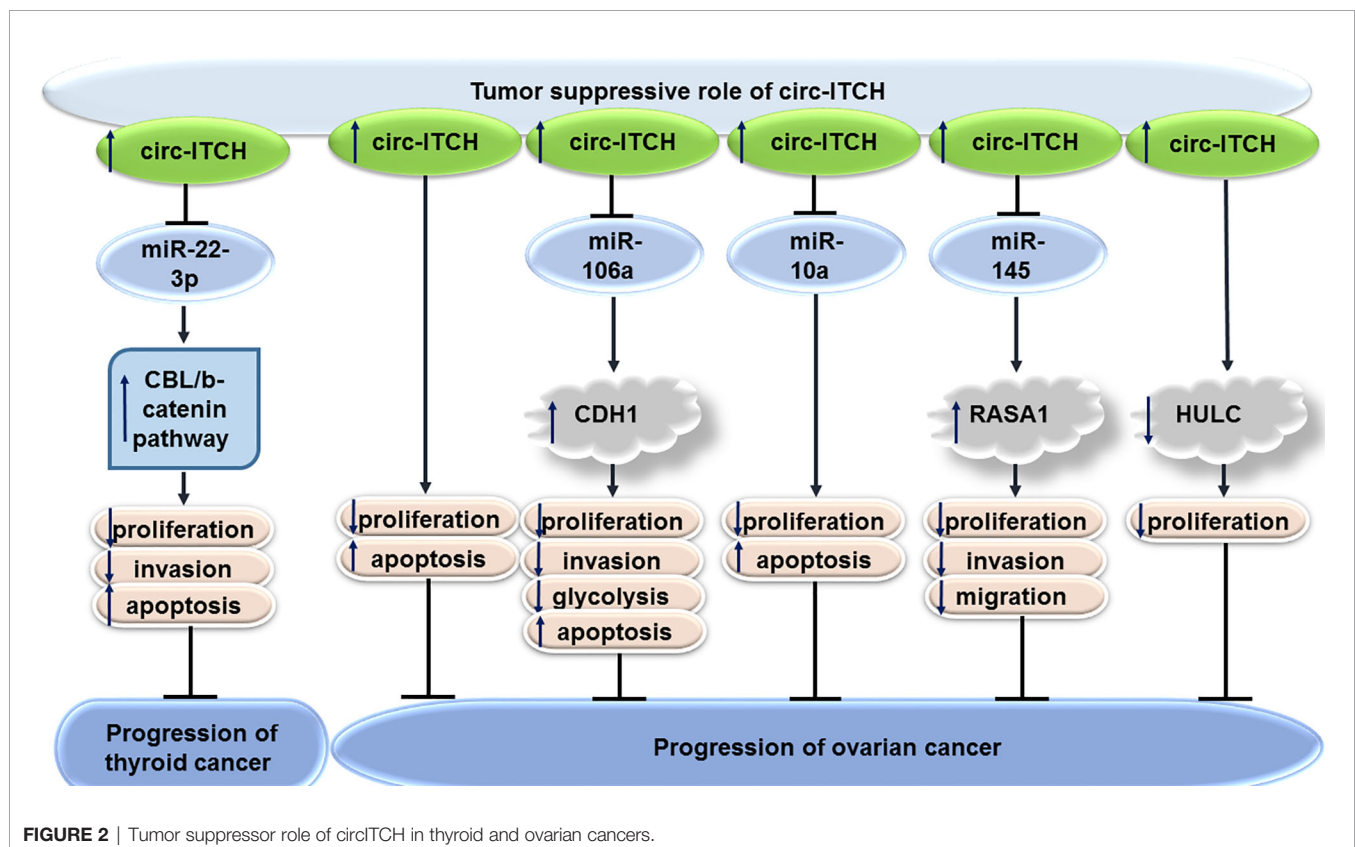
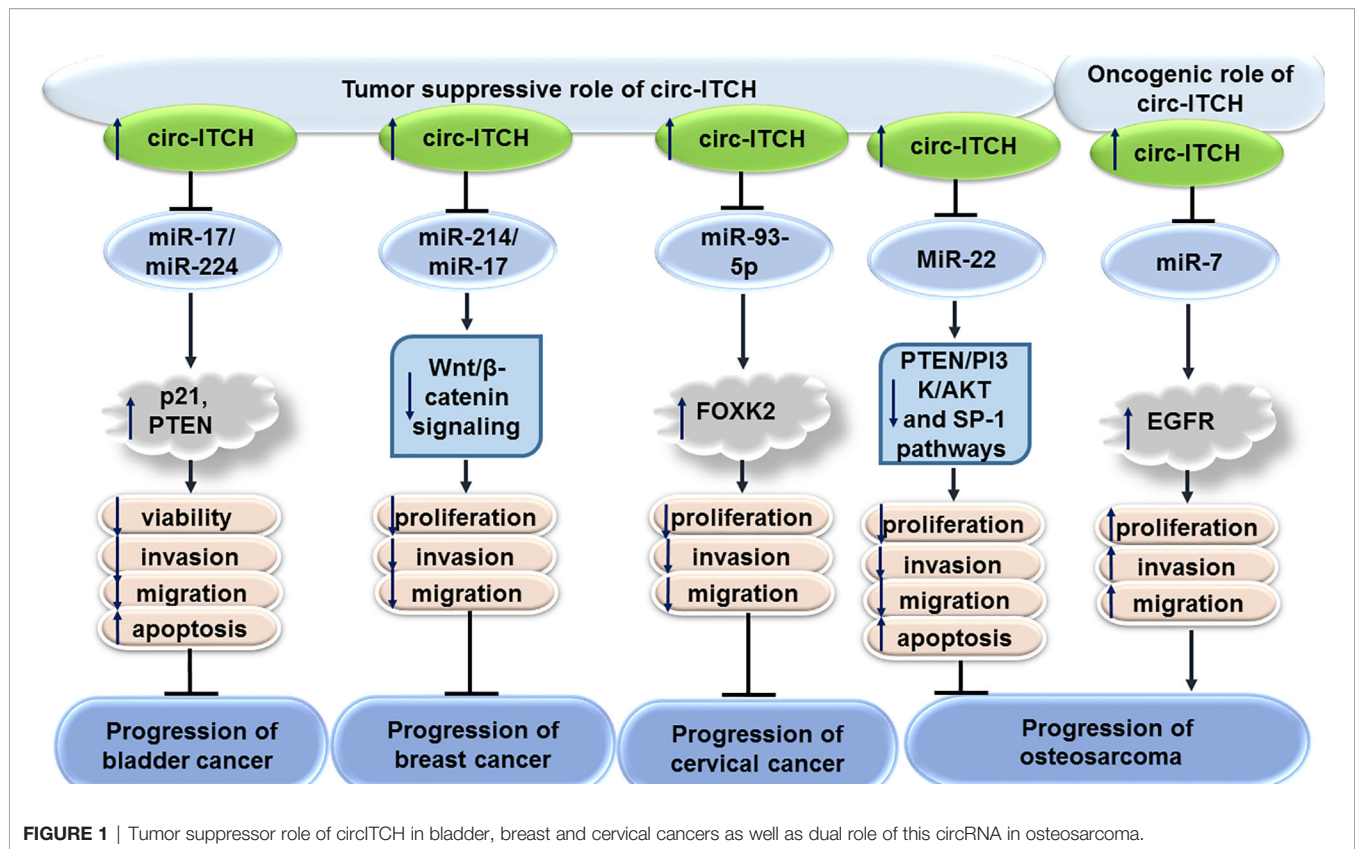
In thyroid cancer cells, forced over-expression of circITCH inhibits cell proliferation and invasive properties, while promoting cell apoptosis. These effects are mediated through sponging miR-22-3p and subsequent up-regulation of levels of CBL, an E3 ligase of nuclear  $\beta$ -catenin. Cumulatively, circITCH affects activity of the Wnt/ $\beta$ -catenin pathway through modulation of CBL levels, therefore suppressing progression of thyroid cancer (13).

### Ovarian Cancer

Expression of circITCH expression has been found to be down-regulated in numerous epithelial ovarian cancer cell lines versus normal ovarian epithelial cells. This circRNA could inhibit proliferation of SKOV3 and OVCAR-3 cells, while enhancing their apoptosis (14). Another study has shown the role of circITCH in suppression of proliferation, invasiveness, and glycolytic process in ovarian cancer cells through sequestering miR-106a and enhancing expression of CDH1 (15). miR-10a-alpha has also been identified as a target of circITCH in ovarian cancer cells through which circITCH exerts its tumor suppressor effects (16). Moreover, circITCH has been shown to suppress progression of this cancer *via* influencing miR-145/RASA1 axis (17). Finally, circITCH has been suggested to suppress proliferation of ovarian cells through decreasing expression of HULC (18). **Figure 2** shows the tumor suppressor role of circITCH in thyroid and ovarian cancers.

### Hepatocellular Carcinoma

CircITCH has also been shown to have tumor suppressor roles in hepatocellular carcinoma. In fact, the effects of lidocaine on inhibition of proliferation of hepatocellular carcinoma cells have been shown to be mediated through restoration of circITCH in these cells. Mechanistically, circITCH modulates expression of CPEB3 through sponging miR-421 (19). CircITCH can also affect progression of hepatocellular carcinoma through sponging miR-224-5p and increasing expression of MafF (20). CircRNA*ITCH* levels have been found to down-regulated in several hepatocellular cancer cell lines compared with normal hepatic L-02 cell line. Up-regulation of circRNA*ITCH* has





inhibited proliferation of these cells, suppressed their colony formation aptitude and induced their apoptosis. CircRNAITCH could be used as a sponge for miR-7 and miR-214. Through this route, it regulates Wnt/ $\beta$ -catenin signals and suppresses c-myc and cyclin D1 levels (21).

## Glioma

CircITCH has also been shown to inhibit proliferation and invasive potential of glioma cells *via* sequestering miR-106a-5p and enhancing expression of SASH1 (22). Moreover, it has been reported to serve as a sponge for miR-214 and promote expression of linear ITCH in glioma cells (23).

## Oral Squamous Cell Carcinoma

The miR-421/PDCD4 axis has been shown as the downstream axis mediating the role of circITCH in modulation of progression of oral squamous cell carcinoma by regulating (24).

**Figure 3** shows the tumor suppressor role of circITCH in hepatocellular carcinoma, glioma and oral squamous cell carcinoma.

## Prostate Cancer

CircITCH exerts tumor suppressor roles in prostate cancer *via* influencing miR-17-5p/HOXB13 axis (25). Moreover, circITCH can inhibit proliferation, migratory aptitude, and invasiveness of human prostate cancer cells through sequestering miR-17. This circRNA can also down-regulate expression levels of several

proteins in the Wnt/ $\beta$ -catenin and PI3K/AKT/mTOR signal transductions in LNCaP and PC-3 cells, as representatives of androgen receptor (AR)-positive and AR-negative cells, respectively (26). miR-197 is another target of circITCH in prostate cancer cells through which it regulates progression of this type of cancer (27).

## Gastric Cancer

In addition, circITCH can suppress gastric carcinogenesis through modulation of miR-199a-5p/Klotho axis (28) as well as the Wnt/ $\beta$ -catenin pathway (29).

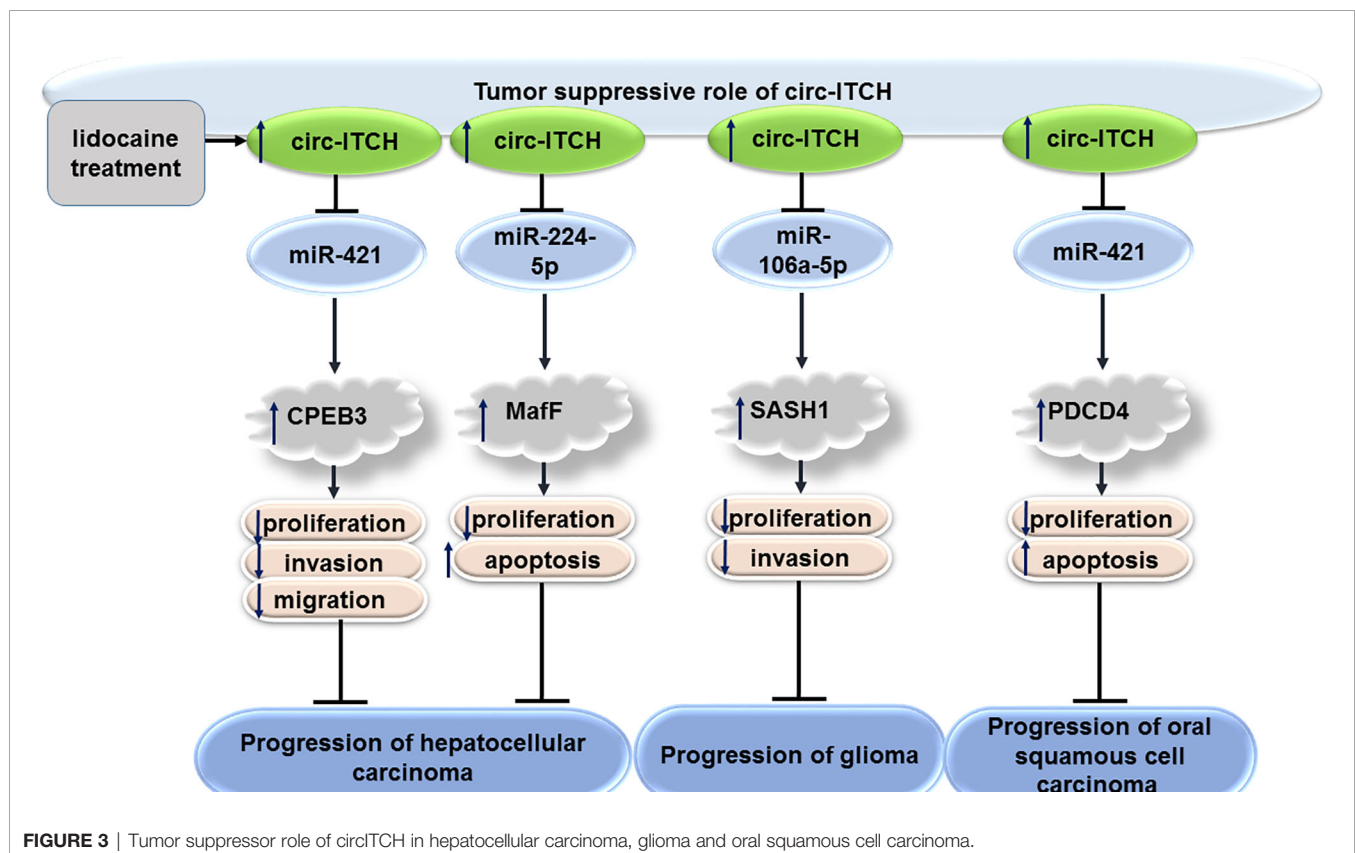
## Melanoma

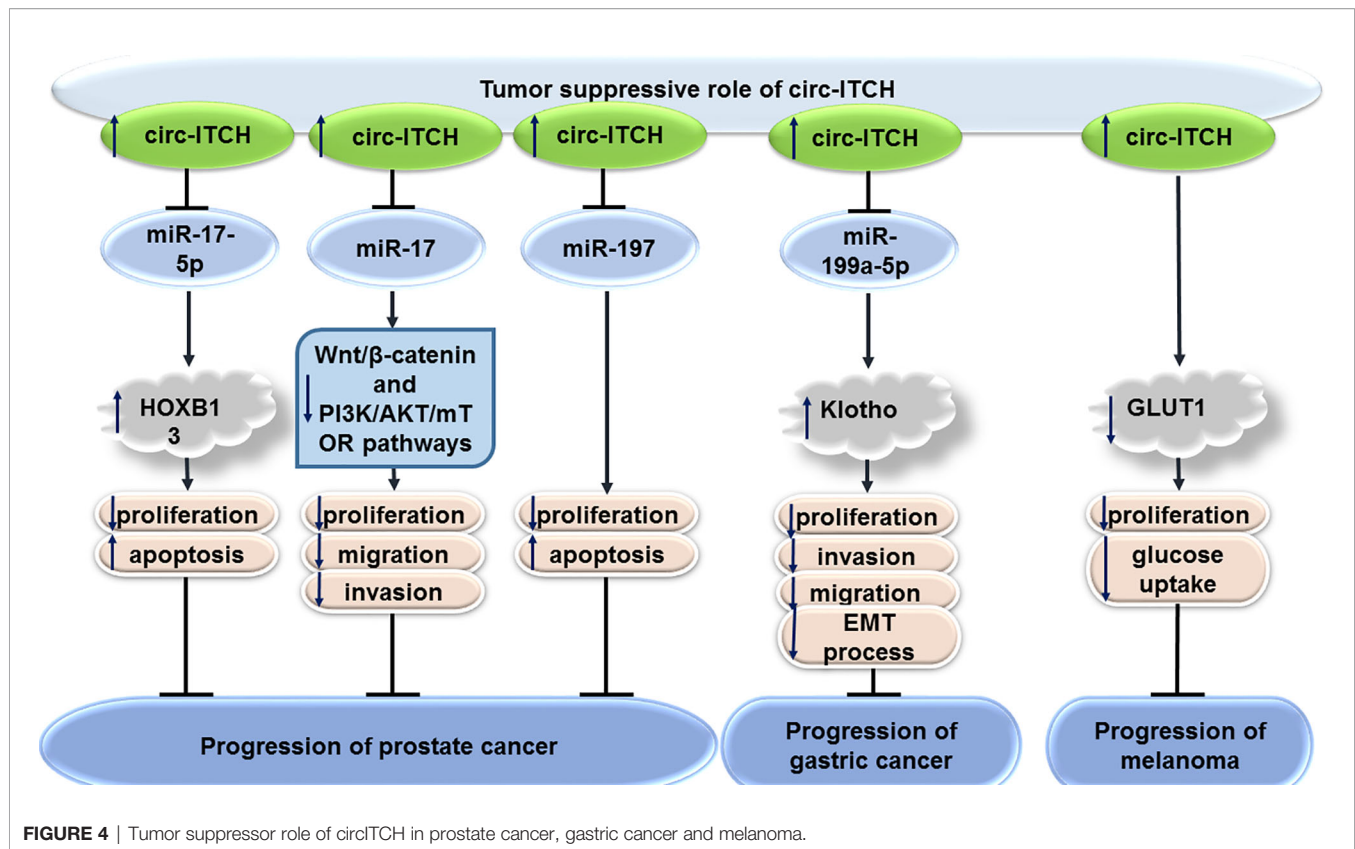
Finally, circITCH decreases expression of GLUT1 and inhibits uptake of glucose by melanoma cells to suppress their proliferation (30).

**Figure 4** shows the tumor suppressor role of circITCH in prostate cancer, gastric cancer and melanoma.

## Other Cancers

miR-7 and miR-214 have been found to be sequestered by circITCH in lung (31) and esophageal cancers (32). In addition to mentioned cancer types, circITCH has tumor suppressor roles in renal cancer (33), multiple myeloma (34) and colorectal cancer (35). **Table 1** summarizes expression and function of circITCH in cancer cell lines





## ANIMAL STUDIES

Subcutaneous injection of T24 bladder cancer cells transfected with circITCH into the nude mice has shown the impact of this circRNA in reduction of tumor volumes and tumor weight. Notably, expressions of p21 and PTEN have been up-regulated in the tumors originated from circITCH overexpressing cells (8). Other *in vivo* studies have consistently verified the tumor suppressor roles of circITCH in different animal models (Table 2). Similarly, over-expression of circITCH has increased sensitivity of bortezomib-resistant multiple myeloma cells to this drug in animal models (34).

## CLINICAL STUDIES

Different studies in samples obtained from patients with diverse types of neoplasms have verified down-regulation of circITCH in neoplastic samples when compared with normal (non-affected) tissues (Table 3). Down-regulation of circITCH in bladder cancer tissues has been correlated with histological grade. In addition, bladder cancer patients who had circITCH down-regulation exhibited poor clinical outcome (8).

Expression of circITCH has also been reported to be lower in ovarian tumor tissues compared with corresponding non-tumoral tissues. Most notably, expression of circITCH has been inversely

correlated with tumor size and FIGO stage in these patients. Based on multivariate Cox analyses, over-expression of circITCH has been identified as an independent predictor of favorable overall survival of patients with ovarian cancer (14).

Cumulatively, decreased levels of circITCH have been correlated with poor outcome in diverse types of cancers, suggesting this circRNA as a prognostic factor in human malignancies.

## DISCUSSION

Except from a single study in osteosarcoma, circITCH has been found to exert tumor suppressor role in diverse cancers. This circRNA is involved in the pathetiology of cancers through regulation of the linear isoform as well as serving as sponge for several microRNAs, namely miR-17, miR-224, miR-214, miR-93-5p, miR-22, miR-7, miR-106a, miR-10a, miR-145, miR-421, miR-224-5p, miR-197 and miR-199a-5p. CircITCH also partakes in the modulation of Wnt/ $\beta$ -catenin and PTEN/PI3K/AKT pathways.

A number of miRNAs have been found to interact with circITCH in diverse tissues. For instance, miR-7 has been found to be sponged by circITCH in osteosarcoma, hepatocellular carcinoma, lung cancer and esophageal squamous cell carcinoma. Meanwhile, miR-17 has been detected as a target of this circRNA in bladder, breast, prostate, gastric and esophageal squamous cell cancers. Moreover, circITCH has been shown to sponge miR-214 in breast,

**TABLE 1 |** Expression and function of circITCH in cancer cell lines ( $\Delta$ : knock-down or deletion, BTZ: Bortezomib).

Tumor type	Targets/Regulators and Signaling Pathways	Cell line	Function	Reference
Bladder cancer	miR-17, miR-224, p21, PTEN	EJ, T24	$\uparrow$ circITCH: $\downarrow$ viability, $\downarrow$ migration, $\downarrow$ invasion, $\uparrow$ G1/S cell cycle arrest, $\uparrow$ apoptosis	(8)
Breast cancer	miR-214, miR-17, Wnt/ $\beta$ -catenin signaling	MCF-10A, MCF-7, T47D, SK-BR-3, MDA-MB-231, BT-549	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(9)
Cervical cancer	miR-93-5p, FOXK2	HeLa	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(10)
Osteosarcoma	miR-22, PTEN/PI3K/AKT and SP-1 pathways	MG63, U2OS, Saos-2, hFOB1.19	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion, $\uparrow$ apoptosis	(11)
Thyroid cancer	miR-7, EGFR	SJSA-1, U2OS, hFOB1.19	$\uparrow$ circITCH: $\uparrow$ proliferation, $\uparrow$ migration, $\uparrow$ invasion	(12)
	miR-22-3p, CBL/b-catenin pathway	K1, IHH4, TPC1	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ invasion, $\uparrow$ apoptosis	(13)
Ovarian cancer	–	SKOV3, OVCAR-3	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis	(14)
	miR-106a, CDH1	A2780 and OVCAR3, ISOE80	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ invasion, $\downarrow$ glycolysis, $\uparrow$ apoptosis	(15)
	miR-10a	SKOV3, A-2780, OVCAR-3, HO-8910, IOSE80	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis	(16)
	miR-145, RASA1	SK-OV-3, Caov-3	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(17)
	HULC	UWB1.289 + BRCA1 and UWB1.289	$\uparrow$ circITCH: $\downarrow$ proliferation	(18)
Hepatocellular carcinoma	miR-421, CPEB3	Huh7, Hep3B, THLE-2	$\Delta$ circITCH: $\downarrow$ suppressive effect of lidocaine on hepatocellular carcinoma development lidocaine treatment: $\uparrow$ circ-ITCH, $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(19)
Glioma	miR-224-5p, MafF	SMMC7721, Huh7, Hep3B	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis, $\uparrow$ MafF levels	(20)
	miR-7, miR-214, Wnt/ $\beta$ -catenin signaling	HCC Huh-7, U251, HB611, SMMC-7721, L-02	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ colony formation ability, $\uparrow$ apoptosis	(21)
	miR-106a-5p, SASH1	U251, U87, SHG44, A172, HEB	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ invasion	(22)
	miR-214, linear ITCH, Wnt/ $\beta$ -catenin pathway	U87, U251, A172, SHG44, LN229, T98G, SHG139, M059K	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion, $\downarrow$ EMT process, $\uparrow$ apoptosis	(23)
Oral squamous cell carcinoma	miR-421, PDCD4	HOK, SCC6, SCC9, SCC25, HN4, HN6	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis	(24)
Prostate cancer	miR-17-5p, HOXB13	C4-2, LNCaP, DU145, 22Rv1, PC-3 and VcaP, RWPE-1	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis	(25)
	miR-17, Wnt/ $\beta$ -catenin and PI3K/AKT/mTOR pathways	RWPE-1, LNCaP, PC-3	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(26)
	miR-197	U 145, 22RV1, VCaP, PC-3, RWPE	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis	(27)
Gastric cancer	miR-199a-5p, Klotho	HGC-27, AGS, MKN-45, MGC-803 and HEK-293 T, GES-1	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion, $\downarrow$ EMT process, effect on anticancer chemotherapy	(28)
	miR-17, ITCH, Wnt/ $\beta$ -catenin pathway	GES-1, AGS, MKN45	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ migration, $\downarrow$ invasion	(29)
Melanoma	GLUT1	A375, M21	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ glucose uptake	(30)
	miR-520f	A375, WM35	$\Delta$ circITCH: $\uparrow$ proliferation, $\uparrow$ colony-forming ability	(36)
Lung cancer	miR-7 and miR-214, linear ITCH, Wnt/ $\beta$ -catenin signaling	A549, NIC-H460	$\uparrow$ circITCH: $\downarrow$ proliferation	(31)
Esophageal squamous cell carcinoma	miR-7, miR-17, miR-214, ITCH, Wnt/ $\beta$ -catenin pathway	Eca-109, TE-1	$\uparrow$ circITCH: $\downarrow$ proliferation, $\downarrow$ colony-forming ability	(32)
Clear cell renal cell carcinoma	miR-106b-5p, PDCD4	HK-2, OSRC-2, A498, SW839, 786-O, Caki-1, GRC-1	$\uparrow$ circITCH: $\downarrow$ metastasis, $\downarrow$ migration, $\downarrow$ invasion	(33)
Multiple myeloma	miR-615-3p, PRKCD	U-266, NCI-H929, RPMI 8226, NCI-H929, RPMI 8226	$\uparrow$ circITCH: $\downarrow$ proliferation, $\uparrow$ apoptosis, $\uparrow$ sensitivity to BTZ	(34)
Colorectal cancer	Linear ITCH, Wnt/ $\beta$ -catenin pathway	HCT116, SW480	$\uparrow$ circITCH: $\downarrow$ proliferation	(35)

lung, hepatocellular carcinoma, glioma and esophageal cancers. Thus, circITCH/miR-7, circITCH/miR-17 and circITCH/miR-214 axes are appropriate therapeutic targets for diverse types of cancers.

The correlation between expression levels of circITCH and clinicopathological data such as tumor size, local invasion, distant metastasis and different staging systems shows the importance of this circRNA in the development or progression

of cancers, representing a novel biomarker role for it. Although the impact of circITCH in determination of prognosis of cancer patients is well established, its function as a diagnostic marker is not studied. Since circRNAs are stable transcripts in the circulation, they are expected to reflect cancer course. Thus, future investigations should focus on evaluation of levels of circITCH in plasma of patients with different stages of cancers

**TABLE 2 |** Summary of studies which assessed impact of circITCH up-regulation or silencing in animal models ( $\Delta$ : knock-down or deletion, BTZ: Bortezomib).

Tumor Type	Animal models	Results	Reference
Bladder cancer	female athymic BALB/C nude mice	$\uparrow$ circITCH: $\downarrow$ tumor volume, $\downarrow$ tumor weight	(8)
Breast cancer	female BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor volume, $\downarrow$ number of lung nodules	(9)
Cervical cancer	nude mice	$\uparrow$ circITCH: $\downarrow$ tumorigenesis	(10)
Thyroid cancer	female BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor size, $\downarrow$ tumor weight	(13)
Ovarian cancer	BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor volume, $\downarrow$ tumor weight	(15)
	female BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor volume, $\downarrow$ tumor weight	(17)
Hepatocellular carcinoma	male BALB/c nude mice	$\Delta$ circITCH: $\uparrow$ tumor volume, $\uparrow$ tumor weight under lidocaine treatment condition	(19)
Glioma	BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor growth, $\downarrow$ tumor weight	(22)
Prostate cancer	female BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor growth, $\downarrow$ tumor volume	(25)
Gastric cancer	male athymic nude mice	$\uparrow$ circITCH: $\downarrow$ tumor growth	(29)
Esophageal squamous cell carcinoma	female BALB/c nude mice	$\uparrow$ circITCH: $\downarrow$ tumor growth	(32)
Clear cell renal cell carcinoma	male BALB/c mice	$\uparrow$ circITCH: $\downarrow$ tumor volume, $\downarrow$ tumor weight	(33)
Multiple myeloma	BALB/c nude mice	$\uparrow$ circITCH + BTZ treatment: $\downarrow$ tumor volume	(34)

**TABLE 3 |** Results of studies that reported dysregulation of circITCH in clinical samples (ANCTs, adjacent non-cancerous tissues; OS, Overall survival; FIGO, International Federation of Gynecology and Obstetrics; DFS, disease-free survival; TNM, tumor-node-metastasis).

Tumor type	samples	Expression (Tumor vs. Normal)	Kaplan-Meier analysis (impact of circITCH down-regulation)	Univariate/Multivariate cox regression	Association of down-regulation of circITCH with clinicopathologic characteristics	Reference
Bladder cancer	72 pairs of tumor tissues and ANCTs	down	shorter OS	—	histological grade	(8)
Breast cancer	275 tumor tissues and 68 ANCTs	down	shorter OS	—	lymph node metastasis, larger tumor size and advanced TNM stage	(9)
Osteosarcoma	22 pairs of osteosarcoma tissues and para-osteosarcoma tissues	down	—	—	—	(11)
Thyroid cancer	37 tumor tissues and 14 ANCTs	down	—	—	clinical stage and lymph node metastasis	(13)
Ovarian cancer	77 tumor tissues and ANCTs	down	shorter OS	CircITCH was found to be an independent predictive factor for favorable OS.	Larger tumor size, increased FIGO stage	(14)
	45 pairs of tumor tissues and ANCTs	down	shorter 5-year OS	—	Larger tumor size, increased FIGO stage	(15)
	20 pairs of tumor tissues and ANCTs	down	shorter OS	—	—	(17)
	75 pairs of tumor tissues and ANCTs	down	—	—	—	(18)
Hepatocellular carcinoma	40 tumor tissues and 34 ANCTs	down	—	—	—	(19)
	288 pairs of tumor tissues and ANCTs	down	shorter OS	—	—	(37)
Glioma	48 pairs of tumor tissues and ANCTs	down	—	—	—	(22)
	60 pairs of tumor tissues and ANCTs	down	poor OS	—	tumor size, WHO grade and Karnofsky Performance Status	(23)
Oral squamous cell carcinoma	103 pairs of tumor tissues and ANCTs	down	shorter OS	—	lymph node metastasis and advanced TNM stage	(24)
Prostate cancer	52 pairs of tumor tissues and ANCTs	down	poor OS	—	preoperative PSA, Gleason score, and tumor stage	(25)
	10 pairs of tumor tissues and ANCTs	down	—	—	—	(26)
	324 pairs of tumor tissues and ANCTs	down	shorter DFS and OS	—	advanced pathologic T stage and high risk of lymph node metastasis	(38)
Gastric cancer	61 pairs of tumor tissues and ANCTs	down	—	—	invasion depth	(28)

(Continued)

**TABLE 3 |** Continued

Tumor type	samples	Expression (Tumor vs. Normal)	Kaplan-Meier analysis (impact of circITCH down-regulation)	Univariate/Multivariate cox regression	Association of down-regulation of circITCH with clinicopathologic characteristics	Reference
Melanoma	30 pairs of tumor tissues and ANCTs	down	–	–	age and tumor grades	(39)
	51 pairs of tumor tissues and ANCTs	down	poor OS	–	lymph node metastasis	(29)
	56 pairs of tumor tissues and ANCTs	down	–	–	–	(30)
Lung cancer	78 pairs of tumor tissues and ANCTs	down	–	–	age	(31)
Esophageal squamous cell carcinoma	684 pairs of tumor tissues and ANCTs	down	–	–	–	(32)
Clear cell renal cell carcinoma	54 pairs of tumor tissues and ANCTs	down	–	–	–	(33)
Multiple myeloma	56 patients with MM and 56 HCs	down	shorter OS	–	–	(34)

**TABLE 4 |** Possible interactions between circITCH and RNA-binding proteins.

CircRNA ID	RNA-binding protein sites matching circRNA junction	RNA-binding protein sites matching flanking regions of circRNA
hsa_circ_0001141	–	EIF4A3, HuR, U2AF65
hsa_circ_0003073	–	DGCR8, EIF4A3, PTB
hsa_circ_0005677	EIF4A3	EIF4A3, PTB
hsa_circ_0005868	–	AGO2, EIF4A3, PTB, U2AF65

to find the suitability of this marker for diagnostic purposes as well as patients' follow-up. The main question in this regard is whether expression level of circITCH is changed after chemo/radiotherapy or tumor excision. If so, it can be used as a marker for early detection of cancer recurrence.

Another question to be answered is the correlation between expression levels of the circular and linear form of ITCH in different types of cancers. The answer to this question can help in better understanding of the mechanism of dysregulation of circITCH in relation to cancer progression.

Since circITCH is mostly considered as a tumor suppressor circRNA, several groups have assessed the impact of forced over-expression of this transcript in cancer cells transplanted into animal models. The results have been mostly promising, yet needing to be approved in clinical settings.

The interactions between circITCH and RNA-binding proteins have not identified in the previous literature. However, the online database circular RNA Interactome (<https://circinteractome.nia.nih.gov/>) has listed a number of RNA-binding proteins possibly interacting with circRNAs originated from *ITCH* locus (Table 4).

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## FUTURE PERSPECTIVES

CircITCH, as a tumor suppressor circRNA can be utilized in therapeutic regimens for cancers. Delivery methods include nanoparticles and exosome-based methods (40). Artificial circRNAs have been successfully used as miRNA sponges in recent years (41). Thus, synthetic circITCH molecules with the potential of sponging oncogenic miRNAs can be used for attenuation of carcinogenic process. Yet, this method should be appraised in cell lines and animal models. Finally, the interactions between circITCH and RNA-binding proteins should be assessed in future investigations.

## AUTHOR CONTRIBUTIONS

SG-F wrote the draft and revised it. MT designed and supervised the study. TK and EJ collected the data and designed the tables and figures. All authors contributed to the article and approved the submitted version.

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# Long Non-coding RNA DANCER in Cancer: Roles, Mechanisms, and Implications

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Long non-coding RNA (lncRNA) DANCER (also known as ANCR)—differentiation antagonizing non-protein coding RNA, was first reported in 2012 to suppress differentiation of epithelial cells. Emerging evidence demonstrates that DANCER is a cancer-associated lncRNA abnormally expressed in many cancers (e.g., lung cancer, gastric cancer, breast cancer, hepatocellular carcinoma). Increasing studies suggest that the dysregulation of DANCER plays critical roles in cancer cell proliferation, apoptosis, migration, invasion, and chemoresistance *in vitro* and tumor growth and metastasis *in vivo*. Mechanistic analyses show that DANCER can serve as miRNA sponges, stabilize mRNAs, and interact with proteins. Recent research reveals that DANCER can be detected in many body fluids such as serum, plasma, and exosomes, providing a quick and convenient method for cancer monitor. Thus DANCER can be used as a promising diagnostic and prognostic biomarker and therapeutic target for various types of cancer. This review focuses on the role and mechanism of DANCER in cancer progression with an emphasis on the clinical significance of DANCER in human cancers.

**Keywords:** long non-coding RNA, DANCER, cancer, biomarker, diagnosis, therapy, target

## INTRODUCTION

lncRNAs are non-coding RNAs (ncRNAs) consisting of longer than 200 nucleotides in length without initiation codon and termination codon. They were initially considered as “junk” transcriptional products without biological functions, thus attracting limited attention among scientists (Anastasiadou et al., 2018). However, in recent decades, large-scale genome-wide sequencing analysis has shown the tissue specificity and essential functions of non-coding RNAs in diverse biological processes (Fico et al., 2019). They can modulate gene expression at epigenetic, transcription, and post-transcription levels (Ma et al., 2019).

lncRNAs also play essential roles in human cancers. The previous studies have indicated that many lncRNAs are dysregulated in cancers (Iyer et al., 2015; Yan et al., 2015). Aberrant expression of lncRNAs is associated with tumor growth (Zhou et al., 2020), metastasis (Liao et al., 2021), angiogenesis (Jin et al., 2020), chemotherapy resistance (Ferretti and León, 2021), and metabolism (Xu et al., 2021). Therefore, lncRNAs are regarded as important regulators of the pathological processes in cancer cells.

Differentiation antagonizing non-protein coding RNA (DANCER or ANCR), located on human chromosome 4q12, was first reported in 2012 to suppress differentiation of epithelial cells, and then proved to promote the stemness features of hepatocellular carcinoma cells (Kretz et al., 2012; Yuan et al., 2016). Subsequently, in recent decades, many studies have been carried out to understand the function of DANCER in cancer. DANCER is aberrantly expressed in various kinds of cancers (Figure 1). The dysregulation of DANCER expression is closely associated with biological functions (Figure 2) and clinical pathological factors (Figure 3). In this review, we summarized the current evidence regarding the function and underlying mechanism of DANCER in numerous cancers. It is suggested that DANCER serve as a tumor promoter or suppressor, representing a promising cancer biomarker or therapeutic target in various cancers.

## REGULATORY MECHANISMS FOR DANCER

The downstream regulatory mechanisms for the biological roles of DANCER in cancers are complicated (Figure 4). The competing endogenous RNA (ceRNA) network, initially proposed in 2011, is one popular regulatory mechanism of DANCER in cancers (Table 1; Salmena et al., 2011). As we know, miRNAs could regulate gene expression at the post-transcriptional level by interacting with the 3'UTR region of mRNA via base-pairing (Fabian et al., 2010). Meanwhile, the miRNA recognition elements (MRE) of ceRNA could compete with relevant miRNAs to bind to mRNAs and regulate their expression (Salmena et al., 2011). Up to now, DANCER has been reported to bind to ~ 50 miRNAs (Table 1).

In addition to binding to miRNAs, DANCER could also interact with mRNAs or proteins. For example, DANCER was verified to interact with 3'UTR of CTNNB1 mRNA in hepatocellular carcinoma, thus competitively blocking miRNA site and reversing miRNA-mediated CTNNB1 suppression (Yuan et al., 2016). Similarly, in sorafenib-resistant HCC, DANCER could bind with PSMD10 mRNA to stabilize its expression via blocking the miRNA binding site (Liu et al., 2020a). In the case of binding to proteins, it is interesting that DANCER could regulate both protein stability (Wen et al., 2020) and protein degradation (Xie et al., 2020).

Another function of DANCER is epigenetic regulation. EZH2, a histone methyltransferase of polycomb repressive complex 2 (PRC2), is a common binding protein for DANCER. By interacting with EZH2, DANCER could epigenetically silence target gene transcription. In cholangiocarcinoma, DANCER-EZH2 interaction promoted FBPI silence by regulating histone methylation of FBPI promoter and then exacerbating tumorigenesis (Wang N. et al., 2019).

What's more, DANCER expression is regulated at both epigenetic and transcriptional levels. It was suggested that in gastric cancer cells, DANCER could be activated by SALL4, which could bind to the promoter of DANCER (Pan et al., 2018). In addition, DANCER has been considered as a drug

target. In nasopharyngeal carcinoma, resveratrol was verified to suppress cancer progression via DANCER/PTEN pathway (Zhang J. et al., 2020). Moreover, IGF2BP2 protein has been shown to regulate DANCER stability by m<sup>6</sup>A modification (Hu et al., 2020). In conclusion, the upstream and downstream mechanisms of DANCER are multifaceted and play vital roles in human cancers.

## DIAGNOSTIC VALUE OF DANCER

It has been verified that DANCER is aberrantly expressed in many cancers. Notably, DANCER is detected in some body fluids, including serum, plasma, and even exosomes (Table 2). This indicates that DANCER may help diagnosis and prognosis in many cancers by non-invasive methods with low cost and real-time monitoring.

Circulating DANCER expression is also positively associated with tissue DANCER level. For example, after HCC surgery treatment, plasma DANCER level decreased dramatically, which indicates that circulating DANCER may drive from tissues (Ma et al., 2016). As expected, the release of RNAs to circulation may reveal disease-associated information of tissue or activation of intercellular signaling pathways (Misawa et al., 2017). Thus, circulating DANCER may have a diagnostic capability. It was reported by Ma et al. (2016) that plasma DANCER had a better diagnostic value than AFP in HCC. As illustrated in Table 2, plasma DANCER (AUC = 0.868) showed a better effect in distinguishing HCC from healthy volunteers (HV), patients with CHB (chronic hepatitis B), and cirrhosis than AFP (AUC = 0.744). Recently, it has been confirmed that DANCER can be transported through exosomes, but the detailed mechanism is unclear. In addition, droplet digital PCR (ddPCR) has been used to detect exosomal DANCER to monitor HCV-HCC recurrence after curative surgical resection, showing a higher AUC (0.88) than qRT-PCR detection (0.831) (Wang S.C. et al., 2020).

These encouraging researches indicate that circulating DANCER may be a novel non-invasive biomarker for cancer diagnosis. However, the lncRNA-based liquid biopsy is still at its early stage. For further application, there is still a long way to go. For instance, standardized sample preparation and reliable endogenous controls need to be considered.

## FUNCTION AND MECHANISM OF DANCER IN HUMAN CANCERS

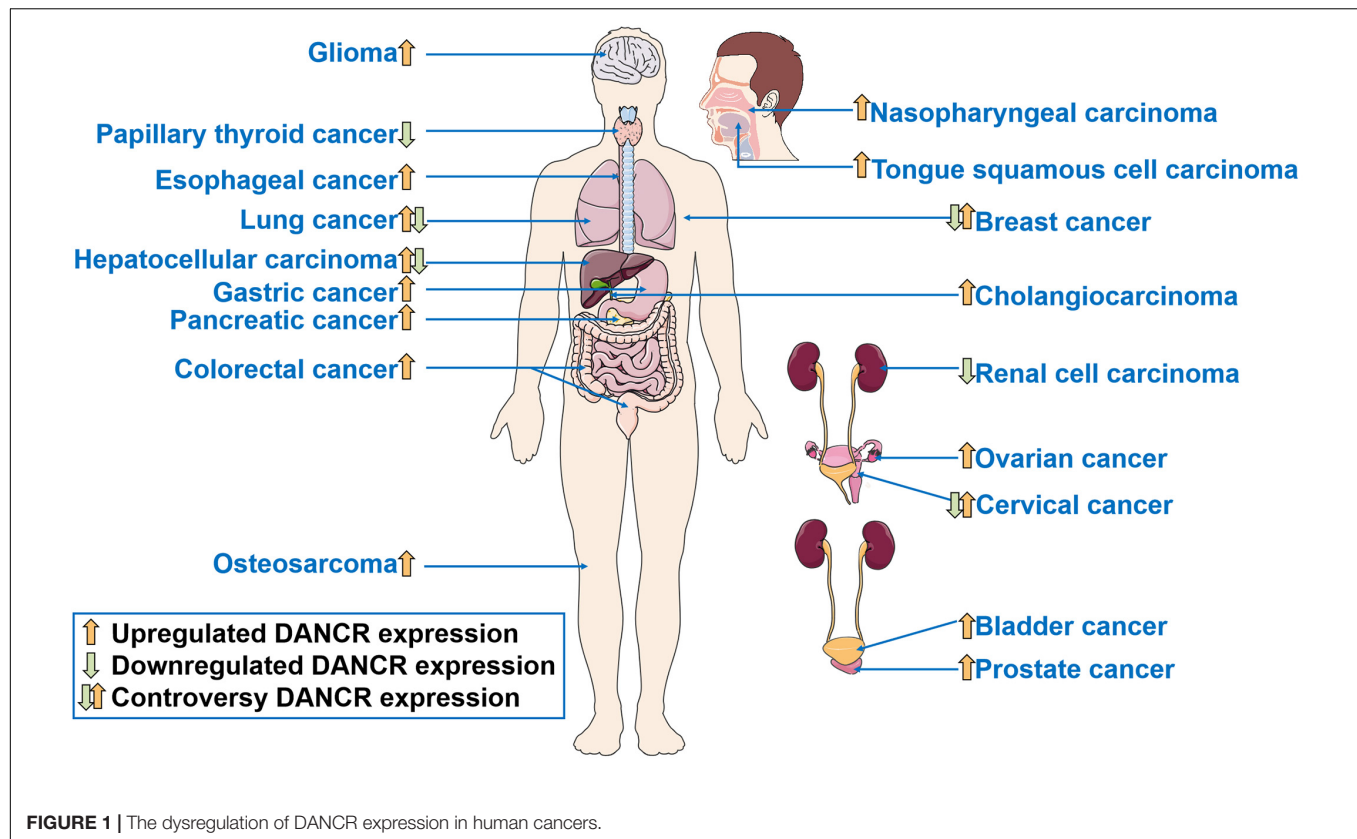
### Digestive System

#### Tongue Squamous Cell Carcinoma

Tongue squamous cell carcinoma (TSCC) is one of the most common oral cavity malignancies, known for its aggressive biological behavior (Boldrup et al., 2017). Although significant progress has been made in treating TSCC, long-term survival does not improve significantly (Hu et al., 2017). Therefore, it would be beneficial to find promising biomarkers for TSCC detection and treatment.

DANCER expression was upregulated in TSCC cells. *In vitro* studies showed that DANCER could enhance TSCC





cell proliferation, migration, and invasion. The molecular mechanism study suggested that DANCR act as a ceRNA to sponge miR-135a-5p and upregulate Kruppel-like Factor 8 (KLF8) expression. Similarly, knockdown of DANCR inhibited tumor growth and KLF8 expression *in vivo*, suggesting that DANCR may play an essential role via miR-135a-5p/KLF8 axis (Zheng et al., 2019). So DANCR may be a diagnostic biomarker and new therapeutic target in TSCC.

### Esophageal Cancer

Esophageal cancer (EC) is one of the most frequent cancers in the digestive system and ranks the seventh leading cause of death in cancers (Siegel et al., 2021). Though the management and treatment of EC patients have improved, there is still no effective treatment, and 5-year post-esophagectomy survival rates are still poor (Hou et al., 2017; Huang and Yu, 2018). In addition, due to the deficiency of early clinical symptoms, it tends to be diagnosed in advanced stages (Encinas de la Iglesia et al., 2016).

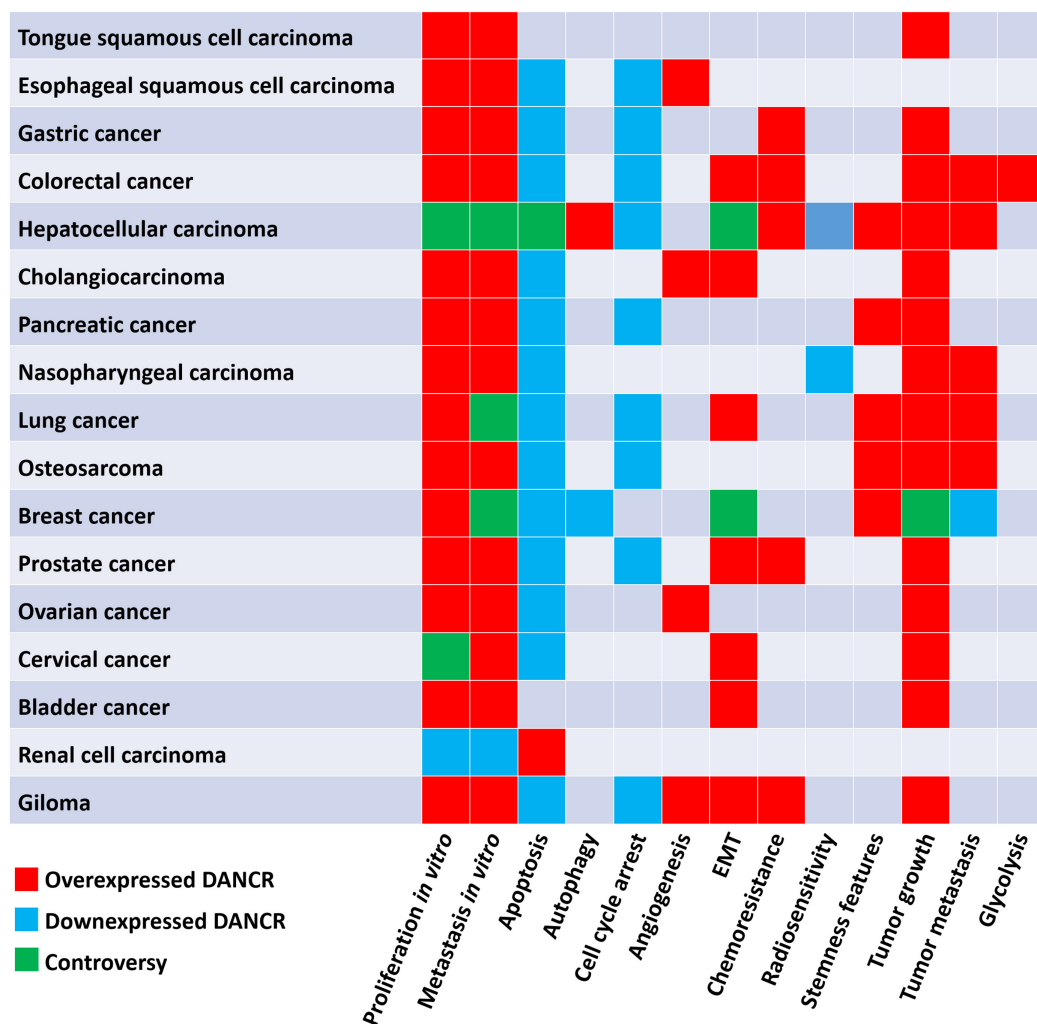
Esophageal squamous cell carcinoma (ESCC) is one histological subtype of EC (Le Bras et al., 2016). Shi et al. (2018) monitored the expression of DANCR in ESCC, and they found that ESCC cell lines and tissues expressed a higher level of DANCR compared with that in adjacent normal counterparts. Its aberrant expression was implicated in accelerating cell proliferation, migration, invasion, and inhibiting apoptosis. Subsequently, Zhang C. et al. (2019) investigated the role of DANCR in ESCC. They found that DANCR sponged miR-33a-5p and upregulated zinc-finger-enhancer binding protein

1 (ZEB1) expression. Recently, Bi et al. (2020) discovered that DANCR was a direct downstream target of a tumor suppressor gene *ZNF750*. The mutations or deletions of *ZNF750* were significantly associated with upregulated DANCR and poor prognosis, especially tumor metastasis in ESCC patients. Their further investigations verified that DANCR could competitively bind with miR-4707-3p and serve as a ceRNA to regulate *FOXC2* expression in ESCC, which provides a novel DANCR/miR-4707-3p/*FOXC2* pathway regulated by *ZNF750* in ESCC. In summary, DANCR is associated with ESCC progression, and it may act as a novel prognostic and therapeutic target for ESCC.

### Gastric Cancer

Gastric cancer (GC) is a complex and heterogeneous disease, regarded as the fourth leading cause of cancer-related death (Serra et al., 2019). Traditional clinical indicators such as CEA, CA199, and CA724 are faced with shortcomings in diagnostic sensitivity and specificity. Therefore, there is an urgent need to find a potential marker for GC with higher diagnostic sensitivity and specificity (Cai et al., 2019).

Recent studies have revealed that DANCR expression was upregulated in GC tissues (Hao et al., 2017) and cell lines (Pan et al., 2018) compared to adjacent normal ones. High DANCR expression promoted cell proliferation, migration, and invasion in GC cells. Its expression was positively associated with tumor size, lymph node metastasis, invasion depth, and TNM stage of GC patients (Pan et al., 2018), suggesting that DANCR may correlate to the malignant progression of GC. For



**FIGURE 2 |** The biological function of DANCR in different cancers.

further function and mechanism analysis of DANCR in GC, Pan et al. (2018) proved that DANCR was regulated by SALL4 (sal-like protein 4), previously shown as a critical transcription factor to regulate the stemness of GC cells. Then DANCR accelerated GC progression via Wnt/ $\beta$ -catenin pathway (Zhang et al., 2014; Pan et al., 2018). A study by Xie et al. (2020) indicated another mechanism showing that DANCR inhibited FOXO1 expression by promoting its ubiquitination. As a result, M1 macrophage polarization was inhibited, which promoted GC cell invasion and metastasis.

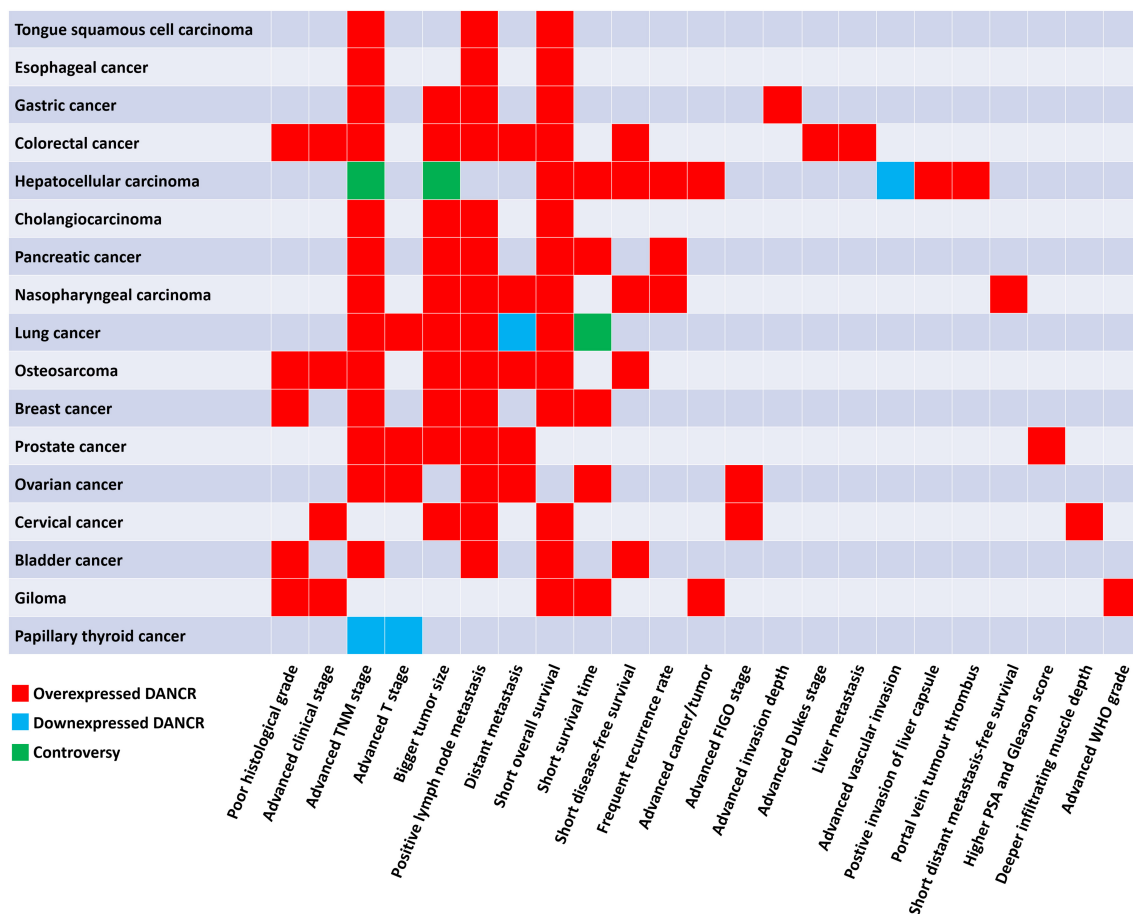
Chemotherapy is the primary therapy for GC patients with unresectable tumors, and cisplatin (DDP) is used as the first-line drug (Zhu et al., 2019). However, DDP resistance is a major obstacle for DDP application. Thus, exploring underlying mechanisms of DDP-resistance development in GC remains an urgent issue (Guo Q. et al., 2019). Xu et al. (2019) found that DANCR was upregulated in DDP-resistant GC cells. Further study showed that the expression of multidrug resistance (MDR) related genes, MDR1 and MRP1, were induced by DANCR in

GC cells. Their studies suggest that DANCR is associated with MDR development and may be a potential therapeutic target for GC with MDR.

### Colorectal Cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed malignant neoplasms among men (Siegel et al., 2019, 2021). Thanks to the improvements in screening tests and treatment, CRC incidence and mortality rates have declined for several years in developed countries (Siegel et al., 2017). However, in some low-income and middle-income regions, CRC incidence and mortality rates are still rising rapidly. Consequently, improvement in treatment options and accessibility is still necessary for economically backward areas (Arnold et al., 2017).

Liu et al. (2015) demonstrated that CRC tissues expressed a higher level of DANCR than adjacent normal ones. In addition, high DANCR expression was associated with worse overall survival and disease-free survival, and further study showed that DANCR might be an independent prognostic factor for CRC.



**FIGURE 3 |** The correlation of DANCR expression level with clinicopathological factors of patients with cancers.

A recent study by Wang et al. (2018b) showed that miR-577 shared the same binding site for HSP27 (heat shock protein 27) with DANCR. They verified DANCR could enhance CRC cell proliferation and metastasis by acting as a miRNA sponge to promote HSP27 expression. Moreover, in *in vivo* study, the elevation of DANCR promoted tumor growth and liver metastasis of CRC. Another mechanism study by Lian et al. (2020) identified that DANCR could bind with lysine acetyltransferase 6A (KAT6A) and then triggered H3K23 acetyltransferase activity to promote CRC development. Intriguingly, DANCR could also promote the expression of an oncogenic lncRNA MALAT1 via enhancing its RNA stability, which suppressed doxorubicin-induced apoptosis in CRC cells (Xiong et al., 2021). In summary, these studies provided a potential therapeutic target for molecular treatment in CRC.

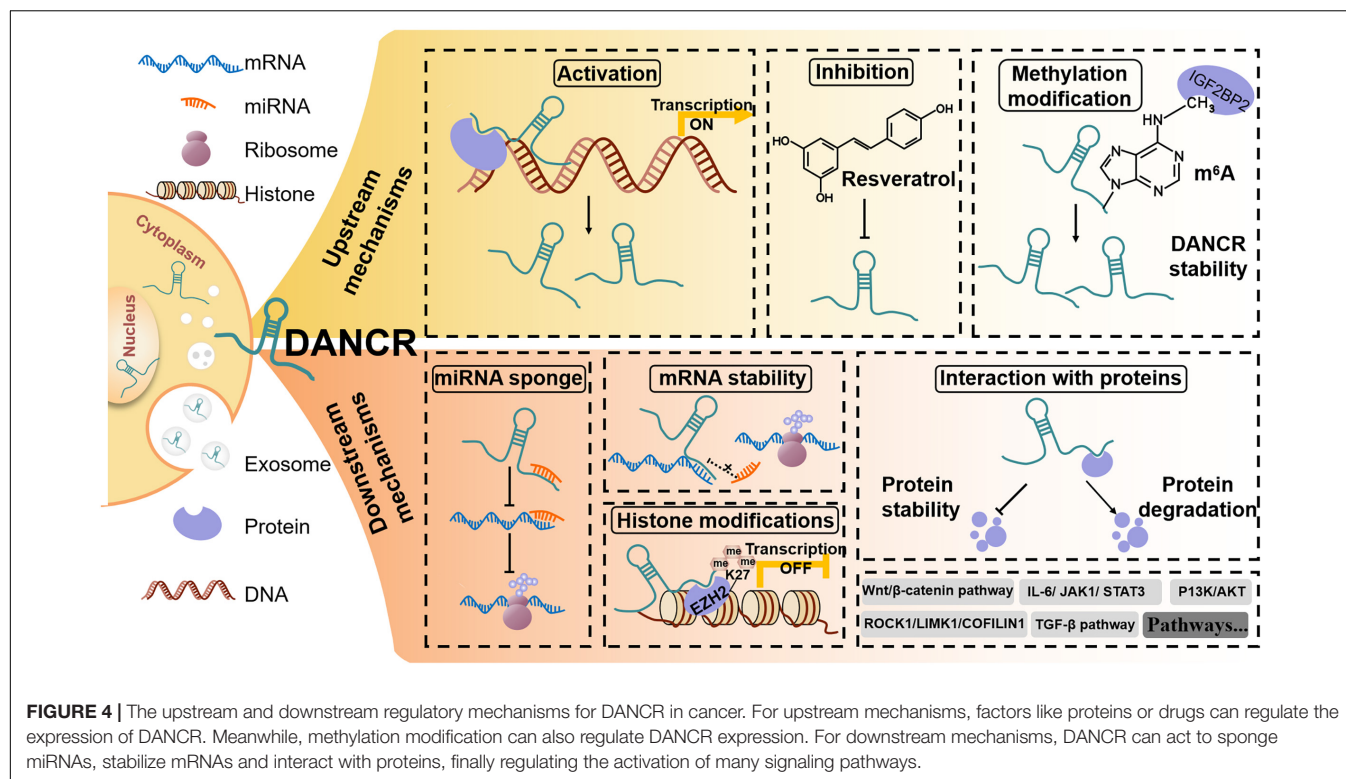
DANCR was also overexpressed in the serum of CRC patients. Serum DANCR level was positively associated with the TNM stage. Surprisingly, serum DANCR showed a better diagnostic ability than CEA and CA199 and a better performance in distinguishing CRC and colorectal polyps. Moreover, the combination of DANCR with CEA and CA199 showed better sensitivity than the single or double

combination (Shen et al., 2020; **Table 1**), suggesting that DANCR has the potential to be a promising biomarker for CRC patients.

### Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and ranks the sixth most common neoplasm and the third leading cause of cancer-related death (Forner et al., 2018). Sorafenib is an anti-angiogenic multi-kinase inhibitor for advanced hepatocellular carcinoma. However, the outcomes after therapy are not encouraging (Llovet et al., 2008). So, it is urgent to find other available opinions for treatment. As shown in recent studies, non-coding RNAs are considered promising biomarkers for diagnosis and treatment (Duan et al., 2019).

DANCR was upregulated in HCC cell lines, tissues, plasma and exosomes (Ma et al., 2016; Wang S.C. et al., 2020; Wen et al., 2020). It was implicated that DANCR played an essential role in HCC progression. For example, a high level of DANCR was associated with stemness features (Yuan et al., 2016), metastasis (Wen et al., 2020), and chemotherapeutic drug resistance in HCC (Liu et al., 2020a). In addition, the ROC (receiver operating characteristic) analysis showed that plasma DANCR



and exosomal DANCER exhibited good discriminatory ability, suggesting it could be a promising diagnostic marker (Ma et al., 2016; Wang S.C. et al., 2020; **Table 2**).

DANCER could act as sponges for multiple miRNAs including miR-216a-5p (Wang J. et al., 2019), miR-27a-3p (Guo D. et al., 2019), miR-125b-5p (Yang et al., 2020), miR-140-3p (Wen et al., 2020) and miR-222-3p (Wang X. et al., 2020) in HCC. DANCER could also stabilize PSMD10 (Liu et al., 2020a) and CTNNB1 (Yuan et al., 2016) mRNAs by binding to their 3'UTR region to prevent its degradation by miRNAs. Wen et al. (2020) found that DANCER could not only improve HNRNPA1 expression via DANCER/miR-140-3p/HNRNPA1 axis but also bind to HNRNPA1 protein and inhibit its degradation. However, DANCER was also considered as a tumor suppressor due to the suppression of Wnt/β-catenin signaling pathway in HCC (Song et al., 2020).

In conclusion, DANCER is a crucial factor in HCC progression and may serve as a new marker for HCC diagnosis and treatment.

### Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a rare but aggressive biliary epithelial tumor (Goyal et al., 2021). Surgery is the best therapeutic option; however, only a minority (35%) of CCA patients are diagnosed early, whereas most of them are diagnosed late due to the “silent” clinical character (Rizvi et al., 2018). Therefore, there is an urgent need for early diagnosis and new treatment methods to help improve CCA survival outcomes.

DANCER was overexpressed in CCA tissues compared with adjacent normal tissues. Its expression level was associated with tumor size, TNM stage, and lymph node metastasis. Moreover,

CCA patients with higher levels of DANCER had a lower survival rate. Knockdown of DANCER impeded cell proliferation, migration, invasion, EMT process, angiogenesis, and enhanced apoptosis *in vitro*. miR-345-5p was predicted and verified as a target miRNA of DANCER, which regulated the expression of Twist1 (Twist-related protein 1) in CCA cells (Zhu et al., 2020). Wang N. et al. (2019) showed that DANCER inhibited FBPI expression epigenetically via interacting with EZH2 and subsequently promoted CCA. In conclusion, DANCER may be a potential biomarker and therapeutic target for CCA.

### Pancreatic Cancer

Pancreatic cancer is the most life-threatening cancer with the lowest 5-year survival rate (10%) (Siegel et al., 2021). What is worse, early detection for pancreatic cancer is lacking, and the available treatment options are limited (Moore and Donahue, 2019).

DANCER showed higher expression in pancreatic cancer cell lines and tissues. Upregulated DANCER expression was associated with poor prognosis and short overall survival time in patients with pancreatic cancer. Knockdown of DANCER suppressed pancreatic cancer cell proliferation, migration, invasion *in vitro*, and tumor growth *in vivo* (Luo et al., 2019; Yao et al., 2019). Mechanistic studies showed that DANCER could be methylated at the N6 position of adenosine and then be stabilized by the m6A reader protein IGF2BP2 (Hu et al., 2020). In addition, DANCER sponged several miRNAs to regulate the expression of target mRNAs in pancreatic cancer cells (Luo et al., 2019; Yao et al., 2019; Tang et al., 2020). Interestingly, Liu et al. (2020b) found that DANCER could downregulate MLL3 expression to



**TABLE 1** | The molecular mechanisms of DANCR in various cancers.

Cancer	Role	Regulated molecules			Related pathway	References
		miRNA	mRNA	Protein		
Tongue squamous cell carcinoma	Oncogene	miR-135a-5p	KLF8		DANCR/miR-135a-5p/KLF8	Zheng et al., 2019
Esophageal squamous cell carcinoma	Oncogene	miR-33a-5p; miR-4707-3p	FOXC2	ZEB1	DANCR/miR-33a-5p/ZEB1; ZNF750 mutation/DANCR/miR-4707-3p/FOXC2	Zhang C. et al., 2019; Bi et al., 2020
Gastric cancer	Oncogene		MDR1 and MRP1	SALL4; FoxO1	SALL4/DANCR/ $\beta$ -catenin pathway; DANCR/FoxO1; P-gp and MRP1 pathways	Pan et al., 2018; Xie et al., 2020; Xu et al., 2019
Colorectal cancer	Oncogene	miR-577; miR-185-5p; miR-518a-3p; miR-125b-5p; miR-145-5p	HSP27; HMG2; MDMA; HK2; NRAS; MALAT1	KAT6A; EZH2; QK	DANCR/miR-577/HSP27; DANCR/miR-185-5p/HMG2; DANCR/miR-518a-3p/MDMA; DANCR/miR-125b-5p/HK2; DANCR/miR-145-5p/NRAS; DANCR/KAT6A acetyltransferase activity; DANCR-EZH2; Doxorubicin/DANCR/QK/MALAT1	Yang et al., 2017; Wang et al., 2018b; Lian et al., 2020; Shi et al., 2020; Sun et al., 2020; Bahreini et al., 2021; Lu et al., 2021; Xiong et al., 2021
Hepatocellular carcinoma	Oncogene	miR-216a-5p; miR-27a-3p; miR-125b-5p; miR-140-3p; miR-222-3p; miR-214, miR-1254, miR-199a, and miR-605; miR-214, and miR-320a	KLF12; LIMK1; HNRNPA1; ATG7; PSMD10; CTNNB1	p-p38/p38, p-ERK1/2/ ERK1/2, p-JNK/JNK	DANCR/miR-216a-5p/KLF12; DANCR/miR-27a-3p/LIMK1; ROCK1/LIMK1/COFILIN1; DANCR/miR-125b-5p; MAPK signaling pathway; DANCR/miR-140-3p/HNRNPA1 DANCR/HNRNPA1; DANCR/miR-222-3p/ATG7; DANCR/miR-214, miR-1254, miR-199a or miR-605; DANCR/PSMD10/IL-6/STAT3/DANCR; DANCR/miR-214, miR-320a/CTNNB1 Wnt/ $\beta$ -catenin signaling pathway	Yuan et al., 2016; Guo D. et al., 2019; Wang J. et al., 2019; Liu et al., 2020a; Wang X. et al., 2020; Wen et al., 2020; Yang et al., 2020
	Tumor suppressor					Song et al., 2020
Cholangiocarcinoma	Oncogene	miR-345-5p	TWIST1; FBP1	EZH2	DANCR/miR-345-5p/Twist1; DANCR-EZH2/FBP1	Wang N. et al., 2019; Zhu et al., 2020
Pancreatic cancer	Oncogene	miR-135a; miR-214-5p; miR-33b	NLRP37; E2F2; MMP16	MLL3; IGF2BP2	DANCR/miR-135a/NLRP37; DANCR/miR-214-5p/E2F2; DANCR/miR-33b/MMP16; DANCR/MLL3 (advanced stages); IGF2BP2/DANCR (m6A modified)	Luo et al., 2019; Yao et al., 2019; Hu et al., 2020; Liu et al., 2020b; Tang et al., 2020
Nasopharyngeal carcinoma	Oncogene	miR-4731	HIF1A; PTEN; SOX2; NMT1	NF90/NF45; EZH2; RBM3; STAT3	DANCR- (NF90/NF45)/HIF-1 $\alpha$ ; DANCR-EZH2/PTEN; Resveratrol/DANCR-EZH2/PTEN; DANCR-RBM3/SOX2; IL-6/JAK1/STAT3; DANCR/miR-4731-5p/NMT1	Ma X. et al., 2018; Wen et al., 2018; Zhang X. et al., 2019; Li et al., 2020; Zhang J. et al., 2020; Ma et al., 2021
Lung cancer	Oncogene	miR-216a; miR-496; miR-1225-3p; miR-758-3p; miR-138; miR-214-5p	MTOR; ERBB2; SOX4; CIZ1; KRAS; HMG2	EZH2; HMG2	DANCR/miR-216a; Wnt/ $\beta$ -catenin signaling pathway; DANCR/miR-496/mTOR; DANCR/miR-1225-3p/ErbB2; DANCR/miR-758-3p; DANCR/miR-138/Sox4; DANCR/miR-214-5p/CIZ1; DANCR-EZH2/p21	Lu Q.C. et al., 2018; Wang and Jiang, 2018; Zhen et al., 2018; Bai et al., 2019; Guo L. et al., 2019; Zhang and Jiang, 2019; Chen Y.R. et al., 2020; Yu et al., 2020; Huang et al., 2021
	Tumor suppressor		TGFB1	TGF- $\beta$ 1	TGF- $\beta$ signaling	Wang et al., 2018a
Osteosarcoma	Oncogene	miR-149; miR-33a-5p; miR-216a-5p; miR-335-5p; miR-1972	MSI2; AXL; SOX5; ROCK1; KRAS and CDKN1B	EZH2; p-p38MAPK	DANCR/miR-149/MSI2; DANCR/miR-33a-5p/AXL; AXL-Akt pathway; DANCR/miR-216a-5p/SOX5; DANCR/miR-335-5p or miR-1972/ROCK1; DANCR-EZH2/p21 and p27; p38MAPK signaling pathway	Jiang et al., 2017; Zhang and Peng, 2017; Wang et al., 2018c; Liu et al., 2019; Pan et al., 2020; Zhang W. et al., 2020

(Continued)

TABLE 1 | (Continued).

Cancer	Role	Regulated molecules			Related pathway	References
		miRNA	mRNA	Protein		
Breast cancer	Oncogene	miR-758-3p; miR-874-3p; miR-4319; miR-216a-5p;	PAX6; SOX2; SOCS3; CD44, ABCG2;	TUFT1; EZH2; RXRA	DANCER/miR-758-3p/PAX6; TUFT1/DANCER/miR-874-3p/SOX2; DANCER/miR-4319/VAPB; DANCER/miR-216a-5p; DANCER-EZH2/SOCS3; DANCER-EZH2/CD44, ABCG2; PI3K/AKT signaling	Sha et al., 2017; Tang et al., 2018; Tao et al., 2019; Jia et al., 2020; Wu et al., 2020; Zhang K.J. et al., 2020; Zhang X.H. et al., 2020
	Tumor suppressor		RUNX2	EZH2	DANCER-EZH2; TGF- $\beta$ /DANCER/RUNX2	Li et al., 2017b,c
Prostate cancer	Oncogene	miR-135a; miR-34a-5p; miR-214-5p; miR-185-5p	JAG1; LASP1; TIMP2/3	EZH2; MYC, and p21	DANCER/miR-135a; DANCER/miR-34a-5p/JAG1; DANCER/miR-214-5p/TGF- $\beta$ ; DANCER/miR-185-5p/LASP1; AK/PI3K/AKT/GSK3 $\beta$ /snail; DANCER-EZH2/TIMP2/3	Jia et al., 2016; Lu Y. et al., 2018; Ma et al., 2019; Zhao et al., 2019; Deng et al., 2021; Sun et al., 2021
Ovarian cancer	Oncogene	miR-145; miR-214	KLF5	SP1; UPF1	DANCER/miR-145/VEGF; TGF- $\beta$ /DANCER/miR-214/KLF5; SP1/DANCER; DANCER/UPF1	Lin et al., 2019; Pei et al., 2019; Cui et al., 2020; Huang et al., 2020
Cervical cancer	Oncogene	miR-335-5p; miR-665; miR-145-3p	TGFBR1; FRAT1 and FRAT2	KLF5, ZEB1; FRAT1 and FRAT2	DANCER/miR-335-5p/ROCK1; DANCER/miR-665/TGFBR1; ERK/SMAD pathway; KLF5/DANCER/miR-145-3p/ZEB1; Wnt/ $\beta$ -catenin signaling pathway	Cao et al., 2019; Liang et al., 2019; Tian et al., 2020; Hu et al., 2021
	Tumor suppressor			HIF-1 $\alpha$	DANCER/HIF-1 $\alpha$	Ta et al., 2020
Bladder cancer	Oncogene	miR-149; miR-335	MSI2; VEGF-C	CCND1	DANCER/miR-149/MSI2; IL-11-STAT3 signaling; DANCER/miR-335/VEGF-C	Zhan et al., 2018; Chen et al., 2019; Ping et al., 2021
Glioma	Oncogene	miR-33a-5p; miR-634; miR-216a; miR-33a-5p, miR-33b-5p, miR-1-3p, miR-206, and miR-613	RAB1A; LGR5; AXL; KLF8	EZH2, PTEN	DANCER/miR-33a-5p; DANCER/miR-634/RAB1A; DANCER/miR-216a/LGR5, PI3K/AKT signaling pathway; AXL/PI3K/Akt/NF- $\kappa$ B; Wnt/ $\beta$ -catenin signaling pathway; DANCER-EZH2/PTEN	Li and Zhou, 2018; Ma Y. et al., 2018; Xu et al., 2018; Yang et al., 2018; Wang W. et al., 2019; Cheng et al., 2020

influence pancreatic cancer progression only at a late stage rather than an early stage.

Hence, DANCER was associated with pancreatic cancer development and regarded as a promising target for pancreatic cancer prognosis and treatment.

## Respiratory System Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is not a common cancer type but notable for its distinctive geographical distribution pattern. Most cases occur in the east and southeast parts of Asia (Chua et al., 2016). NPC patients at an early stage or with locoregional advanced disease could be well treated benefiting from the radiotherapy. However, distant metastasis is the primary cause of treatment failure (Yin et al., 2017).

DANCER was upregulated in NPC and promoted NPC cell proliferation, migration, invasion, and inhibited apoptosis *in vitro*. In addition, DANCER knockdown inhibited NPC tumor growth *in vivo* (Hao et al., 2019; Wen et al., 2018).

Ma X. et al. (2018) found that DANCER could promote NPC proliferation and radiation resistance via DANCER/PTEN pathway. Interestingly, another research by Zhang J. et al. (2020) found that resveratrol could downregulate the expression of DANCER through this pathway. It validated DANCER as a promising target in NPC therapy.

Hypoxia is known as a target for cancer treatment, including NPC, and intratumoral hypoxia could lead to HIF-1 $\alpha$  (hypoxia inducible factor-1 $\alpha$ ) overexpression (Semenza, 2003; Shan et al., 2018). Wen et al. (2018) found that DANCER overexpression was associated with lymph node metastasis and indicated a poor prognosis in NPC. Further study revealed that DANCER could stabilize HIF-1 $\alpha$  mRNA through interacting with NF90/NF45 complex. These data suggest that DANCER might be a potential biomarker and therapeutic target of NPC.

## Lung Cancer

Lung cancer is the leading cause of cancer-related deaths worldwide, both in men and women. It is reported that nearly

**TABLE 2 |** The diagnostic and prognostic value of DANCR in cancer.

Cancer	Marker	Sensitivity	Specificity	AUC	Distinction	Source	Detection method	References
Gastric cancer	DANCR	64.6%	67.7%	0.704	GC ( <i>n</i> = 65)/paired adjacent normal tissues (ANT) ( <i>n</i> = 65)	Tissues	qRT-PCR	Pan et al., 2018
		72.7%	79.5%	0.816	GC ( <i>n</i> = 55)/HV (healthy volunteers) ( <i>n</i> = 39)	Serum		
Colorectal cancer	DANCR	67.5%	82.5%	0.747	CRC ( <i>n</i> = 40)/HV ( <i>n</i> = 40)	Serum	qRT-PCR	Shen et al., 2020
	CEA	40.0%	85%	0.623				
	CA199	32.5%	80%	0.573				
	DANCR + CEA + CA199	87.5%	55%	0.812				
	DANCR	67.5%	87.5%	0.745	CRC ( <i>n</i> = 40)/colorectal polyps ( <i>n</i> = 10)			
	CEA	–	–	0.555				
	CA199	–	–	0.542				
	DANCR	87.5%	72.5%	0.732	CRC tissues ( <i>n</i> = 40)/ANT ( <i>n</i> = 40)	Tissues	qRT-PCR	
Hepatocellular carcinoma	DANCR(> –6.2 dCt) DANCR(T/ANT > 1.1)	91.2%	42.9%	0.69	HCV-HCC recurrence ( <i>n</i> = 126)/without recurrence ( <i>n</i> = 57) after curative surgical resection	Tissues	qRT-PCR	Wang S.C. et al., 2020
		88.9%	49.1%	0.74				
		83.5%	94.6%	0.88	HCV-HCC recurrence ( <i>n</i> = 121)/without recurrence ( <i>n</i> = 56) after curative surgical resection	Exosomes	ddPCR	
	DANCR	68.3%	85.7%	0.831			qRT-PCR	Ma et al., 2016
		83.8%	72.7%	0.868	HCC ( <i>n</i> = 52)/HV, CHB and cirrhosis ( <i>n</i> = 94)	Plasma	qRT-PCR	
	AFP	65.4%	77.7%	0.744				
	DANCR	80.8%	84.3%	0.864	HCC ( <i>n</i> = 52)/CHB and cirrhosis ( <i>n</i> = 51)			
	AFP	55.8%	76.5%	0.650				
	DANCR	–	–	0.9265	NSCLC ( <i>n</i> = 72)/HV ( <i>n</i> = 44)	Tissues	qRT-PCR	Wang et al., 2018a
		–	–	0.8831		Plasma		
Prostate cancer	DANCR	–	–	0.852	PC patients ( <i>n</i> = 53)/HV ( <i>n</i> = 47)	Serum	qRT-PCR	Deng et al., 2021)
Ovarian cancer	DANCR	–	–	0.852	OC tissues ( <i>n</i> = 20)/ANT ( <i>n</i> = 20)	Tissues	qRT-PCR	Pei et al., 2019
Cervical cancer	DANCR	–	–	0.9073	HPV-negative CSCC ( <i>n</i> = 38)/HV ( <i>n</i> = 38)	Tissues	qRT-PCR	Ta et al., 2020
		–	–	0.8740		Serum		
Papillary thyroid cancer	DANCR	85.29%	66.18%	0.8233	PTC tissues ( <i>n</i> = 76)/ANT ( <i>n</i> = 76)	Tissues	qRT-PCR	Zhang K. et al., 2019
		81.54%	82.22%	0.8756	PTC ( <i>n</i> = 49)/HV ( <i>n</i> = 45)	Tissues (GSE33630)		
		83.33%	91.67%	0.9167	PTC ( <i>n</i> = 57)/HV ( <i>n</i> = 9)	Tissues (GSE50901)		
		72.41%	70.83%	0.704	PTC I/II patients ( <i>n</i> = 57)/III/IV patients ( <i>n</i> = 19)	Tissues		
Brain tumors	DANCR	91.67%	0.9231%	0.91	Glioma ( <i>n</i> = 13)/meningioma ( <i>n</i> = 13)	Tissues	qRT-PCR	Malakootian et al., 2018
		91.67%	0.7778%	0.84	Meningioma ( <i>n</i> = 13)/pituitary adenoma ( <i>n</i> = 9)			

**TABLE 3 |** Information of DANCR in breast cancer from six research articles.

Role of DANCR	DANCR expression in BC cell lines	Compared normal cell line	<i>In vitro/in vivo</i> validation	Tissue sample size	DANCR expression in tissues	References
Tumorigenesis	↑: MCF7, T47D, MDA-MB-231, MDA-MB-468	Hs578Bst	Both	63 (TNBC)/63 (ANT)	↑: $P = 0.009$	Sha et al., 2017
Tumorigenesis	↑: MCF7, T47D, BT549, MDA-MB-231, MDA-MB-468, MDA-MB-453	MCF10A	Both	60 (TNBC)/10 (normal breast tissue); 60 (TNBC)/15 (Luminal A); 60 (TNBC)/15 (Luminal B)	↑: $P < 0.001$ ; ↑: $P < 0.05$ ; ↑: $P < 0.01$	Tang et al., 2018
Tumorigenesis	↑: MCF7, MDA-MB-231	MCF10A	Both	57 (TNBC)/57 (ANT)	↑: $P < 0.05$	Tao et al., 2019
Tumorigenesis	↑: MCF7, T47D, MDA-MB-231, MDA-MB-468	MCF10A	Both	46 (BC)/46 (ANT)	↑: $P < 0.01$	Zhang K.J. et al., 2020
Tumor suppression	↓: MCF7, T47D, BT474, MDA-MB-436, MDA-MB-231, MDA-MB-231HM	MCF10A	Both	32 (BC)/32 (ANT)	↓: $P < 0.0001$	Li et al., 2017c
Tumor suppression	↓: MCF7, T47D, MDA-MB-231HM ↑: BT549	MCF10A	Both	25 (BC)/25 (ANT)	↓ ( $P$ -value was not provided)	Li et al., 2017b

a quarter of cancer deaths are related to lung cancer (Siegel et al., 2021). Non-small cell lung cancer (NSCLC) accounts for most types of lung cancer, and lung adenocarcinoma is the most common type of NSCLC. Despite improvements in early detection and standard treatment, NSCLC is often diagnosed at an advanced stage with a poor prognosis (Herbst et al., 2008). Understanding the molecular mechanism of NSCLC may bring better treatment for lung cancer.

Upregulated DANCR expression was detected in NSCLC tissue specimens and cell lines compared with normal counterparts. High level of DANCR was associated with larger tumor size, advanced TNM stage, and lymph node metastasis. Bai et al. (2019) found DANCR could activate EMT and act as a ceRNA to competitively bind to miR-138. And then, Sox4, a vital regulator involving tumor growth and metastasis, was regulated. In addition to miR-138, many other miRNAs have been verified as sponge targets for DANCR (Lu Q.C. et al., 2018; Wang and Jiang, 2018; Zhen et al., 2018; Chen Y.R. et al., 2020; Yu et al., 2020; Huang et al., 2021). Another study by Guo L. et al. (2019) showed that DANCR facilitated carcinogenesis by epigenetically silencing p21 expression via binding to EZH2. Thus, DANCR might be a key regulator of lung cancer progression and used as a promising biomarker.

## Genital System

### Prostate Cancer

Due to PSA testing and advances in early detection and treatment, the death rate for prostate cancer (PCa) dropped by 51% in the past two decades. However, PCa remains the second leading cause of cancer-related deaths in men, and

the high rate of overdiagnosis is widely debated. Androgen deprivation therapy (ADT) is the principal treatment for advanced PCa (Magnan et al., 2015; Siegel et al., 2021). However, the majority of PCa will develop into castration-resistant prostate cancer (CRPC) inevitably over time (Nuhn et al., 2019). Compared with ADT, initial treatment with chemotherapy could improve the survival rate (Kahn et al., 2014; Litwin and Tan, 2017). However, the acquisition of chemoresistance inevitably develops, which is a major reason for therapy failure (Domanska et al., 2012).

Strong evidence has been presented about the oncogenic roles of DANCR in PCa. DANCR was upregulated in PCa tissues and cell lines (Jia et al., 2016; Zhao et al., 2019). Lu Y. et al. (2018) found that DANCR expression was induced by MYC, a common oncogene, which resulted in the reduction of p21, a protein required for cell cycle progression. Enzalutamide, a kind of AR (androgen receptor) inhibitor, was used to treat PCa. However, in some cases, it caused side effects such as PCa metastasis (Lin et al., 2013). DANCR knockdown limited the enzalutamide-induced metastasis. Mechanically, DNACR could inhibit TIMP2/3 expression by binding to EZH2 (Jia et al., 2016). Their studies suggested that DANCR might be a potential target for PCa.

Chemotherapy with Taxol (paclitaxel and its semisynthetic analog docetaxel) is commonly used for CRPC. However, there are obstacles to drug resistance (Gan et al., 2009; Jiang and Huang, 2010). Ma et al. (2019) and Zhao et al. (2019) found that DANCR could function as a sponge to miR-135a and miR-34a-5p, and eventually triggered the resistance to paclitaxel and docetaxel, respectively. Meanwhile, DANCR knockdown could



promote the sensitivity of PCa cells to these drugs. Thus, DANCER provides a promising target to improve the effectiveness of chemotherapy for PCa.

### Ovarian Cancer

Ovarian cancer (OC) is typically diagnosed at an advanced stage and has no effective screening strategy (Matulonis et al., 2016). Searching available biomarkers and developing appropriate targeted therapies are in need (Cortez et al., 2018).

DANCER was detected upregulated in ovarian cancer tissues and cell lines (Lin et al., 2019). Vascular endothelial growth factor (VEGF) is the master regulator of vessel formation, resulting in the growth and metastasis of tumors (Chen et al., 2018). Lin et al. (2019) suggested that DANCER can facilitate angiogenesis by regulating the DANCER/miR-145/VEGF axis in a manner of ceRNA. Additionally, DANCER showed a cancer-promoting property by negatively regulating UPF1. Enhanced UPF1 in OC cells was able to partly reverse the promotion of cell proliferation and migration by DANCER (Pei et al., 2019). Thus, DANCER might serve as a potential therapeutic target for ovarian cancer treatment.

### Cervical Cancer

Infection of specific types of human papillomavirus (HPV) is the primary biological etiology for cervical cancers (CC). Prophylactic vaccination for HPV provides the most effective method of primary prevention against HPV-related diseases (Kessler, 2017). However, the prognosis of advanced patients with cervical cancer is still poor (Siegel et al., 2021).

Liang et al. (2019) found that the expression of DANCER was aberrantly increased in cervical cancer tissues and cell lines and its high expression was associated with bigger tumor size, advanced FIGO stage, and poorer prognosis. Further functional analysis showed that DANCER could regulate ROCK1 expression by competitively binding to miR-335-5p and promote CC progression. Interestingly, another study by Ta et al. (2020) observed the diagnostic value of DANCER in distinguishing different types of CC. Their further investigation indicated that DANCER downregulated HIF-1 $\alpha$  expression and inhibited the growth of HPV-negative CC under hypoxic conditions.

### Breast Cancer

From Cancer statistics 2021, breast cancer (BC) has become the most common type of malignancy among women worldwide, responsible for 15% of deaths in women (Siegel et al., 2021). TNBC (triple-negative breast cancer), with a poor prognosis, is a subtype of breast cancer that does not express ER, PR, and HER2 (Wang X. et al., 2019). Though endocrine and HER2-targeted therapy have made great progress in recent years, targeted therapies for TNBC remain unsatisfactory (Costa et al., 2018). Therefore, it is necessary to identify new molecular targets for breast cancer therapy.

DANCER was significantly upregulated in TNBC tissues and cell lines compared with normal ones (Tang et al., 2018). Sha et al. (2017) found that DANCER expression was increased in TNBC tissues compared with that in adjacent normal tissues. Patients with higher DANCER expression tended to have worse

TNM stage and poorer overall survival (OS). Tao et al. (2019) also found that DANCER knockdown significantly suppressed cancer cell proliferation and invasion, while the opposite phenomena were observed when DANCER was overexpressed. Inhibition of DANCER expression also impaired the growth of breast tumors *in vivo*.

The molecular mechanism analysis showed that DANCER played oncogenic roles by targeting miR-216a-5p as a ceRNA in MDA-MB-231 cells (TNBC cell line) (Tao et al., 2019). In addition, DANCER knockdown was associated with reduced expression of CD44, ABCG2, and ALDH1 in TNBC cells (Sha et al., 2017). Similarly, Tang et al. (2018) showed that DANCER could activate PI3K/AKT signaling through the activation of serine phosphorylation of RXRA by binding of GSK3 $\beta$  and RXRA, which promotes breast cancer progression. Zhang K.J. et al. (2020) verified that DANCER regulated EMT and cancer stemness in BC cells by binding to EZH2, which suppressed SOCS3 (suppressor of cytokine signaling 3) expression. Thus, DANCER was validated as an oncogene in breast cancer, and targeting DANCER may have therapeutic value in BC.

Interestingly, DANCER has also been suggested as a tumor suppressor in BC. Li et al. (2017c) revealed that DANCER expression was downregulated in BC cells as well as tumor tissues. It was verified that DANCER mediated EZH2 degradation and attenuated EMT and metastasis in BC. Another study from the same lab also showed that DANCER could inhibit TGF- $\beta$ -induced EMT progress and BC metastasis by downregulating RUNX2 expression (Li et al., 2017b; **Figure 5**).

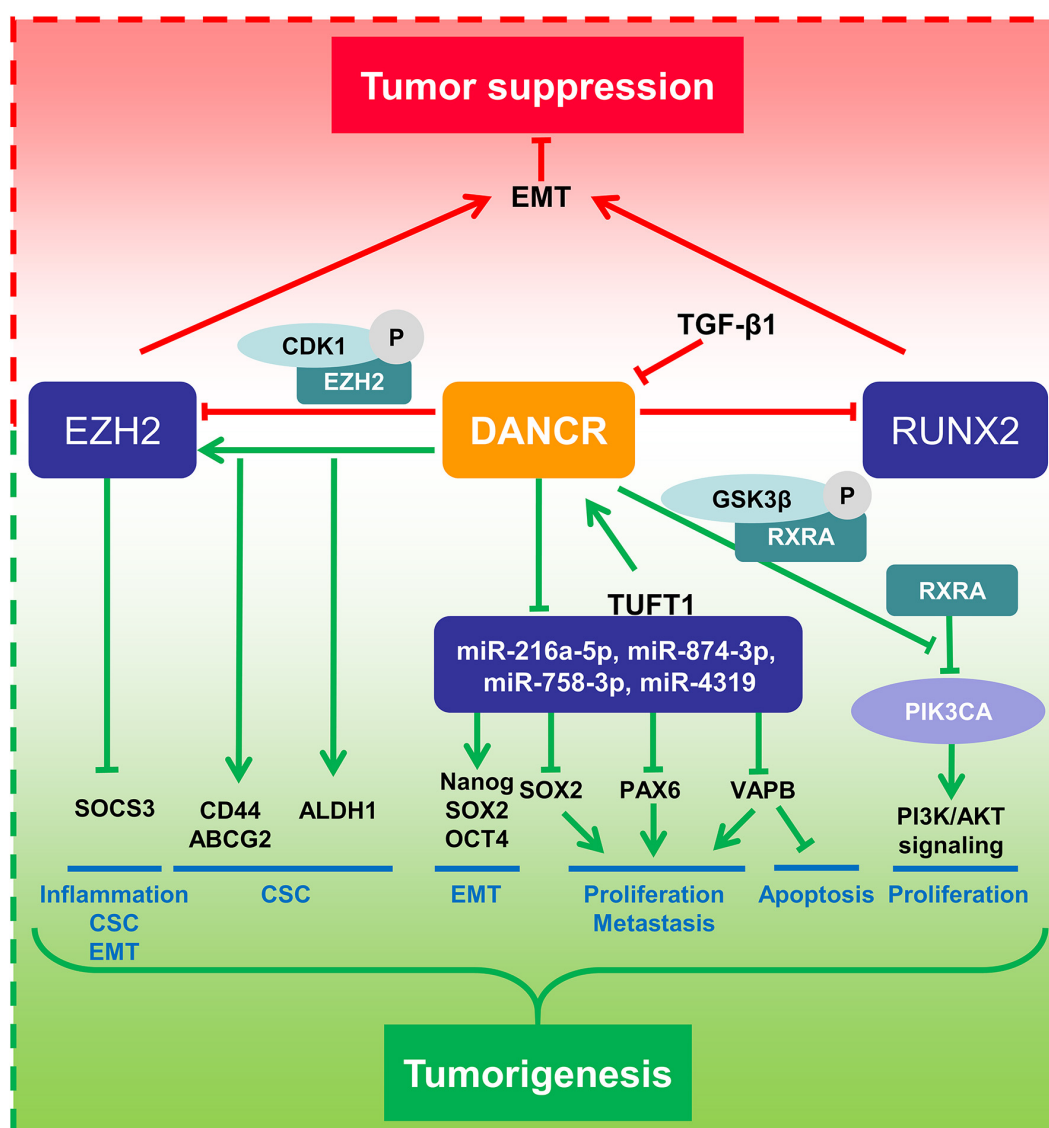
## Urinary System

### Bladder Cancer

Bladder cancer (BCa) is among the top ten most common cancer types and the second most common genitourinary malignancy globally, with approximately 550,000 new cases annually (Bhanvadia, 2018; Richters et al., 2019). Diagnosis often occurs too late, particularly in women, due to their misinterpret of hematuria (Grayson, 2017). Thus, it is essential to find potential biomarkers to monitor tumorigenesis, development, and progression in BCa.

Zhan et al. (2018) showed that DANCER was aberrantly upregulated in BCa tissues compared to adjacent normal tissues. Moreover, increased DANCER expression was positively related to higher histological grade and advanced TNM stage. DANCER played as a miRNA sponge to positively regulate the expression of MSI2 (musashi RNA binding protein 2) via sponging miR-149 and subsequently promoted the malignant phenotypes of BCa cells.

Chen et al. (2019) also found that DANCER was significantly upregulated in bladder cancer with lymph node (LN) metastasis. Aberrant expression of DANCER in BCa was associated with LN metastasis and poor prognosis. DANCER guided leucine-rich pentatricopeptide repeat containing (LRPPRC) to stabilize target mRNAs, including IL-11, PLAU, and CCND1. Further investigations indicated that DANCER/LRPPRC/IL-11/STAT3 signaling pathway played an important role in BCa metastasis.



**FIGURE 5 |** Mechanism of DANCR in breast cancer.

These studies suggested that DANCR might be a valuable target for clinical intervention in LN-metastatic BCa.

### Renal Cell Carcinoma

Renal cell carcinoma (RCC) is one of the most common cancers in the urological system, originating from the renal tubular epithelial system. Surgical resection is an available choice for patients with localized RCC. However, due to the lack of sensitivity to radiotherapy and chemotherapy in RCC, targeted therapy is necessary for individuals without conditions for surgery (Lu et al., 2020).

Jin et al. (2017) found that DANCR was down-regulated in RCC tissues compared to adjacent normal tissues. Overexpression of DANCR caused the suppression of RCC cell proliferation, migration and invasion, and the induction of cell apoptosis. miR-3646 and miR-634 were predicted as the

downstream targets of DANCR. However, biological validation studies are needed to confirm.

### Endocrine System

#### Papillary Thyroid Cancer

Papillary thyroid cancer (PTC) is the most prevalent form of thyroid cancer with a rapidly increasing incidence without a concomitant rise in mortality (Jegerlehner et al., 2017; Abdullah et al., 2019). Though the mean survival rate after 10 years is higher than 90%, disease recurrence cannot be ignored (Li et al., 2017a). An efficient and accurate diagnosis of PTC is still needed (Zhang H. et al., 2018).

Zhang K. et al. (2019) found that DANCR expression was lower in PTC tissues compared to that in normal thyroid tissues. Its expression was negatively associated with the clinical stage. ROC curve and AUC suggested the clinical diagnosis value of

DANCR in PTC, which indicated DANCR might be a potential marker for PTC diagnosis. However, few mechanism studies about DANCR in PTC have been conducted.

## Nervous System

### Glioma

Glioma is the most frequent and lethal central nervous system (CNS) tumor occurring both in children and adolescents (Sturm et al., 2017). DANCR expression was significantly higher in glioma cells than in normal human astrocytes (Wang W. et al., 2019). DANCR served as a ceRNA to modulate tumorigenesis, growth and metastasis by sponging miR-33a-5p (Yang et al., 2018), miR-634 (Xu et al., 2018), miR-216a (Wang W. et al., 2019) and miR-135a-5p (Feng et al., 2020) via regulating miR-33a-5p axis, miR-634/RAB1A axis, miR-216a/LGR5 axis, and miR-135a-5p/BMI1 axis, respectively. Li and Zhou (2018) also verified that high expression of DANCR might be a poor prognostic factor in glioma patients. Moreover, Ma Y. et al. (2018) found that DANCR promoted cisplatin resistance via activating AXL/PI3K/Akt/NF- $\kappa$ B signaling pathway through competitively binding with miRNAs, including miR-33a-5p, miR-33b-5p, miR-1-3p, miR-206, and miR-613 in glioma. These studies suggested that DANCR would be a potential biomarker for predicting cisplatin sensitivity and a therapeutic target for enhancing cisplatin efficacy in glioma.

## Motor System

### Osteosarcoma

Osteosarcoma is one of the most common primary solid malignancies of bone and mainly occurs in adolescence. Though the cure rate for conventional treatment of osteosarcoma is close to 70%, once osteosarcoma has spread to distant organs such as lungs, the survival rates are disappointing (Ritter and Bielack, 2010; Fan et al., 2020). Understanding the molecular mechanisms of osteosarcoma might provide new chances for early diagnosis and targets for therapy.

Increased expression of DANCR could be detected in osteosarcoma tissues and cell lines. Enhanced DANCR was found to have a positive correlation with poor prognostic outcomes (Jiang et al., 2017). DANCR suppression could restrain osteosarcoma progression by inhibiting autophagy (Pan et al., 2020). In recent studies, DANCR was found to function as a ceRNA to promote osteosarcoma progression by sponging miR-33a-5p (Jiang et al., 2017), miR-216a-5p (Pan et al., 2020), miR-149 (Zhang W. et al., 2020), miR-335-5p, and miR-1972 (Wang et al., 2018c). The interaction between DANCR and EZH2 was also found in osteosarcoma, which led to the inhibition of p21 and p27 expression. In conclusion, these studies indicated that DANCR could be utilized as a potential therapeutic target for the treatment of osteosarcoma.

## Other Diseases

Not only in cancer, DANCR could also participate in various biological processes and other diseases. Many research indicated that DANCR might play important role in the differentiation of mesenchymal stem cells (MSCs). Zhang J. et al. (2018) found

DANCR was downregulated in human bone marrow-derived MSCs (BD-MSCs) during osteogenic differentiation. Their further investigation revealed DANCR could inhibit proliferation and osteogenic differentiation through p38 MAPK pathway. Similarly, Weng et al. (2021) observed the same function of DANCR in osteogenic differentiation and proposed another mechanism hypothesis. They considered that DANCR might suppress osteogenic differentiation via miR-1301-3p/PROX1 axis (Weng et al., 2021). With a similar situation to BD-MSCs, osteogenic differentiation capacity in periodontal ligament stem cells could also be inhibited by DANCR (Wang Z. et al., 2020). Apart from that, DANCR suppressed vascular smooth muscle cells transforming into osteoblast-like cells, thus attenuating arterial calcification (Zhang X. et al., 2020). Moreover, odontoblast differentiation in human dental pulp cells (Chen et al., 2016; Chen L. et al., 2020) and chondrogenic differentiation in human synovium-derived stem cells (Zhang et al., 2017) was inhibited and promoted by DANCR, respectively. To sum up, our increasing knowledge of DANCR indicated that targeting DANCR may be a novel therapeutic method in many diseases.

## DISCUSSION AND CONCLUSION

Dysregulation of lncRNAs is involved in regulating diverse malignant behaviors of cancer cells, leading to cancer progression and metastasis. It indicates that developing new diagnostic methods and therapeutic options targeting lncRNAs may be a new answer to the fight against cancers. LncRNA-DANCR, a booming researching topic in recent years, has been demonstrated to regulate many cellular functions such as proliferation, apoptosis, EMT, and CSC in various human cancers. The mechanism by which DANCR promotes tumor development is extremely complicated, including serving as a ceRNA for miRNAs, interacting with mRNAs or proteins, activating signaling pathways, and regulating epigenetic modulations. This review depicts a comprehensive picture of the biological roles of DANCR and its underlying mechanisms in cancer.

In most cancers, DANCR was upregulated and acted as an oncogene. However, a minority of studies reported that DANCR functioned as a potent tumor suppressor. Even in the same cancer type, the role of DANCR contradicted with each other (Li et al., 2017c; Zhang X.H. et al., 2020). This may be due to the heterogeneity in different cell lines, clinical sample selection, and experimental design (Table 3). Moreover, some of the confusing results in these studies may be explained by dynamic cancer progression. As in pancreatic cancer, MLL3 was only downregulated by DANCR at an advanced stage (Liu et al., 2020b). In addition, the study of gene expression always focuses on bulk analysis, only showing the information of the dominant cellular subset (Kanzaki and Pietras, 2020). Fortunately, emerging single-cell sequencing can reveal the uniqueness of individual cell and provide individualized therapy for patients (Ding et al., 2020).

As shown in **Figure 4**, the downstream mechanisms are complicated. So when focusing on different targets of DANCER, we may come to different conclusions. These conjectures indicate that more studies on DANCER are needed to further clarify its role in specific cancer types and under distinct conditions.

DANCER is considered as a powerful biomarker not only in discriminating cancer patients from healthy people or patients with benign diseases but also in helping to predict the prognosis for cancer patients. Moreover, the combination of DANCER and other traditional biomarkers may enhance diagnostic efficiency. For cancer treatment, DANCER may be a promising target due to its essential role in cancers. Though DANCER was a promising biomarker and therapeutic target in cancer, more comprehensive and systematic clinical studies and more extensive sample tests are needed to further explore these complex issues. Further studies in DANCER mechanistic investigations and clinical application are still needed. Only after the mechanisms of DANCER in specific cancers have been elucidated can it likely be used for therapeutic purposes.

## AUTHOR CONTRIBUTIONS

JY, XiZ, and XF conceived the project and supervised the writing. MW and JG wrote the draft of the review. XuZ and XF provided

funds and assisted with preparation of the manuscript. All authors are involved in the revision and approved the final version of the manuscript.

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# Long Non-Coding RNA: A Potential Strategy for the Diagnosis and Treatment of Colorectal Cancer

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Colorectal cancer (CRC), being one of the most commonly diagnosed cancers worldwide, endangers human health. Because the pathological mechanism of CRC is not fully understood, there are many challenges in the prevention, diagnosis, and treatment of this disease. Long non-coding RNAs (lncRNAs) have recently drawn great attention for their potential roles in the different stages of CRC formation, invasion, and progression, including regulation of molecular signaling pathways, apoptosis, autophagy, angiogenesis, tumor metabolism, immunological responses, cell cycle, and epithelial-mesenchymal transition (EMT). This review aims to discuss the potential mechanisms of several oncogenic lncRNAs, as well as several suppressor lncRNAs, in CRC occurrence and development to aid in the discovery of new methods for CRC diagnosis, treatment, and prognosis assessment.

**Keywords:** long non-coding RNA, colorectal cancer, drug resistance, proliferation, metastasis, occurrence, invasion

## INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. In the USA, CRC is the third leading cause of cancer mortality in both men and women. There will be over 10,000 new cases in 2020, and the proportion of young patients is increasing (1). In addition, the incidence rate of CRC in transitioned countries is approximately 4-fold higher than that in transitioning countries (2). Since the pathological mechanism of CRC is not yet fully understood, further studies are urgently needed to identify and develop new effective biomarkers and targets for its diagnosis and treatment.

Long non-coding RNAs (lncRNAs) are non-coding transcripts composed of more than 200 nucleotides that have a variety of regulatory modes. lncRNAs interact with proteins, RNA, and DNA and form RNA-RNA, RNA-DNA, and RNA-protein complexes, allowing them to participate in many important biological processes, such as transcription, intranuclear transport, and genomic imprinting (3, 4). Recent studies have shown that lncRNAs are involved in the regulation of CRC occurrence and development. Some oncogenic lncRNAs promote the occurrence, proliferation, invasion, metastasis, and drug resistance of CRC cells, while some lncRNAs suppress the proliferation and metastasis of CRC cells. The potential mechanism of lncRNAs in CRC are combining with proteins to form complexes to regulate the target downstream, affecting miRNA translation, acting as miRNA sponges (ceRNA) or scaffolds, regulating relevant signalling pathways



and cell cycle progression, as well as the expression of transcriptional factors, ribosomal biogenesis factors, and anti-oncogenes.

Understanding the potential roles of lncRNAs in CRC occurrence, proliferation, invasion, metastasis, and drug resistance, as well as the effects of some suppressor lncRNAs related to CRC, can provide new ideas and countermeasures for the diagnosis, assessment, and treatment of CRC.

## LNCRNAs IN COLORECTAL CANCER

LncRNAs can regulate the occurrence and development of CRC. Some lncRNAs tend to promote cancer cell proliferation, invasion, metastasis, and drug resistance, while others suppress cancer cell proliferation and metastasis. We discuss their roles and related molecular mechanisms in the following sections.

### LncRNAs in Colorectal Cancer Occurrence

LncRNA ubiquitin-like plant homeodomain (PHD) and really interesting new gene (RING) finger domain-containing protein 1 (UHRF1) Protein Associated Transcript (UPAT) expression is significantly upregulated in highly tumorigenic CRC cell lines compared to that in weak tumorigenic and normal cell lines, as evaluated through quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. UPAT stabilizes the UHRF1 protein by interfering with the ubiquitination and degradation mediated by  $\beta$ -TrCP E3 ubiquitin ligase ( $\beta$ -TrCP1 and  $\beta$ -TrCP2), thus promoting the expression of stearyl-coenzyme A desaturase-1 (SCD1) and sprouty RTK signaling antagonist 4 (SPRY4). UPAT can also upregulate the expression of phosphoglucomutase 1 (PGM1) and G protein-coupled receptor class C group 5 member A (GPRC5A), but the specific mechanism is not yet clear (5). The epigenetic factor UHRF1 regulates transcription by regulating DNA methylation and histone modification and plays a key role in tumor cell proliferation and survival (6). SCD1, SPRY4, PGM1, and GPRC5A are necessary for the transformation of normal cells into cancer cells and their survival and development (7–9). The UHRF1-UPAT axis may be a promising molecular target for the treatment of CRC. However, the regulatory mechanism of UPAT on the expression of PGM1 and GPRC5A requires further study.

Polycomb repressive complex 2 (PRC2) and DEAD box protein 5 (DDX5) associated lncRNA (PRADX), which acts as a cancer driver, is highly expressed in CRC cells and tissues and is mainly distributed in the nucleus. Enhancer of zeste homolog 2 (EZH2), a histone methyltransferase that catalyzes histone H3 lysine 27 trimethylation (H3K27me3) and epigenetically silences target genes (10), is overexpressed in many cancer types and has been shown to act as an oncogene (11, 12). PRADX can bind to the EZH2 protein through its 1-500 bp 5' end sequence to recruit PRC2 and DDX5, forming a PRC2/DDX5 complex (13). Activation of the nuclear factor kappa B (NF- $\kappa$ B) pathway promotes the occurrence of colorectal adenocarcinoma. UBXN1 is a UBX domain protein that can inhibit the degradation of I $\kappa$ B $\alpha$

thus blocking the NF- $\kappa$ B pathway (14). Because the PRC2/DDX5 complex can inhibit the expression of UBXN1, the NF- $\kappa$ B pathway is activated thereby promoting the occurrence of colon adenocarcinoma (15).

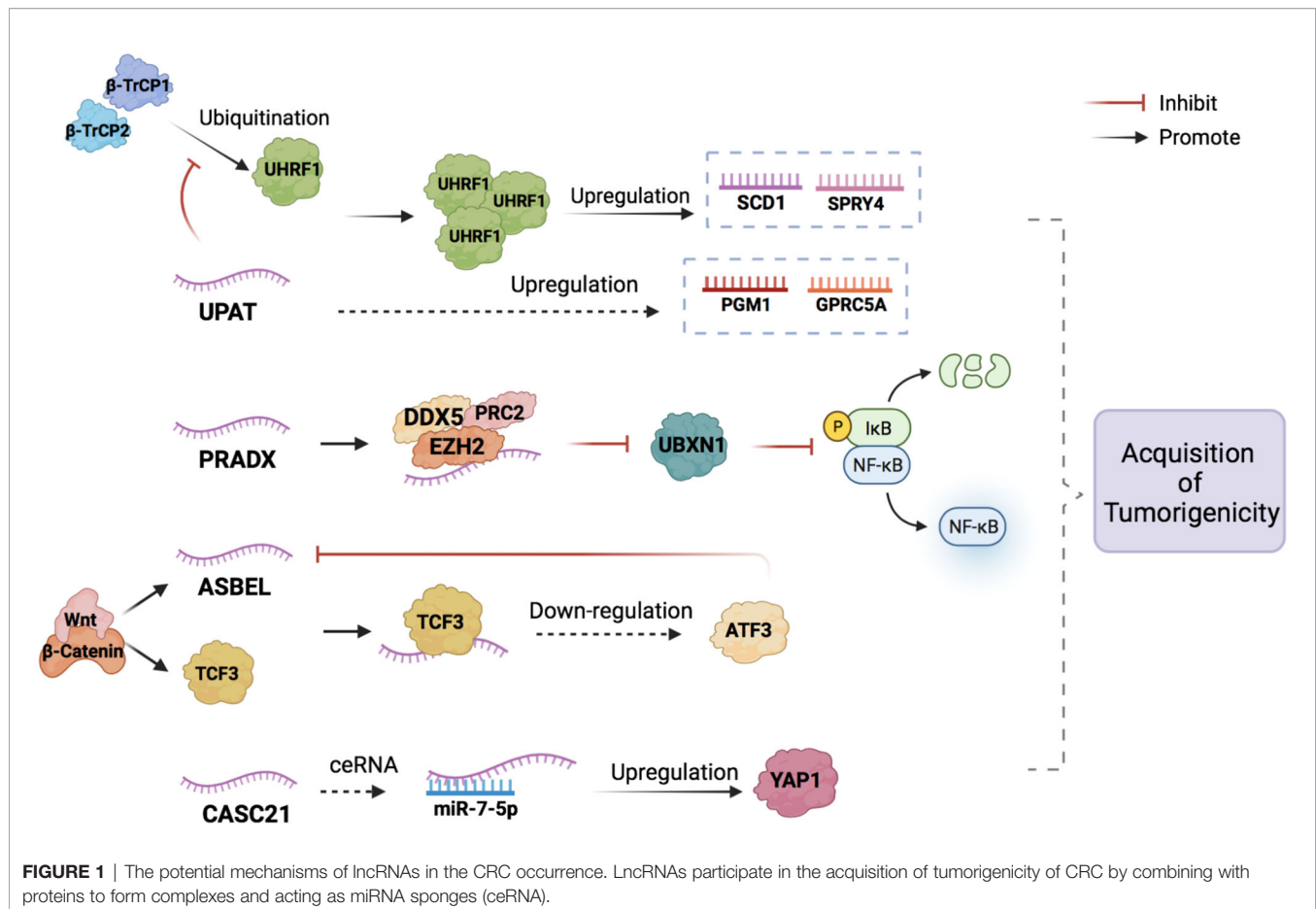
LncRNA antisense ncRNA in the abundant in neuroepithelium area (ANA)/B-cell translocation gene 3 (BTG3) locus (ASBEL) is a tumorigenic gene that can be directly activated by the Wnt/ $\beta$ -catenin pathway. At the same time,  $\beta$ -catenin, can also activate the transcription factor TCF3 (16). TCF3 can form a complex with ASBEL to downregulate the expression of the target transcription factor ATF3 and inhibit the development of CRC. The classic Wnt/ $\beta$ -catenin signaling pathway plays an important role in regulating proliferation, cell fate, stem and progenitor cell self-renewal, and tumorigenesis (17–19). The role of this signaling pathway in carcinogenesis was first described in the context of adenomatous polyposis coli (APC) gene mutations. APC mutations are usually acquired early in the onset of most colon cancers (over 80%), leading to the cytoplasmic accumulation of  $\beta$ -catenin, which binds to TCF/Lef1 and shuttles to the nucleus, where it acts as a transcription factor and promotes cell proliferation (20). Thus, the  $\beta$ -catenin-ASBEL-TCF3-ATF3 pathway may be a promising target for colon cancer treatment.

Cancer susceptibility 21 (CASC21) is significantly upregulated in CRC tissues. Yes1 Associated Transcriptional Regulator (YAP1), a well-studied transcriptional coactivator, is a main downstream effector of the Hippo pathway that plays a critical role in controlling organ size in animals (21). YAP1 is known to be upregulated in some solid tumors, including CRC, and acts as an oncogene that promotes tumor cell proliferation, migration, and invasion (22, 23). Therefore, the upregulation of YAP1 promotes the occurrence of CRC. LncRNAs can regulate the mRNA expression of many functional target genes by functioning as competing endogenous RNAs (ceRNAs) that sponge microRNAs (miRNAs) and competitively inhibit the binding of miRNAs to targets (24, 25). miRNAs are short non-coding RNA molecules that inhibit the expression of target genes by cutting down mRNA or inhibiting translation (26). CASC21 acts as a ceRNA sponging miR-7-5p to upregulate YAP1 expression and thus promote the occurrence of CRC (27).

The finding of lncRNA tumorigenicity helps us detect CRC at an early stage, and related molecular signaling pathways may provide us with potential targets for treatment (Figure 1, Table 1).

### LncRNAs in Colorectal Cancer Cell Proliferation

Glycolysis-associated lncRNA of colorectal cancer (GLCC1) is an oncogene in CRC, which is involved in the glycolysis of CRC cells (30). Abnormal activation of glycolytic pathways in cancer cells is considered a sign of malignancy (31). GLCC1 stabilizes c-Myc by binding to HSP90 (HSP90AA1) chaperone and preventing cytoplasmic ubiquitination degradation, thereby increasing the transcription level of lactate dehydrogenase A (LDHA) and activating glycolytic metabolism. HSP90, as a protein chaperone, can stabilize transcription factors, protein kinases, and oncoproteins in the tumor signaling pathway (32). C-myc is an important oncogene involved in regulating glucose metabolism



and is the key on/off switch in cancer cell metabolism. High levels of LDHA expression are associated with poor clinical outcomes of CRC due to its regulation of glycolytic metabolism in cancer cells (41, 42). GLCC1-c-Myc-LDHA, as a cascade reaction coordinated by GLCC1 under glucose starvation, may be a promising metabolic blocker target for antitumor therapy.

LncRNA X-inactive specific transcript (XIST) expression in CRC tissues is abnormally high. XIST can target and downregulate miR-486-5p, leading to CRC cell proliferation (43). The miRNA miR-486-5p plays a protective role against CRC, and it acts by obstructing the expression of neuropilin-2 (NRP-2) (44), which is known to interfere with the epithelial-mesenchymal transition (EMT) of CRC cells *via* crosstalk with transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) (33).

The lncRNA colorectal differentially expressed (CRNDE) was originally found to be highly expressed in colorectal adenomas and adenocarcinomas (34). CRNDE targets and upregulates miR-181a-5p. Overexpression of miR-181a-5p reduces the expression of endogenous  $\beta$ -catenin and TCF4, leading to the inhibition of the Wnt/ $\beta$ -catenin signaling pathway. CRNDE binds to miR-181a-5p and blocks its inhibitory effect on Wnt/ $\beta$ -catenin signaling, resulting in the proliferation of CRC cells (35). From this perspective, we can hypothesize that it is possible to search for miRNA agonists that can compete against miR-

181a-5p in binding with CRNDE to inhibit the Wnt/ $\beta$ -catenin signaling pathway on the proliferation of colon cancer cells.

LncRNA small nuclear RNA host gene 3 (SNHG3) expression in CRC tissue has been found to be significantly upregulated compared to that in adjacent normal tissues. The binding of SNHG3 and miR-539 can upregulate the expression of its target gene, runt-related transcription factor 2 (RUNX2), to promote cancer cell proliferation (45). RUNX2 is involved in the occurrence and development of a variety of cancers, including CRC, and plays a role as an oncogene (36, 37). The authors believe that interfering with the ceRNA mechanism of SNHG3 and blocking its inhibitory effect on miRNA may help suppress the development of CRC.

The expression of lncRNAs BRAF-activated non-coding RNA (BANCR) and chromosome segregation like 1 (CSE1L) has also been found to be significantly upregulated in CRC tissues. BANCR acts as a molecular sponge for miR-203 and separates miR-203 from CSE1L in CRC cells, thus upregulating the expression of CSE1L (46). The upregulation of CSE1L expression, in turn, can promote the proliferation and invasion of cancer cells (38, 47). BANCR knockdown suppressed CRC cell proliferation, and CSE1L overexpression reversed the anti-proliferation and anti-invasion effects of BANCR silencing (46). Thus, the upregulation of BANCR may promote CRC development through the miR-203/CSE1L axis.

**TABLE 1 |** LncRNAs in the occurrence, proliferation, invasion, and metastasis of CRC cells.

lncRNAs	Expression	Function	Downstream targets	Reference
UPAT	↑	Promotes tumorigenesis	SCD1, SPRY4 PGM1, GPRC5A	(5)
PRADX	↑	Promotes tumorigenesis	EZH2	(6)
ASBEL	↑	Promotes tumorigenesis	ATF3	(15)
CASC21	↑	Promotes tumorigenesis	mir-7-5p/Yap1	(14)
GLCC1	↑	Promote glucose metabolism, proliferation	c-Myc	(16)
XIST	↑	Promote proliferation, EMT	mir-486-5p	(28)
CRNDE	↑	Promote proliferation, Drug resistance	mir-181a-5p	(17)
SNHG3	↑	Promote proliferation, metastasis	mir-539/Runx2	(18)
BANCR	↑	Promote proliferation, Drug resistance	mir-203/CSE1L	(19)
CCAT2	↑	Promote proliferation, drug resistance	mir-145, BOP1 MYC	(20, 29)
P14AS	↑	Promote proliferation	lncRNA ANRIL	(22)
00659	↑	Promote proliferation	/	(30)
LOC441461	↑	Promote proliferation, metastasis	RhoA/ROCK MLC, LIMK1	(31)
PRNCR1	↑	Promote proliferation, Regulates cell cycle	/	(32)
SNHG1	↑	Promote proliferation	mir-154-5p/EZH2	(33)
CYTOR	↑	Increases migration, invasion	Wnt/β-Catenin	(34)
RAMS11	↑	Promote metastasis	TOP2α	(35)
LDLRAD4-AS1	↑	Promote metastasis, EMT	snail, E-cadherins	(36)
CALIC	↑	Promote metastasis	AXL	(37)
GSEC	↑	Promote metastasis	DHX36	(38)
ZEB1-AS1	↑	Enhances growth, metastasis	mir-455-3p/PAK2	(39)
LINCO1578	↑	Promote metastasis	NF-κB/YY1	(40)

The lncRNA colon cancer-associated transcript 2 (CCAT2), which is overexpressed in CRC tissues, can promote cancer cell proliferation (39). Yu et al. found that miR-145 could modulate the proliferation and differentiation of colon cancer stem cells (CSCs). CCAT2 can selectively inhibit miR-145 maturation by preventing the export of pre-miR-145 to the cytoplasm (39).

P14AS, which binds to AU-rich binding factor 1 (AUF1), is a novel lncRNA transcribed from the antisense strand of the CDKN2A/P14 gene (48). AUF1 is an RNA-binding protein that promotes the expression of many cancer-related RNAs, including c-Myc, P16, and NEAT1 (40, 49, 50). The carcinogenicity of lncRNA ANRIL (CDKN2B-AS1) has been confirmed in various studies (51, 52), and the binding of lncRNA P14AS and AUF1 can increase the levels of ANRIL, resulting in cancer cell proliferation.

Recently, many studies have found that the knockdown of some lncRNAs, such as lncRNA 00659, LOC441461, and lncRNA prostate cancer-associated non-coding RNA 1 (PRNCR1), can inhibit cancer cell proliferation by interfering with the process of the cell cycle (53–55). Among these, the knockdown of LOC441461 expression inhibits the phosphorylation of MLC and LIMK1 by inhibiting RhoA/ROCK signaling, thereby affecting the cell cycle and inducing apoptosis to prevent the proliferation of cancer cells (54).

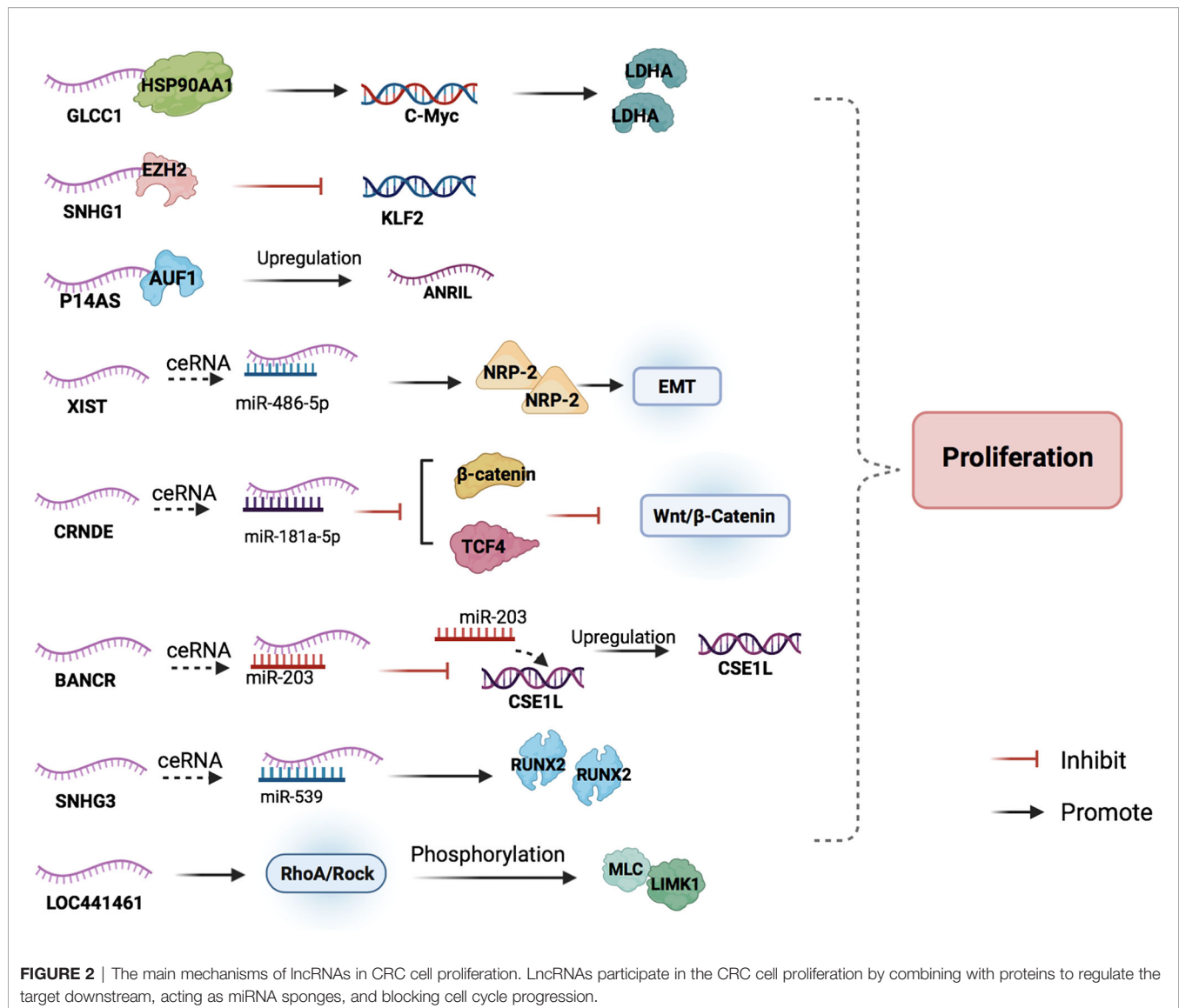
SNHG1 expression was found to be significantly upregulated in CRC tissues while Kruppel-like factor 2 (KLF2) was downregulated. KLF2 possesses tumor-suppressor features, such as inhibition of cell proliferation and enhancement of DNA damage-associated apoptosis in many cancers (56).

SNHG1 can directly interact with EZH2 to silence KLF2 expression and promote CRC proliferation (29).

The discovery that lncRNAs promote CRC cell proliferation may help improve the prognosis of patients. Studying their specific mechanisms may contribute to the development of new therapeutic targets to inhibit the proliferation of tumor cells (Figure 2, Table 1).

## LncRNAs in Colorectal Cancer Cell Invasion and Metastasis

The lncRNA cytoskeleton regulator RNA (CYTOR) is involved in CRC cell invasion and metastasis *via* the Wnt/β-catenin pathway. CYTOR binds to cytoplasmic β-catenin and inhibits its phosphorylation induced by kinase 1 (CK1), resulting in β-catenin accumulation and translocation to the nucleus. At the same time, β-catenin enhances CYTOR transcriptional activity in the nucleus, thus forming a positive feedback loop. The wound-healing assay showed that CYTOR knockdown inhibited the migration of HCT8 and SW620 cells, and the depletion of CYTOR colon cancer cells failed to induce further invasion. The nude mouse model for lung metastases inoculated with CYTOR-expressing cancer cells showed that the CYTOR knockdown group had a decrease in the size and number of metastatic tumor nodules compared with the control group. This illustrated that CYTOR promoted CRC cell invasion and metastasis both *in vivo* and *in vitro*. The upregulation of CYTOR accelerates β-catenin nuclear translocation and increases the transcription activity of the β-catenin/TCF complex in the nucleus, activating the Wnt/β-catenin pathway to promote cancer cell invasion and metastasis (57).



RNA associated with metastasis 11 (RAMS11) is a lncRNA that can induce tumor formation and promote tumor growth and metastasis. Compared to that in primary tumor tissues, the expression level of RAMS11 was increased in metastatic colon cancer cells. Upon measuring samples from two independent cohorts of colon cancer patients through qPCR, the expression of RAMS11 in metastatic samples was upregulated compared with that in primary cancer tissues. RAMS11 promotes topoisomerase II alpha (TOP2α) expression by binding to Chromobox protein 4 (CBX4). Silencing CBX4 or TOP2α can slow down the invasion and metastasis of LoVo colon cancer cell lines (58). TOP2α is used as a proliferation marker for many cancer types, including CRC (59, 60), and its increased expression levels are associated with prostate cancer, pancreatic cancer, and breast cancer metastases (61–64). In patients with primary and metastatic CRC (mCRC), the expression of TOP2α is elevated (65–67). RAMS11 promotes the resistance of colon cancer cells to

topoisomerase inhibitors, which has become the basic principle for the use of anthracyclines to treat certain mCRC patients (58).

LDLRAD4 antisense RNA 1 (LDLRAD4-AS1) expression levels were reported to be higher in rectal cancer tissues than in adjacent normal tissues. Using the Transwell assay, Mo et al. found that the overexpression of the lncRNA LDLRAD4-AS1 promoted the migration and invasion of highly invasive CRC cell lines (RKO and LoVo) *in vitro*. LDLRAD4-AS1 also enhanced the migration ability of RKO and LoVo cells in a wound-healing assay. The upregulation of lncRNA LDLRAD4-AS1 expression destabilizes LDLRAD4 mRNA and decreases LDLRAD4 mRNA expression at the protein level, thus reducing the transcription factor snail and E-cadherins, which then promotes epithelial interstitialization and metastasis (68). EMT, marked by the loss of E-cadherin, enables the epithelial cells of a primary tumor to lose cell polarity and break the cellular adhesion constraints, allowing cancer cells to acquire migratory and invasive



characteristics and be mesenchymal-like towards aggressive malignancy (69–71).

The lncRNA cancer metastasis-associated long intergenic non-coding RNA (CALIC) was significantly upregulated in subpopulations of HCT116 cells that were selected for their elevated metastatic activity. The RNA-Seq and gene ontology (GO) analysis on HCT116 cells that used small interfering RNA (siRNA) to knock down CALIC showed that CALIC target genes are enriched in genes involved in “cell movement” and “cell localization” and that CALIC knockdown inhibits high-level expression CALIC’s WiDr colon cancer cell migration. In contrast, the knockdown had little effect on the migration of Caco-2 and Caco-320 colon cancer cells that expressed low levels of CALIC (72). The receptor tyrosine kinase AXL, which regulates FAK1, RHO family GTPases, and GTP exchange factor Vav1, is important in cancer cell migration and invasion. CALIC associates with the RNA-binding protein heterogeneous nuclear ribonucleoprotein L (hnRNP-L) and upregulates AXL, thereby promoting migration and metastasis of colon cancer cells (73, 74).

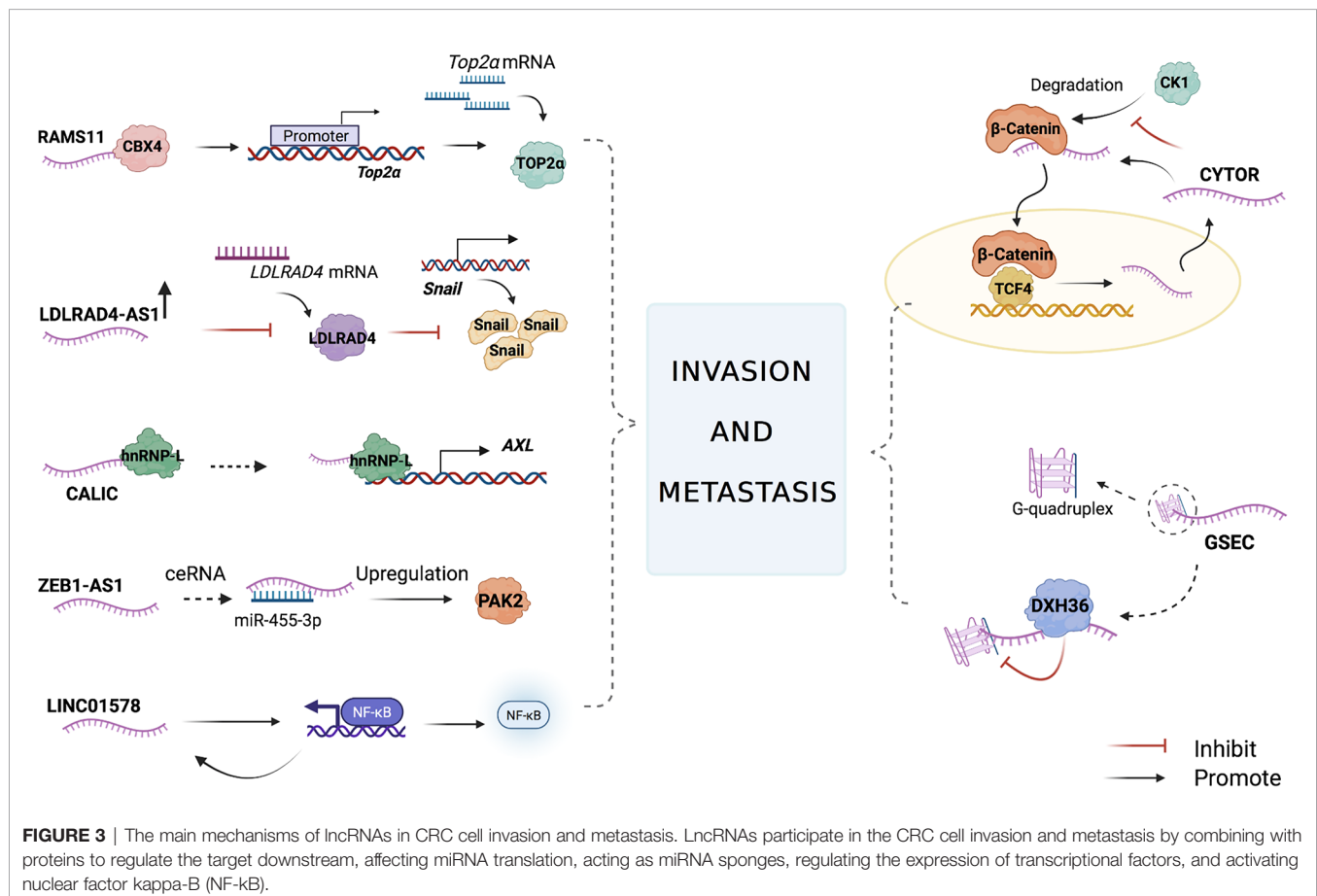
G-quadruplex forming sequence containing lncRNA (GSEC) is enriched in the cytoplasm of colon cancer cells, and its expression is significantly higher than that in the surrounding normal colon tissue. DHX36 is a GSEC-related protein that has the ability to unfold G-quadruplex. Overexpression of DHX36

inhibits the motility of the colon cancer cell line DLD-1. GSEC is a G-quadruplex-containing lncRNA that can bind to DHX36, thus inhibiting the unwinding activity of G-quadruplexes to promote colon cancer cell metastasis (28).

The knockdown of the lncRNA zinc finger E-box binding homeobox 1 antisense 1 (ZEB1-AS1), whose expression level increased in colon adenocarcinoma tissues, suppressed the invasion and migration of SW480 and HT29 cells (75). P21-activated kinase 2 (PAK2), a member of the P21-activated kinase (PAK) family of serine/threonine kinases, engages in many signaling pathways related to malignant progression. A study showed that miR-455-3p exhibits anti-cancer effects in colon adenocarcinoma cells by targeting PAK2. ZEB1-AS1 inhibits miR-455-3p and dampens the inhibition of PAK2, thereby promoting cancer cell metastasis (76).

LncRNA LINC01578 activates NF- $\kappa$ B, which, in turn, promotes the expression of LINC01578. Therefore, a positive feedback loop forms between LINC01578 and NF- $\kappa$ B/YY1 (77). NF- $\kappa$ B has been shown to promote colon cancer cell invasion and metastasis (78, 79), and LINC01578 also exerts a similar effect due to the feedback loop (77).

Studying the signaling pathways of lncRNAs in promoting CRC cell metastasis may help us find potential therapeutic targets for treatment and predict the metastasis rate of the disease (Figure 3 and Table 1).



## LncRNAs in Colorectal Cancer Cell Drug Resistance

Currently, improvements in screening, surgical techniques, radiation therapy, and chemotherapy largely contribute to the reduction in the mortality rate of colorectal cancer; however, over 40% of colorectal cancer patients die from recurrence and metastasis, and multiple-drug resistance in chemotherapy is the main reason for treatment failure. There is strong evidence that supports the effect of lncRNA on the increase in drug resistance in CRC cells (80) (**Table 2**).

LncRNA H19 is overexpressed in CRC cells and is associated with the immunostaining score of acetaldehyde dehydrogenase 1A1 (ALDH1A1) in H19-high and H19-low CRC specimens (81). ALDH1A1 is a cancer stem cell marker in colon cancer cells (82), which suggests that H19 is also associated with the malignant potential of CRC stem cells. Studies have evaluated the chemosensitivity of CRC cells in the routine and widespread use of oxaliplatin, and it has been found that H19 overexpression promotes oxaliplatin resistance in SW480 and HCT116 cells. In addition, an apoptosis assay confirmed that H19 overexpression enhanced oxaliplatin resistance in SW480 cells. Cancer-associated fibroblasts (CAFs) are the main type of stromal cells in the CRC tissue matrix, which can transfer H19 into cancer cells by secreting H19-containing exosomes. H19 can then activate the  $\beta$ -catenin pathway by acting as a ceRNA to sponge miR-141, thus increasing drug resistance in cancer cells (81).

The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is associated with a poor response to oxaliplatin-based chemotherapy in CRC patients. A study measured the expression level of MALAT1 in the serum of 53 patients with metastatic CRC using qRT-PCR. According to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, patients were divided into two groups: responsive (CR + PR) and non-responsive (SD + PD) groups. The results showed that the non-responsive group had significantly higher MALAT1 expression levels compared with the responsive group (83). During oxaliplatin treatment, MALAT1 binds EZH2 to the CDH1 promoter and inhibits miR-128 to promote drug resistance in colorectal cancer cells (84).

The lncRNA CCAT2 promotes the expression of genes involved in ribosome biogenesis and protein synthesis. A CRC cohort study found that lncRNA CCAT2 was positively associated with the expression of BOP1 ribosomal biogenesis factor (BOP1). Chromosomal instability (CIN) can increase chemotherapy sensitivity in colon cancer cells (85). BOP1 can

increase the active form of aurora B kinase, which is responsible for regulating chromosomal segregation and promoting CIN, thus increasing drug resistance (86). lncRNA CCAT2 directly interacts and stabilizes BOP1. In addition, it activates the expression of BOP1 by increasing MYC expression.

Lnc00152 is highly expressed in SW620 and HT29 cells at the basal level compared to SW480 and Caco2 cells. As for apoptosis-related genes, Western blot analysis of cleaved poly (ADP-ribose) polymerase (PARP) and cleaved Caspase 3 revealed that SW480 and Caco2 cells are more sensitive to oxaliplatin-induced apoptosis than SW620 and HT29 cells. It has been shown that Lnc00152 upregulates ERBB4 by competitively binding to miR-193a-3p and then activates the AKT pathway, thereby leading to the development of resistance to oxaliplatin (87).

LncRNA MIR4435 expression in cisplatin-resistant colon cancer HCT116 cells is seven to eight times higher than that in normal colon cancer cells, as determined through PCR analysis (88). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that responds to oxidative stress and plays a crucial role in redox homeostasis (89). Heme oxygenase (HO-1), which is downstream of Nrf2, also plays a role (90). Both are associated with drug resistance and poor prognosis. The lncRNA MIR4435 may increase the cisplatin resistance of colon cancer cells by promoting the expression of Nrf2/HO-1 (91).

LncRNA POU6F2-antisense 2 (POU6F2-AS2) knockdown in colon cancer cell lines leads to the downregulation of the expression of the anti-cancer genes P-gp, MRP2, and BRCA2. Hence, knockdown of lncRNA POU6F2-AS increases the sensitivity of colon cancer cells to cisplatin (92).

Studies on lncRNAs' regulation of chemoresistance in CRC can help predict drug sensitivity to different chemotherapy treatments in patients with CRC and can serve as a guide in adjusting drug use, modifying treatments, and ultimately improving chemotherapy (**Figure 4**, **Table 2**).

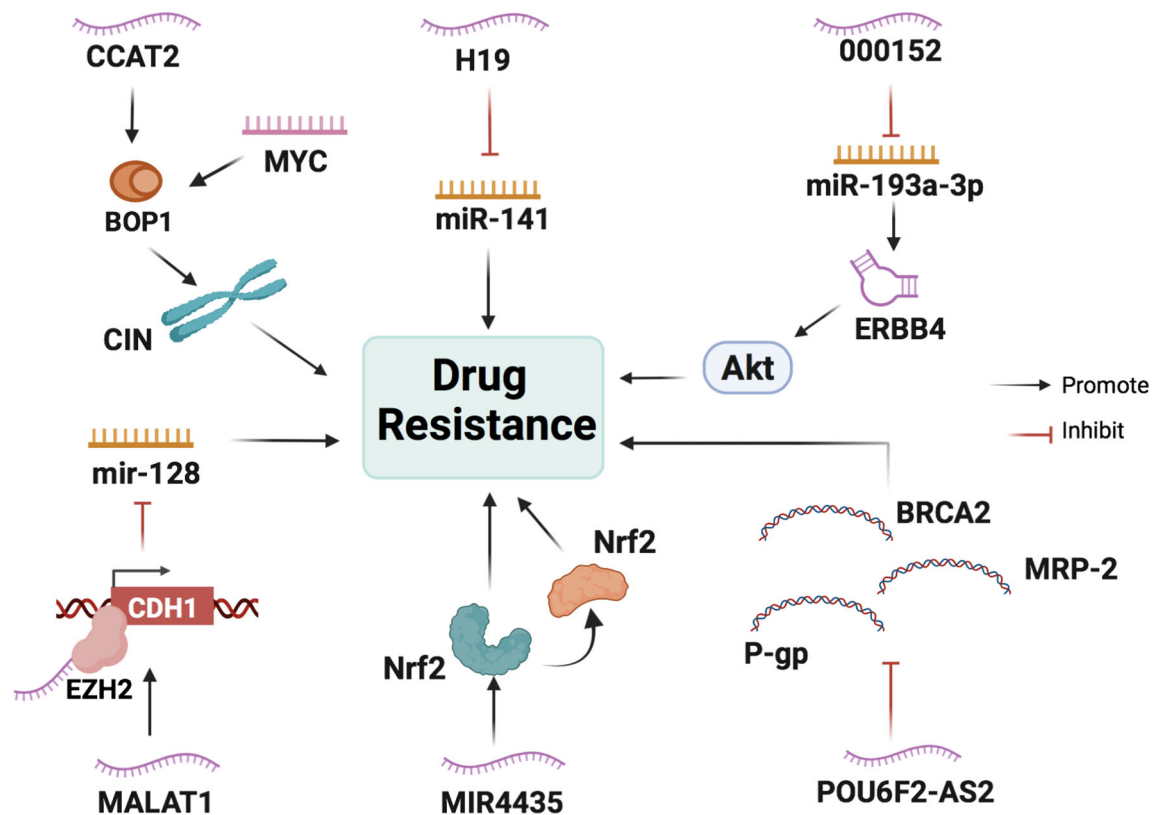
## Inhibitory lncRNAs in Colorectal Cancer

LncRNAs function not only as oncogenes but also as tumor suppressor genes. Promoting the expression of tumor suppressor lncRNAs may provide a new direction for the treatment of CRC.

LncRNA P53 induced transcript (PINT) expression in primary CRC cells is downregulated compared to that in normal colorectal tissues. The lncRNA PINT is the target of the transcription factor P53, which plays a crucial role in cancer suppression. An *in vitro* study showed that colon cancer HCT0116 cells overexpressing

**TABLE 2** | LncRNAs in CRC cell drug resistance.

LncRNAs	Expression	Function	Downstream targets	Reference
CCAT2	↑	Promoted drug resistance, proliferation	mir-145, BOP1 MYC	(20, 29)
H19	↑	Promotes drug resistance	mir-141	(52)
MALAT1	↑	Promotes drug resistance	mir-128	(54)
Linc00152	↑	Promotes drug resistance	mir-193a-3p, ERBB4	(57)
MIR4435	↑	Promotes drug resistance	Nrf2/HO-1	(58)
POU6F2AS	↑	Promote proliferation, drug resistance	P-gp, MRP2, BRCA2	(62)



**FIGURE 4 |** The main mechanisms of lncRNAs in CRC cell drug resistance. LncRNAs participate in CRC cell drug resistance by acting as scaffolds or miRNA sponges and regulating the expression of transcriptional factors, ribosomal biogenesis factors, and anti-oncogenes.

PINT had a slower growth rate than control cells, but the underlying anti-cancer mechanism has not been revealed (93).

LncRNA loc285194, which is significantly downregulated in CRC cells compared with normal tissues, can also be induced by transcription factor P53. Overexpression of loc285194 inhibited colon cancer HCT-116 and MCF-7 cell proliferation by negatively regulating mir-211 (94).

Maternally expressed 3 (MEG3) is a lncRNA that enhances the sensitivity of CRC cells to chemotherapeutic agents. Its expression in CRC tissues is increased compared with that in adjacent normal tissues. MEG3 knockdown promotes the proliferation, migration, and colony formation of CRC cells and induces G0/G1 cell cycle arrest in these cells (95). MEG3 also acts as a sponge miR-141 to increase the expression of PDCD4 in CRC, which enhances the sensitivity of CRC HCT116 and HT29 cells to oxaliplatin (96).

LncRNA overexpressed in colon carcinoma-1 (OCC-1) knockdown in CRC cells promotes the growth of cancer cells (97). Human antigen R (HuR) is an RNA-binding protein that can stabilize mRNAs involved in a variety of biological processes. OCC-1 binds to the HuR protein and increases its interaction with E3 ubiquitin ligase  $\beta$ -TrCP1, leading to the ubiquitination and degradation of HuR protein, which suppresses the progression of CRC (98).

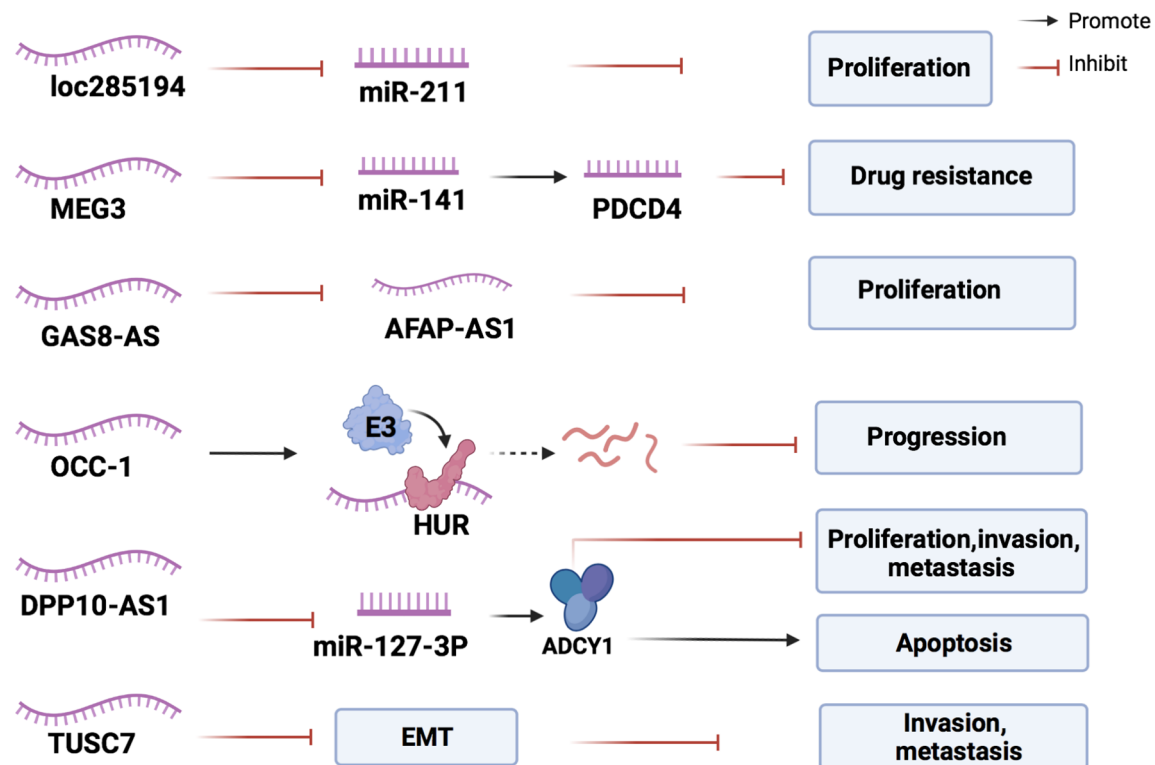
The lncRNA tumor suppressor candidate 7 (TUSC7) is involved in inhibiting the migration and invasion of CRC cells. EMT is a key process in inducing migration and invasion (99, 100). Zhang et al. detected EMT biomarkers through qRT-PCR and Western blot analysis in CRC cells and discovered that the expression level of TUSC7 is positively associated with the expression of E-cadherin and negatively associated with the expression of vimentin. This indicates that TUSC7 can inhibit EMT in CRC cells to suppress invasion and metastasis (101).

The lncRNA DPP10 antisense RNA 1 (DPP10-AS1) exerts anti-tumor effects on colon cancer cells. It can downregulate the expression of its target gene, miR-127-3p, to increase adenylate cyclase 1 (ADCY1). Thus, it can suppress the proliferation, migration, and invasion of CSCs and increase apoptosis (102).

By identifying more inhibitory lncRNAs in CRC, we can explore the possibility of inhibiting tumor progression by upregulating their expression (Figure 5 and Table 3).

## CONCLUSION

Numerous studies have shown that lncRNAs play an important role in the occurrence and development of CRC. Studying the effects of lncRNAs on cancer cell proliferation, invasion,



**FIGURE 5** | The mechanisms of lncRNAs in inhibiting the development of CRC. Inhibitory lncRNAs influence the expression of miRNAs, lncRNAs, and proteins associated with CRC proliferation, and inhibit the intraepithelial mesenchymal transformation of CRC cells to resist cancer progression.

metastasis, and drug resistance, with a focus on the related signaling pathways, can help us to further understand CRC and develop better treatment strategies. For example, lncRNA HOX antisense intergenic RNA (HOTAIR) is involved in cancer cell proliferation, apoptosis, invasion, and metastasis, and its increased expression in the blood is associated with a high mortality rate (103–105). Propofol can inhibit lncRNA HOTAIR and is a potential drug for CRC treatment (106). lncRNA urothelial carcinoma associated 1 (UCA1) also plays a critical role in tumorigenesis (107), and it can be inhibited by metformin resulting in the apoptosis of CRC cells (108). Curcumin, which exists in the rhizomes of *Curcuma longa*, has been approved for the treatment of CRC (109, 110), and it can inhibit tumor occurrence by inducing the expression of a suppressor lncRNA neighbor of BRCA1 gene 2 (NBR2).

Recent studies have reported that lncRNAs can be encapsulated in exosomes and transmitted among tumor cells, regulating the occurrence and development of tumors. Exosomal lncRNAs show high organ specificity in the blood, urine, saliva, and tumor tissue and have the advantages of being non-invasive, repeatably detectable, and real-time monitoring. Thus, exosomal lncRNAs are expected to function as meaningful biomarkers (111–113). CRNDE-h is an exosomal lncRNA that can effectively distinguish CRC patients from benign colorectal diseases and NC subjects with significantly high sensitivity and specificity. In addition, the combination of several tumor markers, such as lncRNA ZFAS1, SNHG11, LINC00909, and LINC00654, can improve the accuracy of CRC diagnosis. Moreover, increased lncRNA SNHG11 helps to further screen and diagnose CRC (109). The SLS model

**TABLE 3** | Inhibitory lncRNAs in CRC.

lncRNAs	Expression	Function	Downstream targets	Reference
Pint	↓	Inhibits proliferation	/	(63)
Loc285194	↓	Inhibits proliferation	miR-211	(64)
MEG3	↓	Inhibits proliferation, metastasis, drug resistance	miR-141/PDCD4	(65, 66)
OCC-1	↓	suppresses growth	HUR	(69)
TUSC7	↓	Inhibits metastasis	EMT	(73)
DPP10-AS1	↓	Inhibits metastasis, proliferation	miR-127-3p	(74)



based on 52 lncRNAs can also predict the risk of CRC occurrence and mortality (110).

However, most studies at present can only indicate that there is a correlation between lncRNAs and CRC, but less clearly describes the specific mechanism between them. For the lncRNAs that have been studied, it is still difficult to determine which pathway is dominant, leading to increased challenges in choosing between targeted blocking or activation. A comprehensive understanding of the effects of lncRNAs on CRC has not been established, and studies on inhibitory lncRNAs are limited. It is necessary to consider the mechanism leading to the abnormal expression of lncRNAs in CRC. The upstream regulatory mechanisms of lncRNAs have not yet been fully elucidated. Therefore, new directions for future research may focus on the upstream regulatory factors of lncRNAs in CRC. These efforts may address the gaps in knowledge on lncRNA-related mechanisms and build a framework for understanding the effects of lncRNAs on CRC.

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## AUTHOR CONTRIBUTIONS

SC, BH, and SZ were involved in the conception of the study. SC, YF, LS, and RH were involved in writing the article. SC, BH, and SZ critically revised the manuscript. All authors read and approved the final manuscript. SC, YF, and LS contributed equally to this work.

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# Inhibition of Wnt- $\beta$ -Catenin Signaling by ICRT14 Drug Depends of Post-Transcriptional Regulation by HOTAIR in Human Cervical Cancer HeLa Cells

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**Background:** In Cervical cancer (CC), in addition to HPV infection, the most relevant alteration during CC initiation and progression is the aberrant activation of Wnt/ $\beta$ -catenin pathway. Several inhibitory drugs of this pathway are undergoing preclinical and clinical studies. Long non-coding RNAs (lncRNAs) are associated with resistance to treatments. In this regard, understanding the efficiency of drugs that block the Wnt/ $\beta$ -catenin pathway in CC is of relevance to eventually propose successful target therapies in patients with this disease.

**Methods:** We analyzed the levels of expression of 249 components of the Wnt/ $\beta$ -catenin pathway in a group of 109 CC patients. Three drugs that blocking specific elements of Wnt/ $\beta$ -catenin pathway (C59, NSC668036 and ICRT14) by TOP FLASH assays and qRT-PCR were tested *in vitro* in CC cells.

**Results:** 137 genes of the Wnt/ $\beta$ -catenin pathway were up-regulated and 112 down-regulated in CC patient's samples, demonstrating that this pathway is dysregulated. C59 was an efficient drug to inhibit Wnt/ $\beta$ -catenin pathway in CC cells. NSC668036, was not able to inhibit the transcriptional activity of the Wnt/ $\beta$ -catenin pathway. Strikingly, ICRT14 was neither able to inhibit this pathway in HeLa cells, due to HOTAIR interaction with  $\beta$ -catenin, maintaining the Wnt/ $\beta$ -catenin pathway activated.

**Conclusions:** These results demonstrate a mechanism by which HOTAIR evades the effect of ICRT14, a Wnt/ $\beta$ -catenin pathway inhibitory drug, in HeLa cell line. The emergence of these mechanisms reveals new scenarios in the design of target therapies used in cancer.

**Keywords:** cervical cancer, Wnt- $\beta$ -catenin, HOTAIR, ICRT14 drug, post-transcriptional regulation

## INTRODUCTION

Cervical cancer (CC) constitutes a major health concern worldwide since it is the fourth most common cancer in women (1). Epidemiological and molecular studies have shown that high-risk Human Papilloma Virus (HR-HPV) is a causal agent but not sufficient (2). Hence, the malignant progression of HR-HPV infected cells is a casual event that requires the emergence of other genetic, epigenetic, phenotypic and micro-environmental alterations (3–5). Among the most relevant alterations during CC initiation and progression is the aberrant activation of Wnt/ $\beta$ -catenin pathway, which is essential in cervical oncogenesis (6, 7). Wnt/ $\beta$ -catenin pathway has a critical role in development, differentiation, and tissue homeostasis. However, in several types of cancer this pathway is dysregulated promoting poor patient prognosis, so it is attractive to be pharmacologically blocked (8). Only a few drugs have made it into Phase I clinical trials, such as Ipafricept and vantictumab (WNT antibodies), LGK974 and ETC-159 (PORN inhibitors), PRI-724 and CWP232291 ( $\beta$ -catenin inhibitors); however, none has been approved yet (9). Other drugs are still in pre-clinical studies, for example; C59, acts at the extracellular level inhibiting the function of PORCN, which is a membrane-bound O-acyltransferase required for palmitoylation, secretion and activity of WNTs ligands (10). NSC668036, is an organic molecule that acts at the cytoplasmic level binding to DVL protein, that inhibits the Wnt3A induced signaling (11). Another drug is ICRT14, which acts at the nuclear level inhibiting direct interactions between  $\beta$ -catenin and TCF4, antagonizing the transcriptional function of nuclear  $\beta$ -catenin and consequently shutting down the signaling pathway (12). Due to the existence of an arsenal of drugs blocking Wnt/ $\beta$ -catenin pathway, some characteristics that determine their efficacy are becoming apparent (13).

Recent studies have revealed that the dysregulation of multiple pathways by long-non coding RNAs (lncRNAs) results in drug resistance (14, 15). HOX transcript antisense intergenic RNA (HOTAIR) is the best example, as its overexpression induced cellular resistance to cisplatin through Wnt/ $\beta$ -catenin pathway activation in ovarian cancer (16). Likewise, HOTAIR upregulation was associated with drug resistance by Wnt/ $\beta$ -catenin pathway activation in lung cancer (17), and colorectal cancer (18).

In the present study, we validated the dysregulation of Wnt/ $\beta$ -catenin pathway signaling in CC patients. Next, since there are several drugs to inhibit this pathway, we performed *in vitro* assays to determine the efficacy of C59, ICRT14 and NSC668036 in order to inhibit Wnt/ $\beta$ -catenin signaling pathway in CC cell lines (HeLa, SiHa and CaSki). C59 was an efficient drug, NSC668036 showed no inhibitory effect while, ICRT14 turned out to have an inhibitory effect in SiHa and CaSki cell lines but did not inhibit the Wnt/ $\beta$ -catenin pathway in the HeLa cell line. HOTAIR overexpression was verified in HeLa cells, and its potential interaction with  $\beta$ -catenin, was associated with Wnt/ $\beta$ -catenin activation, decreasing ICRT14 drug efficiency. These data revealed a new resistance mechanism, hence, some target therapies are not convenient against cancer due to the lncRNAs-mediated regulation in order to promote drug-resistance. The

description of these mechanisms provides new insights into further therapeutic opportunities in CC.

## MATERIALS AND METHODS

### Cervical Samples

We included 109 cervical cancer patients from 2010 to 2013 through Instituto Nacional de Cancerología of Mexico City (INCan). This study was approved by INCan's Review Board and Ethics Committee (015/012/IBI-CEI/961/15). All patients of this study agreed and signed the consent form. In order to identify mRNAs deregulated and involved in the Wnt/ $\beta$ -catenin pathway, gene expression microarray assay was performed for which 89 samples were used and 20 were selected to perform validation by qRT-PCR. After surgical excision, tumor biopsies were segmented into two pieces, one for pathological confirmation and another for nucleic acid separation. Twenty non-pathological cervical tissues were obtained from patients who had undergone a hysterectomy due to uterine myomas.

### RNA Purification and Microarray Hybridization

RNA was extracted from 89 cervical cancer samples and 6 cervical non-tumor tissues to perform the microarray assay and its quality was measured using the 18S:28S ratio. Microarray was performed as previously reported and the raw data are publicly available at the GEO database (Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE56303.

### RT-qPCR

RNA from 20 samples tumor tissues and CC cell lines, was extracted with Trizol reagent (Ambion) according to the manufacture's protocol. The total cDNA was generated by reverse transcription from 1  $\mu$ g of total RNA using the kit High-Capacity cDNA Reverse Transcription (Applied Biosystems) with a final volume of 20  $\mu$ l. To amplify, c-Jun, c-Myc, MMP7, Cox2, CyD1, MMP10, CTNNB, CSNK1, FZD5, DVL, LRP5, NKD2 Klotho (KL), Cerberus (CER1), NKD1, Wnt11 and HOTAIR, a Luminaris Color HiGreen qPCR Master Mix was used along specific primers and specific amplification conditions for each gene (**Supplementary File 1**). Reactions were performed in Step One System. Relative expression levels were calculated using the  $\Delta\Delta C_t$  method (Applied Biosystems).  $\beta$ -actin mRNA was used as a reference gene for normalization. At this section it is important to emphasize that the idea was to evaluate the expression of each of these genes in each tumor and normal sample. However, the amount of RNA obtained from each of the samples (tumor) was insufficient to do so. Therefore, of the 20 tumor tissue samples, only 19 were evaluated for c-Jun expression, 14 for NKD expression and 13 for DVL expression.

### Cell Culture, Transfection, and Reagents Drugs

All cell lines were obtained from ATCC. Human CC cell lines, HeLa (ATCC CRM-CCL-2) and SiHa (ATCC HTB-35) were

cultured in DMEMF12 (Gibco) medium while CaSki cell line (ATCC CRL-1550) was cultured in RPMI (Gibco) medium, both mediums were supplemented with 10% (v/v). All cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The drugs C59 (Bio-vision 2063-5) and ICRT14 (Toronto Research Chemical Canada I163900) were purchased from Sigma Aldrich and were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). NSC668036 (TOCRIS BIOSCENSE 5813) was purchased from TOCRIS and was dissolved in water. All reagents were stored at -20°C. The IC<sub>50</sub> (concentration resulting in a 50% inhibition of cell growth) for each drug is provided by the supplier.

### Viability MTT Assay

In order to verify the half maximal inhibitory concentration (IC<sub>50</sub>) provided by the supplier for C59, NSC668036 and ICRT14 in HeLa, SiHa and CaSki cell lines, an MTT assay was employed to determine cell viability. Briefly, 4x10<sup>3</sup> cells were seeded in a 96-well plate. After 24 hrs of incubation, the cells were exposed to different concentrations close to those given by the supplier of C59, NSC668036, ICRT14 and DMSO as a control in fresh medium for 24 hrs. Cells were washed with PBS and were exposed to MTT (300  $\mu$ L/well, 1 mg/mL; Sigma) for 3 hrs at 37°C. Then, cells were washed and incubated with 100  $\mu$ L of DMSO for 10-15 min. Finally, the optical density (OD) was recorded at 540 nm in an Epoch Microplate Spectrophotometer (Biotech).

### TOP/FOP Flash Assay

To determine the activity of Wnt/ $\beta$ -catenin pathway, TOP/FOP flash assay (TCF Reporter Plasmid Kit Merck Millipore) was performed following the manufacturer's instructions. Briefly, 4x10<sup>5</sup> cells were seeded in a 6-well plate and co-transfected with 2.5  $\mu$ g of TOP and FOP plasmids. After 24 hrs, cells were incubated with the IC<sub>50</sub> of each inhibitor (C59, NSC668036, ICRT14) or 30  $\mu$ M DsiHOTAIR (IDT; San Diego, CA, USA) and a scramble sequence (scramble silencer negative control Ambion AM4611); using Lipofectamine 2000 transfection agent (Invitrogen). After incubation for 24 hrs, the cells of each group were collected, and then the activity of Wnt/ $\beta$ -catenin signaling pathway was measured by Dual Luciferase Reporter Assay Kit (Promega) in GloMax<sup>®</sup> 96 Microplate Luminometer (Promega; Madison, WI, USA).

### Flow Cytometry for Annexin V/Propidium Iodide (PI)

Apoptosis was assessed by staining cells with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Briefly, HeLa cells were washed with PBS, and suspended in serum-free, phenol red-free medium. HeLa cells were seeded in 6-well plates at a density of 3x10<sup>5</sup> cells/well. After 24 hrs, the cells were incubated either with the scramble (30  $\mu$ M for 48 hrs), Dsi HOTAIR (30  $\mu$ M for 48 hrs), ICRT14 (IC<sub>50</sub> 12.9  $\mu$ M for 24 hrs) or the combination DsiHOTAIR (30  $\mu$ M for 48 hrs) plus ICRT14 (IC<sub>50</sub> 12.9  $\mu$ M incubated for this condition for 24 hrs). Then, the cells were washed with PBS. The level of annexin V binding was determined by using a commercially available annexin V apoptosis detection kit (FITC Annexin V Apoptosis Detection Kit with PI, BioLegend), according to the manufacturer's

instructions. The cells were subsequently analyzed by a flow cytometer (FACScalibur). Approximately 10,000 events were collected for each sample. The percentage distributions were calculated by Expo32 ADC software (Beckman Coulter, Inc., Miami, FL). Cells were classified as apoptotic (positive annexin V and negative PI), late apoptotic/necrotic (positive annexin V and positive PI) or viable (negative annexin V and PI). Unstained HeLa cells were used as negative fluorescence controls. The same procedures were performed for 30  $\mu$ g/ml etoposide treated cells. Moreover, we captured a photography for each condition (Microcopy leica 090-135.002).

### RNA Binding Protein Immunoprecipitation (RIP) assay

RNA immunoprecipitation (RIP) was performed using Magna RIP RNA-Binding Protein Immunoprecipitation kit (17-704, EMD Millipore) according to the manufacturer's instructions. HeLa cells were lysed in complete RIP lysis Buffer, after the lysate was incubated with RIP buffer containing magnetic beads conjugated with 2.5  $\mu$ g to human Anti-  $\beta$ -catenin (Abcam, ab227499) and negative control normal rabbit IgG (Millipore). Samples were incubated with proteinase K and the immunoprecipitated RNA was isolated. Finally, HOTAIR was amplified by qRT-PCR as mentioned before.

### Protein Expression Analysis

Protein extracts from cultured cells were achieved by homogenization in RIPA buffer (Santa Cruz Biotechnology), later dissipated by centrifugation at 12,000 rpm for 20 min. For immunodetection, 50  $\mu$ g total protein from cultured cells were mixed with Laemmli sample buffer, boiled, separated in 12 or 15% SDS-PAGE, and transferred in a PVDF membrane (Amersham-GE Healthcare). Membranes were incubated overnight using a 1:1,000 (v/v) dilution of the anti-caspase 3 (Cell signaling), anti-PARP46D11 (Cell signaling), anti-c-Jun (Cell signaling) and anti-c-Myc (Cell signaling). For detection, 1:2,500 (v/v) dilutions of HRP anti-rabbit or anti-mouse conjugate antibodies (Santa Cruz Biotechnology) were used. Finally, using the Super Signal West Femto chemiluminescent substrate (Thermo Scientific), the membranes were scanned in the C-Digit blot scanner (Li-Cor) and the images were analyzed for densitometry in the associated Image Studio software (LiCor). Membranes were stripped and re-probed for detection of actin (anti-actin, Sc-47778) as a loading control. A representative image from three independent experiments is shown.

### Bioinformatics Analysis

RPISeq software (from website <http://pridb.gdcb.iastate.edu/RPISeq/>) was used to predict the interaction probability between HOTAIR and  $\beta$ -catenin protein. The interaction probability accepted was  $\geq 0.8$  in both classifiers of random forest (RF) and support vector machine (SVM).

### Statistical Analysis

In order to obtain a list of significant genes from Wnt signaling pathway aberrantly expressed in tumor tissues *versus* their normal counterparts, we used significance analysis of



microarrays (SAM) software. This software assigned a score based on the change of expression relative to the standard deviation of repeated measurements of each Wnt pathway-dysregulated components. Genes with scores higher than the threshold are considered potentially significant, in this way, we contemplated as positively or negatively regulated genes those with a delta score  $>0.3$  and less than  $-2.0$ , respectively (19). All data are expressed as the mean  $\pm$  S.D. from three independent experiments. Statistical analyses were performed using one-way ANOVA.  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*) was considered to indicate statistical significance.

## RESULTS

### Patients

One hundred nine patients were recruited. Of these, 89 patients were used to assess mRNA profile expression through a microarray assay and 20 were selected for RT-qPCR validation of the data generated by the microarray. The mean age of the patients was 48 years (range, 29–70 years). All patients were diagnosed with CC and the most common histologic subtype was squamous cell carcinoma (90.8%). According to the clinical stage classification (FIGO), patients' specimens were categorized as follows; 60.5% stage IIB, 24.7% stage IIIB, 12% stage IB2 and 0.91% stage IIA and IIIA.

### Wnt Signaling Pathway Is Deregulated in Cervical Cancer Patients

As a first approach, in order to identify the differential expression genes involved in Wnt/ $\beta$ -catenin signaling pathway of CC specimens, microarray data was analyzed with SAM algorithm (<https://statweb.stanford.edu/~tibs/SAM/>), which detects genes with important expression changes using Delta Score (Score (d)  $\geq 1.5$  and  $\leq -1.5$  and a false discovery rate (FDR)  $< 10\%$ ). Thus, we analyzed 249 genes and isoforms involved in Wnt signaling pathway which expression was significantly altered in CC specimens compared to normal cervical tissues (137 were up-regulated and 112 down-regulated) (Supplementary File 2). In Figure 1 is shown a hierarchical clustering in which Pearson correlation distance and complete linkage clustering were used to display differences and similarities based on the expression profiles obtained from Genesis 2.1 software (20).

To confirm the microarray data, we perform RT-qPCR to validate the expression of key upregulated genes such as: NKD2, c-Jun, DVL, FZD5 and c-Myc as well as key downregulated genes such as: Cerberus (CER1), Klotho (KL), NKD1 and Wnt11. The expression was evaluated in an independent cohort of 20 cervical cancer specimens and 10 normal cervical tissues. As shown in Figure 2, the expression levels of each gene obtained by RT-PCR correlated and were consistent with the microarray data analysis.

### C59, NSC668036 and ICRT14 Reducing the Cell Viability in a Dose-Dependent Manner

One of our main goals was to probe the efficacy of three drugs (C59, NSC668036 and ICRT14) to inhibit the Wnt/ $\beta$ -catenin pathway at three different levels (extracellular, cytoplasm and nucleus) into the CC cells (Figure 3). For that purpose, first we

verify the IC50 provided by the supplier of each drug. Thus, we tested a range of concentrations for each drug in HeLa (epithelial adenocarcinoma CC cell line infected with HPV18), SiHa and CaSki (both are squamous cancer cell line infected with HPV16 cells). As expected, the results shown in Figure 4 indicate that these drugs reduced cell survival in a dose-dependent manner. According to the findings, IC50 for C59 and ICRT14 were similar in HeLa, SiHa and CaSki cell lines (Figures 4A, C), whereas IC50 for NSC668036 was higher. Therefore, C59, ICRT14 and NSC668036 were selected for subsequent experiments.

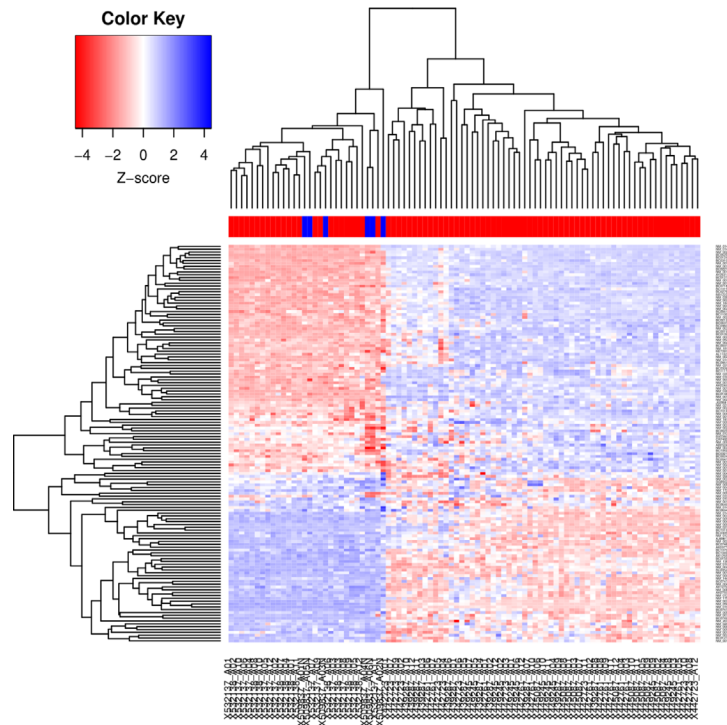
### C59 and ICRT14 Inhibit the Transcriptional Activity of Wnt/ $\beta$ -Catenin Pathway in CC Cell Lines

In order to determine if C59, NSC668036 and ICRT14 inhibit the Wnt/ $\beta$ -catenin pathway in CC cell lines, we conducted a TOP-flash assay, the standard assay for assessing Wnt/ $\beta$ -catenin pathway activity. The transcriptional activity of Wnt/ $\beta$ -catenin pathway was determined in HeLa, SiHa and CaSki using the IC50 of C59, NSC668036 and ICRT14. As we expected, the results demonstrated that C59, was able to significantly inhibit the transcriptional activity of Wnt/ $\beta$ -catenin in HeLa, SiHa and CaSki cell lines (Figure 5A). Moreover, the expression of c-Myc and c-Jun, two main targets of the Wnt/ $\beta$ -catenin pathway, was downregulated when we used C59 in SiHa and CaSki cell lines. In HeLa treated with C59, c-Myc expression protein decreased but not c-Jun expression protein, it is presumed that alternative pathways may be activating its expression (Supplementary File 3A). Conversely, NSC668036, was not able to inhibit the transcriptional activity of the Wnt/ $\beta$ -catenin pathway in HeLa, SiHa and CaSki cell lines (Supplementary File 4). Regarding ICRT14, was able to inhibit the transcriptional activity of Wnt/ $\beta$ -catenin pathway in SiHa and CaSki cell lines but not in HeLa cell line (Figure 5B). Furthermore, to corroborate whether ICRT14 was unable to inhibit the Wnt/ $\beta$ -catenin pathway in HeLa cell line, we analyzed the expression of main targets of the Wnt/ $\beta$ -catenin pathway such as c-Myc, c-Jun, MMP7 and MMP10 in HeLa, SiHa and CaSki treated with ICRT14. The results showed that the expression of c-Myc, c-Jun and MMP10 was downregulated in SiHa cells treated with ICRT14. In CaSki cells treated with ICRT14 was downregulated c-Myc and MMP7. Nevertheless, in HeLa cells treated with ICRT14, c-Myc expression was maintained but c-Jun, MMP7 and MMP10 expression was upregulated (Figure 5C). Moreover, the expression protein of c-Myc and c-Jun also was overexpressed when HeLa cells were treated with ICRT14 (Supplementary File 5A). These data suggested that the Wnt/ $\beta$ -catenin pathway continues to be active, despite the use of ICRT14. This ICRT14 does not inhibit Wnt/ $\beta$ -catenin pathway in HeLa cells.

### HOTAIR Maintains Active Wnt/ $\beta$ -Catenin Pathway in HeLa Cells Despite Treatment With ICRT14 Drug, by Interaction With $\beta$ -Catenin

Several reports have described that upregulation of HOTAIR stimulates the Wnt/ $\beta$ -catenin pathway in several types of cancer including lung (21), pancreatic (22), ovarian (23) and cervical





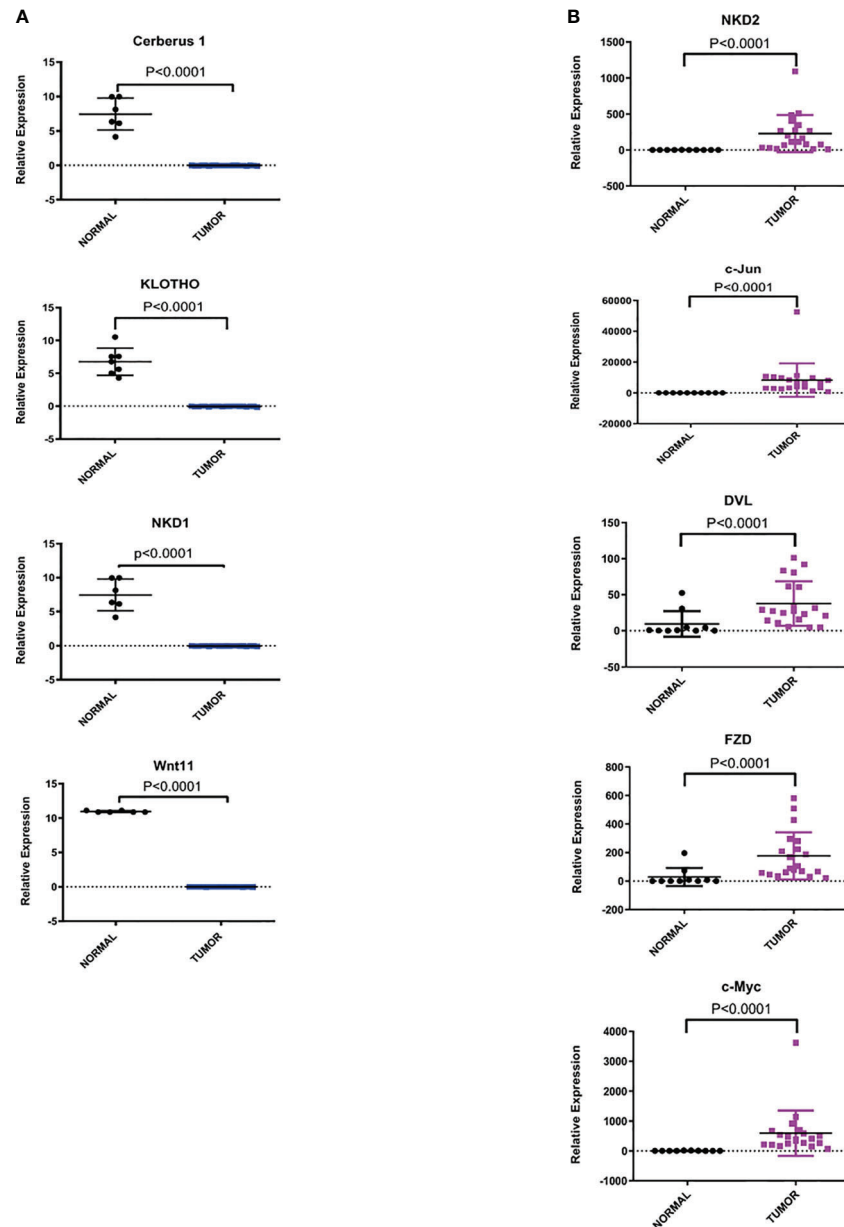
**FIGURE 1** | Hierarchical cluster generated from 89 LACC and 6 non-tumor tissue samples. Cluster analysis of the microarray data. The microarray data were analyzed by the Genesis program. The cluster shows 249 genes (137 up-regulated and 112 down-regulated). Each row represents a gene, whereas each column corresponds to a tissue sample, the color line above the tissue samples indicates the sample type: normal samples (blue) and tumor samples (red). The relative abundance of each gene in the tissue correlates with the color intensity (red, induced; blue, repressed; white, no change). In the dendrogram, all six normal cervical samples clustered together, indicating their similarity based on the expression profile.

cancer (17); mainly in HeLa cells (24). Therefore, we explored if this mechanism was responsible of the ICRT14 drug inefficiency to inhibit Wnt/ $\beta$ -catenin pathway in the HeLa cell line. First, we aimed to quantify HOTAIR expression by RT-qPCR in CC cell lines and CC biopsies samples. As expected, we found that HOTAIR was significantly upregulated in CC cells lines and CC biopsies samples compared to the non-tumor cell line HaCat and normal tissues samples patients, respectively (**Figure 6** and **Supplementary File 3B**). Moreover, HOTAIR expression was higher in HeLa cell line *versus* SiHa and CaSki cells (**Figure 6A**). Nonetheless, ICRT14 treatment increased HOTAIR expression in HeLa cells (**Supplementary File 3C**). To verify this data, we used a DsiRNA to perform a HOTAIR knockdown in HeLa cell line (**Figure 6B**) and evaluated the effect of ICRT14. Indeed, HOTAIR's downregulation led to the inhibition of Wnt/ $\beta$ -catenin pathway, also when the ICRT14 drug was added (**Figure 6C**). Besides, three main targets of the Wnt/ $\beta$ -catenin pathway such as; c-Myc, c-Jun and MMP10 expression was downregulated when we used a DsiRNA to perform a HOTAIR knockdown in HeLa cell (**Figure 6D**). Moreover, the expression protein of c-Myc and c-Jun was not downregulated when we used ICRT14 in HeLa cells *versus* SiHa and CaSki cells lines (**Supplementary Files 5A–C**), but a modest reduction in expression was observed when we used a DsiRNA to perform a HOTAIR knockdown in HeLa cell (**Supplementary File 5A**).

These findings indicate that HOTAIR expression in HeLa cells activates or maintains active Wnt/ $\beta$ -catenin and inhibited the blocking effect of ICRT14 on this pathway. Since ICRT14 acts at the nuclear level inhibiting direct interactions between  $\beta$ -catenin and TCF4, blocking the transcriptional function of nuclear  $\beta$ -catenin, it was feasible to hypothesize that HOTAIR was maintaining the interaction between  $\beta$ -catenin and TCF4, blocking the effect of ICRT14. To support this idea, we explored RPIseq tool, that predicts protein-RNA interactions. We found that RPIseq tool predicted interactions between HOTAIR and  $\beta$ -catenin, as well with TCF, PYGO2 and BCL9 (**Figure 6E**). Next, to demonstrate at least one of these interactions, we performed a RIP assay. We found that HOTAIR was highly enriched in  $\beta$ -catenin-RNA precipitates compared to input precipitates (**Figure 6F** and **Supplementary File 6**). These findings suggested a potential interaction between  $\beta$ -catenin and HOTAIR, which could prevent the blocking effect of ICRT14 on Wnt/ $\beta$ -catenin pathway.

### HOTAIR Knockdown Induce Necrosis in HeLa Cell Line Incubated With ICRT14

To identify the mechanisms by which DsiHOTAIR plus ICRT14 decrease Wnt/ $\beta$ -catenin pathway in HeLa cells, we analyzed cell death by flow cytometry assay. As in **Figure 7** is shown, untreated HeLa cells, and HeLa cells transfected with scramble,



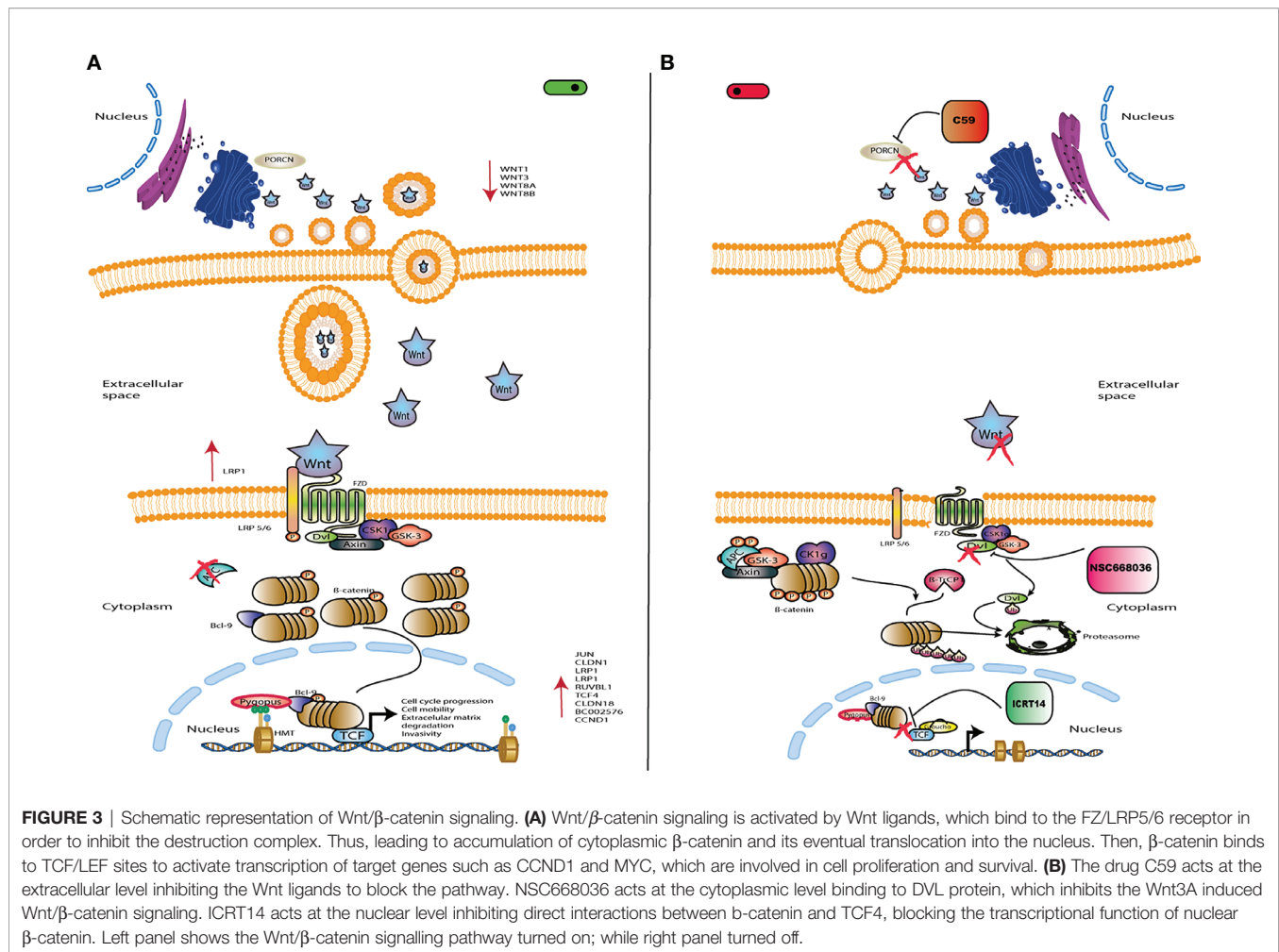
**FIGURE 2** | Relative expression of dysregulated genes of Wnt/ $\beta$ -catenin pathway in CC samples versus normal cervix tissues. The expression level of each gene by RT-qPCR was determined as described in the methods section. Statistical analysis to compare the mRNA expression levels between normal and tumor tissues was performed using an unpaired two-tailed t-test. **(A)** Representative downregulated genes of the Wnt/ $\beta$ -catenin pathway. **(B)** Representative upregulated genes of the Wnt/ $\beta$ -catenin pathway.

the rate cell viability was 93% and 89%, respectively. Similarly, when HeLa cells were treated independently with the ICRT14 drug for 24 hrs, and transfected with DsiHOTAIR, the 85% and 83% of cells were viable, respectively (**Figure 7**). These data confirmed our finding obtained with the TOP-flash assay (**Figure 6C**). However, when HeLa cells were incubated with DsiHOTAIR plus ICRT14 drug, only 14% of the cells were underwent apoptosis, and 74% necrosis. (**Figure 7** and **Supplementary File 5D**). Taken together, HeLa cells treated

with DsiHOTAIR in combination with ICRT14 drug, induces cell death mainly by necrosis.

## DISCUSSION

Accumulating evidence has reported dysregulation of Wnt/ $\beta$ -catenin signaling in several types of cancer (8). Specifically, in

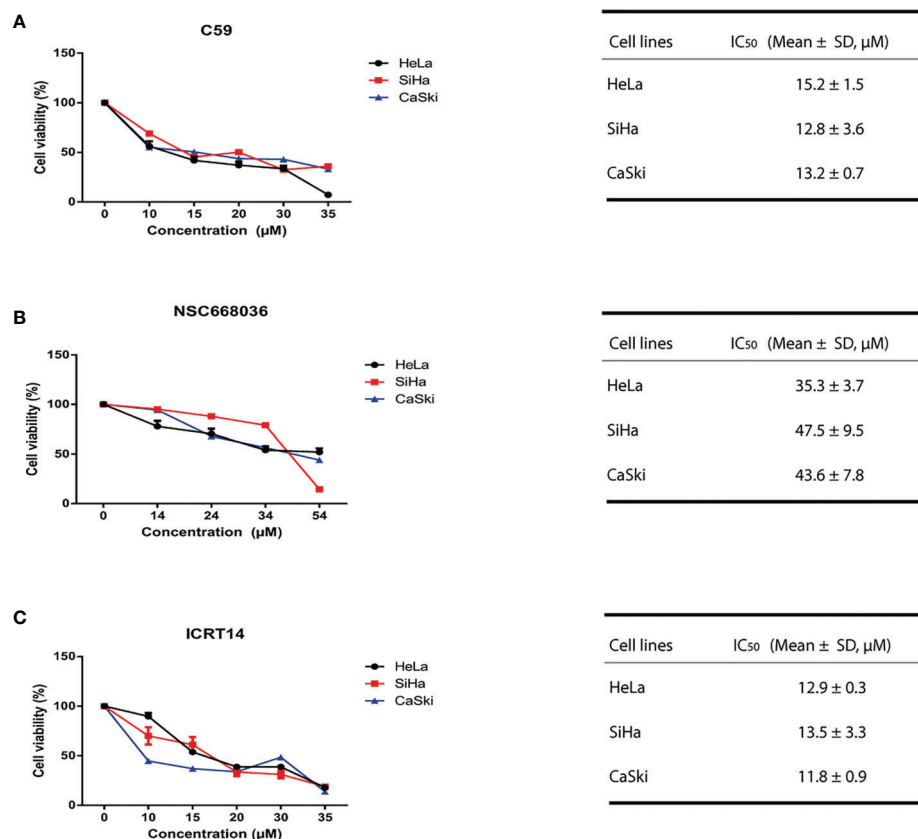


CC, activation of this pathway is a second hit to develop the disease (6, 7), since the transformation of HPV expressing human keratinocytes requires activation of the Wnt/ $\beta$ -catenin pathway (25). In the present study, through a transcriptome exploration on 89 CC samples and 6 non-tumor tissues, we found that Wnt/ $\beta$ -catenin signaling pathway was significantly altered, confirming previous reports (6, 7). We validated the expression of some Wnt/ $\beta$ -catenin pathway involved genes by qRT-PCR in CC specimens. Similar to our findings, some studies have reported the altered expression of DVL (26), FZD5 (27), c-Myc (28), Cox2 (29), c-Jun (30) and Klotho (31). These data support that members of Wnt/ $\beta$ -catenin pathway may be attractive upcoming therapeutic targets (32).

Despite the existence of many drugs to block Wnt/ $\beta$ -catenin (9), it seems that not all of them are effective. This lack of efficacy may be due to drugs acting on different elements of the pathway. It can also be explained by the cellular context in which drugs are being used, even, drug efficacy may be subject to regulation by ncRNAs. C59, NSC668036 and ICRT14 act at extracellular, cytoplasmic and nuclear level, respectively. In the current study, we found that C59 was a highly efficient drug in HeLa, SiHa and CaSki cell lines. Consistent with our results, it has been reported

that C59 blocked Wnt/ $\beta$ -catenin pathway, and in consequence, migration and invasion of triple negative breast cancer cells were inhibited (33, 34). In mice, C59 displayed good bioavailability, it did not exhibit toxicity and blocked progression of mammary tumors, suggesting that C59 is a safe and feasible strategy to block Wnt/ $\beta$ -catenin signaling (35). In colorectal cancer, Koo BK and collaborators, demonstrated that C59, attenuated hyperplasias in mouse-small intestinal stem cells (36). Additionally, mice with nasopharyngeal carcinoma treated with C59 did not develop visible tumors. Moreover, it was demonstrated that this agent inhibited the generation of cancer stem cells (CSCs), activity responsible of Wnt/ $\beta$ -catenin signaling (37). It is significant to mention that this is the first study that evaluated the effect of C59 in CC cells. Taken together, is reasonable to consider that small molecule Wnt/ $\beta$ -catenin pathway inhibitors open a new therapeutic window for what should be tested in clinical trials of patients carrying malignant tumors.

Contrary to C59, we found that NSC668036 did not inhibit Wnt pathway in CC cells. Although, NSC668036 has been less explored compared to C59, it was important for us to consider NSC668036 in our study. In concordance to our results, Shin J and collaborators reported that among several agents that block Wnt pathway,



**FIGURE 4 |** C59, NSC668036 and ICRT14 decrease proliferation of CC cells. HeLa, SiHa and CaSki cells were treated with different doses of **(A)** C59, **(B)** NSC668036 and **(C)** ICRT14 for 24 hrs. Cell viability was analyzed using MTT. IC<sub>50</sub> was determined by non-linear regression.

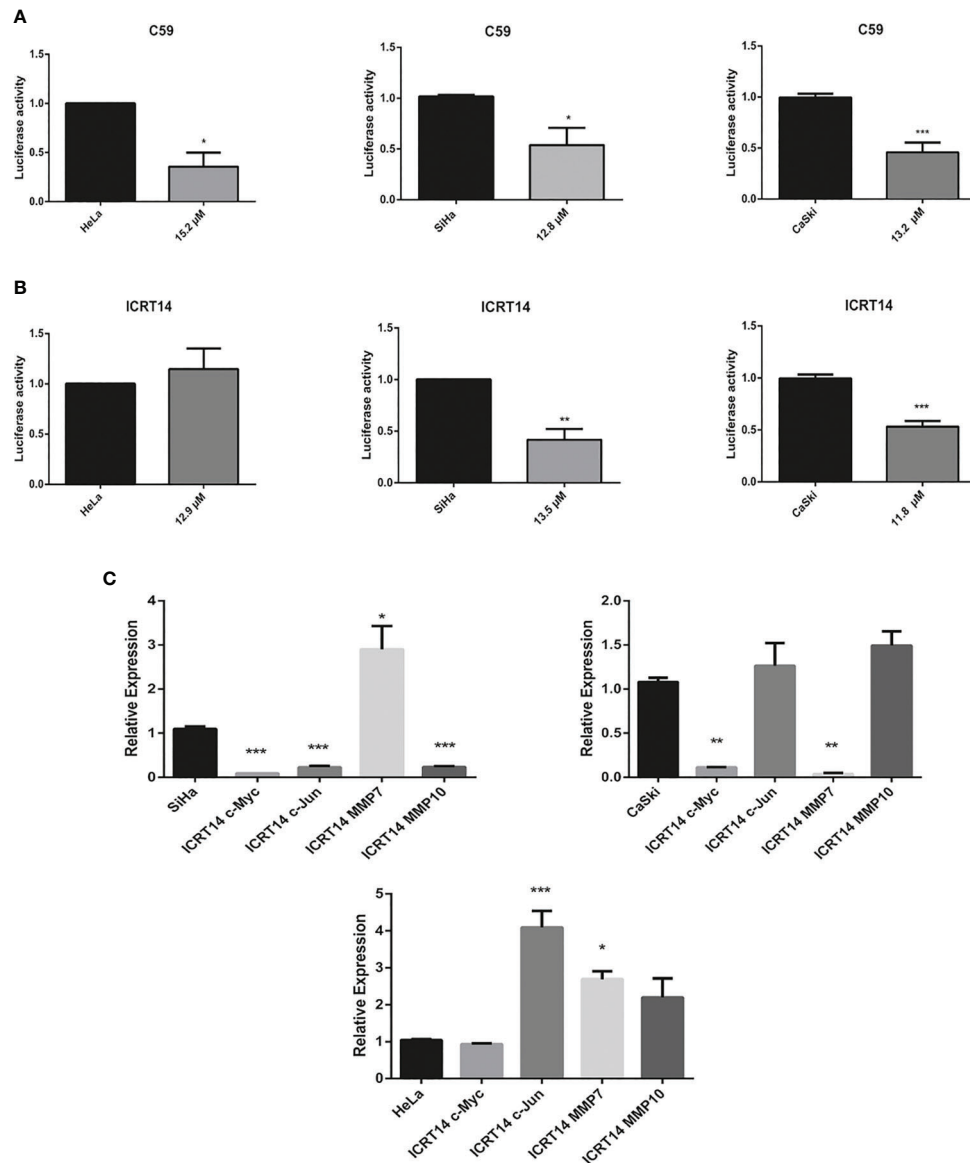
NSC668036 was not an efficient drug to block Wnt/ $\beta$ -catenin pathway in HeLa cells (38). Conversely, NSC668036 blocked Wnt/ $\beta$ -catenin signaling in experimental diabetic peripheral neuropathy rats provided with neuroprotection (39). Furthermore, Reshma K. and Sharma S. reported that in rats treated with paclitaxel and NSC668036, their behavioral pain thresholds and nerve functional parameters were significantly improved by inhibition of Wnt/ $\beta$ -catenin signaling (40). Therefore, we speculate that these findings suggest that each inhibitor had differential effects depending on the cellular context.

Interestingly, the agent ICRT14 inhibited Wnt/ $\beta$ -catenin pathway in SiHa and CaSki but not in HeLa cells. The positive effect of this agent is similar to previously reported results. For instance, ICRT14 inhibited c-Myc and cyclin D1 expression in breast cancer cells as well as it decreased migration and invasion (41–43). In colon cancer cells, ICRT14 inhibited Wnt pathway and sensitized cells to radiation treatment (12, 44, 45). In leukemic cell lines, ICRT14 led to significant downregulation of Wnt target genes (46). In the case of lung cancer, ICRT14 is efficient, and even, is used as a positive control to validate new drugs (47). In pancreatic cancer (48), head and neck cancer (49) and Gallbladder carcinoma (50) cells, ICRT14 has also been demonstrated to be an efficient agent.

Surprisingly, we found that ICRT14 had no effect in HeLa cells due to HOTAIR overexpression maintains Wnt/ $\beta$ -catenin pathway activated. Consistent with our results, it was recently reported that HOTAIR is involved in overactivation of Wnt/ $\beta$ -catenin pathway in HeLa cell line (24). Likewise, HOTAIR maintains Wnt/ $\beta$ -catenin activated in esophageal squamous cell carcinoma (51). Additionally, in line with previous reports, we found that HOTAIR is overexpressed in HeLa cells compared to SiHa and CaSki cell lines (52, 53). Thus, HeLa cells have been used as a model to study mechanisms involving HOTAIR in CC (54–57).

We noticed that HOTAIR knockdown in combination with ICRT14 downregulated Wnt/ $\beta$ -catenin pathway in HeLa cells. These results suggest that HOTAIR overexpression conducted to Wnt inhibitors-resistance through Wnt/ $\beta$ -catenin pathway activation. In this regard, it was already known that HOTAIR induces chemoresistance activating Wnt pathway in other types of cancer such as ovarian (16), colorectal (18) and lung cancer (55). In CC cells and in pancreatic ductal adenocarcinoma, HOTAIR knockdown enhanced sensitivity to radiotherapy through Wnt signaling pathway suppression (22, 55). One of the mechanisms of resistance is that HOTAIR promotes  $\beta$ -catenin transportation to the nucleus to maintain the pathway



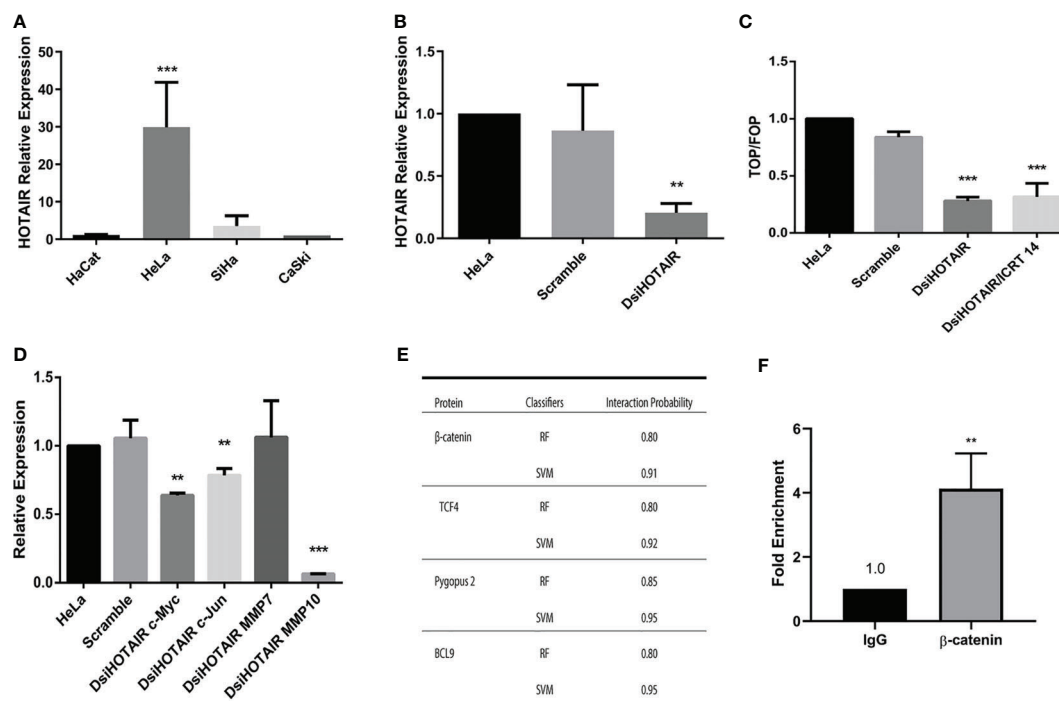


**FIGURE 5** | Effect of C59 and ICRT14 drugs on Wnt/ $\beta$ -catenin pathway in CC cell lines. HeLa, SiHa and CaSki cells were co-transfected with 2.5  $\mu$ g FOPFlash-Luc (mutant reported vector) and TOPFlash (Wnt/ $\beta$ -catenin reporter vector). After 24 hrs, they were incubated with IC50 of (A) C59 and (B) ICRT14. After 24 hrs, the luciferase activity and the expression of the main targets of wnt pathway was measured in order to determine the Wnt/ $\beta$ -catenin pathway activation by luciferase assay (A, B) and (C) RT-qPCR, respectively. The bars represent the mean  $\pm$  standard deviation from at least three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

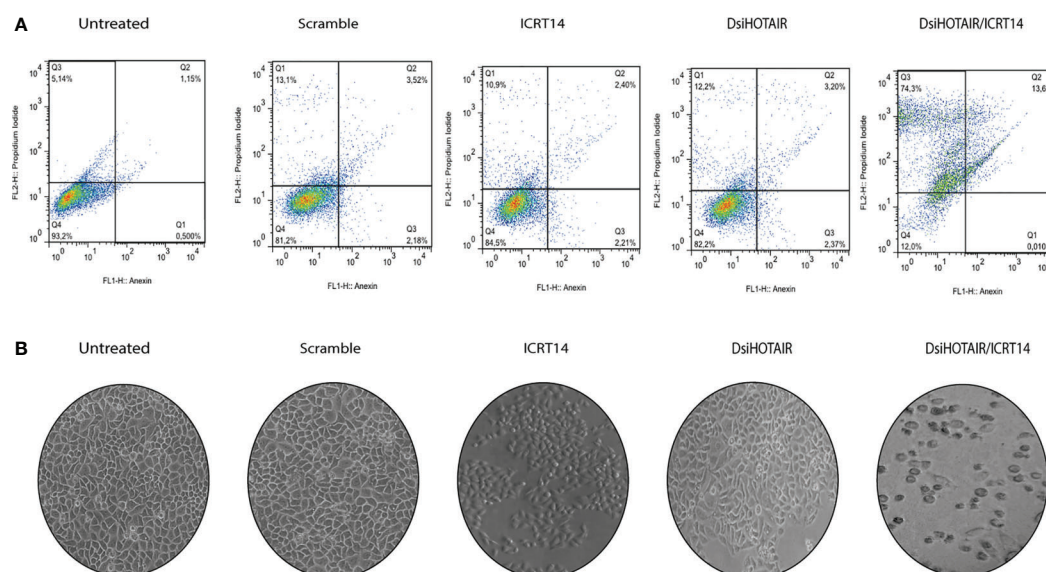
activated (19). In this work, we observed that in HeLa cells line, ICRT14 did not reduced the activation of the Wnt/ $\beta$ -catenin pathway. Since, ICRT14 inhibit the direct interaction between  $\beta$ -catenin and TCF4, blocking the transcriptional function of nuclear  $\beta$ -catenin, we hypothesize that in HeLa cell line, ICRT14 has no effect because HOTAIR is binding to  $\beta$ -catenin to retain it in the nucleus and preserve the pathway active. Although a direct interaction between HOTAIR and  $\beta$ -catenin has not been reported yet, this work is the first to suggests the interaction between them, thus confirming the role of lncRNAs

as protein binding scaffolds sustaining the tumoral phenotype and therapy-resistance (20).

We also found that HOTAIR knockdown plus ICRT14 induced cell death mainly by necrosis. Regarding this, it has been reported that apoptosis machinery is defective in numerous cancers (58, 59). Moreover, it is well described that Wnt/ $\beta$ -catenin pathway regulates early and late apoptosis in cancer (60–64). In our study, Wnt/ $\beta$ -catenin pathway was inhibited by ICRT14 in combination of HOTAIR knockdown, consequently, it is reasonable to contemplate that, since the apoptosis machinery was disturbed,



**FIGURE 6** | HOTAIR maintains Wnt/β-catenin pathway active in HeLa cells by avoiding the effect of ICRT14. **(A)** Relative expression of HOTAIR was determined by RT-qPCR on CC cells. **(B)** HeLa cells were transiently transfected with 30 μM of DsiHOTAIR. After 48 hrs of post-transfection, the expression of HOTAIR was measured. **(C)** HeLa cells were transiently transfected with 30 μM of DsiHOTAIR alone or in combination with 12.9 μM of ICRT14 and the activity of Wnt/β-catenin pathway was detected by TOPFlash assay at 48 hrs. **(D)** The relative expression of c-Myc, c-Jun, MMP7 and MMP10 was determined by RT-qPCR from HOTAIR knockdown HeLa cells. **(E)** Interaction probability between HOTAIR and β-catenin detected by RPIseq tool. RPIseq uses the classifiers of random forest (RF) and support vector machine (SVM) for calculation. **(F)** Relative RIP assays using qPCR to detect binding between β-catenin and HOTAIR in HeLa cell line. The bars represent the mean ± standard deviation from at least three independent experiments. \*p < 0.05; \*\*p < 0.01 and \*\*\*p < 0.001.



**FIGURE 7** | DsiHOTAIR plus ICRT14 induces cell death by necrosis in HeLa cells **(A)** Flow cytometry percentage distributions after annexin V/propidium iodide staining of HeLa cells incubated with the scramble, DsiHOTAIR (30 μM), ICRT14 (12.9 μM) and the combination DsiHOTAIR/ICRT14. **(B)** Representative images of HeLa cells incubated with the scramble, DsiHOTAIR, ICRT14; and the combination DsiHOTAIR/ICRT14.

alternative pathways of cell death such as necrosis took place. As in ICRT14, it has been reported that anticancer drugs, such as  $\beta$ -lapachone, apoptolidin and honokiol, induce cancer cell death through necrosis (65–67). In this way, necrosis induced by drugs and lncRNAs downregulation, may play an important therapeutic role as the main goal of cancer treatment is, irrevocably, cell death. In conclusion, our results indicate that C59 is a good option as a treatment in CC, although further studies are still required in clinical trials. Moreover, we determined that the effect of ICRT14 in CC depends on the cellular regulation by HOTAIR. These findings indicate that not all target therapies can be efficient and that regulation by lncRNAs should be considered as an alternative treatment for drug resistance mechanisms.

## CONCLUSIONS

This is the first study to report the inhibitory effect of C59 on cervical cancer, which was an efficient target therapy for Wnt/ $\beta$ -catenin, *in vitro*. Clinical trials are needed to validate its effectiveness. On the other hand, ICRT14 inhibits direct interactions between  $\beta$ -catenin and TCF4 shutting down the signaling pathways; however according to our results the presence of HOTAIR affected the inhibitory effect of the drug by the potential interaction with  $\beta$ -catenin. These findings demonstrate that the effectiveness of target therapies can be affected by lncRNAs, which have been shown to play an important role in treatment resistance.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Instituto Nacional de Cancerología. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AC-P and CP-P conceived and designed the study. ST-C, JC-H, ID-W, and OM-C performed the experiments. AC-P wrote

manuscript. DH-S and CL-C contributed to the discussion and analysis of results. DC recollected samples and were responsible for all clinical data of the patients. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2021.729228/full#supplementary-material>

**Supplementary File 1** | Primer sequences.

**Supplementary File 2** | Genes and isoforms involved in Wnt signaling pathway from CC specimens compared to normal cervical tissues.

**Supplementary File 3** | (A) Relative expression of c-Myc and c-Jun in HeLa, SiHa and CaSki cells lines treated with C59 drug. (B) Relative expression of HOTAIR in normal cervix tissues and CC samples. (C) Relative expression of HOTAIR in HeLa, SiHa and CaSki cells treated with C59 and ICRT14 drugs.

**Supplementary File 4** | Effect of NSC668036 on Wnt/ $\beta$ -catenin pathway in CC cell lines.

**Supplementary File 5** | Protein levels of c-Myc and c-Jun detected by western blot, in HeLa (A), SiHa (B) and CaSki (C) treated with ICRT14, C59 and DsiHOTAIR.  $\beta$ -actin was used as loading control. (D) Protein levels of PARP1 and Caspase 3 detected by western blot in HeLa cells treated with ICRT14, DsiHOTAIR and DsiHOTAIR plus ICRT14 combination.  $\beta$ -actin was used as loading control.

**Supplementary File 6** | Western blot to IP  $\beta$ -catenin confirmation of RIP assays. Anti-IgG served as negative control.

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# HK2 Is a Crucial Downstream Regulator of miR-148a for the Maintenance of Sphere-Forming Property and Cisplatin Resistance in Cervical Cancer Cells

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The acquisition of cancer stem-like properties is believed to be responsible for cancer metastasis and therapeutic resistance in cervical cancer (CC). CC tissues display a high expression level of hexokinase 2 (HK2), which is critical for the proliferation and migration of CC cells. However, little is known about the functional role of HK2 in the maintenance of cancer stem cell-like ability and cisplatin resistance of CC cells. Here, we showed that the expression of HK2 is significantly elevated in CC tissues, and high HK2 expression correlates with poor prognosis. HK2 overexpression (or knockdown) can promote (or inhibit) the sphere-forming ability and cisplatin resistance in CC cells. In addition, HK2-overexpressing CC cells show enhanced expression of cancer stem cell-associated genes (including *SOX2* and *OCT4*) and drug resistance-related gene *MDR1*. The expression of HK2 is mediated by miR-145, miR-148a, and miR-497 in CC cells. Overexpression of miR-148a is sufficient to reduce sphere formation and cisplatin resistance in CC cells. Our results elucidate a novel mechanism through which miR-148a regulates CC stem cell-like properties and chemoresistance by interfering with the oncogene *HK2*, providing the first evidence that dysregulation of the miR-148a/HK2 signaling plays a critical role in the maintenance of sphere formation and cisplatin resistance of CC cells. Our findings may guide future studies on therapeutic strategies that reverse cisplatin resistance by targeting this pathway.

**Keywords:** HK2, miR-148a, sphere formation, cisplatin resistance, cervical cancer

## INTRODUCTION

Cervical cancer (CC) is ranked the fourth most commonly diagnosed cancer in women and is the second-leading cause of cancer-related death among women in developing countries (1). Surgery followed by a combination of platinum/paclitaxel-based chemotherapy is currently considered the standard of care for CC (2). Although the majority of CC patients have an initial response to chemotherapy, such as cisplatin (CDDP), a substantial proportion of patients eventually develop chemoresistance and relapse (2). Therefore, a better understanding of the mechanisms underlying CDDP resistance is required to retrieve the chemosensitivity in CC patients.

Most cancers contain a small subpopulation of cells (known as “cancer stem cells”) that drives the persistence of malignant tumors by producing new cancer cells (3). The cancer stem cell theory explains numerous clinical observations, such as the recurrence of tumors after initially successful chemotherapy and/or radiation therapy, and metastasis (3). Cancer stem cells have been found in many cancer types, including CC (4). In CC, cancer stem cells have been associated with resistance to commonly used anti-cancer drugs such as CDDP (4). Several potential stem cell markers, including MSI1, ALDH1, SOX2, and OCT4, have been used to identify putative cancer stem cells of CC (5). However, the molecular mechanisms underlying the induction and maintenance of cancer stem cells in CC remain to be explored.

In addition to several key genetic alterations in oncogenes and tumor suppressor genes, several epigenetic mechanisms, including microRNAs (miRNAs), have also been shown to regulate the progression and stemness of CC (6). Hexokinase 2 (HK2) is an enzyme that catalyzes the first committed step in glucose metabolism and converts glucose to glucose-6-phosphate (7). After knocking down HK2 expression, CC cells demonstrated significantly attenuated proliferation ability and glycolysis (8). Deletion of HK2 inhibits the growth and migration of CC cells (9). The same study has also demonstrated that knockdown of HK2 decreases the phosphorylation of AKT and mTOR, and increases the expression of p53 in CC cells (9). Another report indicated that HK2 promotes the proliferation of CC cells *in vitro* and tumor formation *in vivo* by regulating the Raf/MEK/ERK signaling pathway (10). Moreover, a previous study demonstrated that HK2 expression was suppressed by miR-9-5p by directly binding its 3'-untranslated region (3'-UTR) in CC cells (9). To the best of our knowledge, there have been no reports on the function and the underlying mechanism of HK2 in regulating CC stemness and CDDP resistance.

Here, we have explored for the first time that HK2 is overexpressed in CC, and its overexpression promotes sphere formation and CDDP resistance of CC cells. Moreover, we have demonstrated that the induction of HK2 expression in CC is partly due to the downregulation of miR-145, miR-148a, and miR-497. In particular, miR-148a acts as a key tumor suppressor that controls sphere formation and CDDP resistance by targeting HK2. Together, we have unveiled a novel epigenetic mechanism that controls sphere formation and CDDP resistance *via* the

miR-148a/HK2 axis in human CC. Thus, therapeutic targeting this axis may reverse CDDP resistance in CC patients.

## MATERIALS AND METHODS

### Human Tissue Specimens

This study was approved by the Research Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. Written informed consent was obtained from all patients. A total of 30 CC patients with no preoperative chemotherapy, immunotherapy, or radiotherapy were enrolled in this study. Samples were collected from patients who were diagnosed with CC and underwent surgical resection in the Affiliated Hospital of Inner Mongolia Medical University. After surgery, CC tissues and corresponding adjacent normal tissues were immediately frozen by liquid nitrogen and stored at -80°C for further analysis.

### Cell Lines and Cell Culture

Two human CC cell lines (HeLa and SiHa) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The HPV16 E6/E7-immortalized human cervical epithelial cell line Ect1/E6E7 (ATCC) was considered to be a non-cancerous ectocervical epithelial cell line (11, 12). All cells were cultured in DMEM/F12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) at 37°C with a humidified air containing 5% CO<sub>2</sub>. These cells were tested for mycoplasma contamination using the DAPI staining method.

### Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA from CC cell lines and tissues was isolated using TRIzol (Invitrogen). Complementary DNA (cDNA) was obtained from 500 ng of the total RNA using the PrimeScript II cDNA Synthesis Kit (TaKaRa, Beijing, China). The qRT-PCR analysis was conducted with a PRISM 7700 (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Invitrogen). The primers were as follows: *HK2*-F: 5'-GAGCCACCACTCACCCTACT-3' and *HK2*-R: 5'-CCAGGCATTCGGCAATGTG-3'; *MDR1*-F: 5'-TTGCTGCTTACATTCAGGTTTCA-3' and *MDR1*-R: 5'-AGCCTATCTCCTGTCGCATTA-3'; *SOX2*-F: 5'-GCCGAGTGGAACTTTTGTGCG-3' and *SOX2*-R: 5'-GGCAGCGTGTACTTATCCTTCT-3'; *OCT4*-F: 5'-CTGGGTTGATCCTCGGACCT-3' and *OCT4*-R: 5'-CCATCGGAGTTGCTCTCA-3'; *GAPDH*-F: 5'-AATCCCATCACCATCTTC-3' and *GAPDH*-R: 5'-AGGCTGTTGTCATACTTC-3'; *U6*-F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and *U6*-R: 5'-CGCTTCACGAATTTGCGTGTGCAT-3'. The NCode SYBR GreenER miRNA qRT-PCR analysis kit (Invitrogen) was used to validate the expression of miR-145, miR-148a, and miR-497 as previously reported (13). The expression of mRNA or miRNA was normalized to the expression of *GAPDH* mRNA or U6.

## Western Blotting Analysis

Total proteins were isolated from CC cells, which were lysed on ice with a RIPA lysis buffer (Cell Signaling Technology, MA). The protein concentration was determined using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Extracted protein (30 µg) was separated by 12% SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare Life Sciences, Piscataway, NJ). The membranes were blocked with 5% non-fat milk for 1 h and then incubated with anti-HK2 antibody (1:1000, #2106, Cell Signaling) and anti-GAPDH antibody (1:5000, #2118, Cell Signaling) at 4°C overnight. Then, the membranes were subsequently incubated with secondary antibodies for 2 h at room temperature. The protein bands were detected using an ECL detection kit (Amersham Pharmacia Biotech, UK).

## Sphere Formation Assay

Sphere formation assay was performed as previously described (6). CC cells (3000) cells were seeded in 6-well ultra-low attachment plates (Corning Incorporated, Corning, NY), where contain serum-free medium DMEM/F12 (Sigma-Aldrich) supplemented with N2 plus media supplement (Invitrogen), 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), and 4 mg/ml heparin (Sigma-Aldrich) for 14 days. The images were captured after 2 weeks of cell culture, and the number of spheres larger than 50 µm was counted.

## Cell Viability Assay

Cell viability was determined using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). In brief, CC cells (5000 cells per well) were seeded into 96-well plates and cultured for 24 h. The medium was refreshed and cells were exposed to varying concentrations of CDDP. Twenty-four hours after the addition of CDDP, CCK-8 solution (10 µl per well, Dojindo, Japan) was added to each well. Cells were incubated at 37°C for 3 h. The absorbance value was measured at a wavelength of 450 nm.

## Xenograft Mouse Model

The study on animal subjects was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. *In vivo* xenograft mouse experiments were performed as previously described (14, 15). Female mice (BALB/c nude, 4–6 weeks old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). CC cells transfected with HK2 overexpression vector (OriGene, Rockville, MD) or HK2 shRNA (Santa Cruz Biotechnology; Santa Cruz, CA), were subcutaneously injected into the flank of nude mice under aseptic conditions. After a 7-day administration, mice were treated with CDDP (30 mg/kg) intraperitoneally. The tumors were measured in two dimensions by using manual calipers every 3 days. The tumor volume was calculated using the following formula: volume =  $0.5 \times \text{length} \times \text{width}^2$ . After 30 days, all mice were sacrificed and tumor samples were weighted.

## Cell Transfection

Transfection was conducted using the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. The control vector, HK2 overexpression vector, control shRNA, or HK2 shRNA was obtained from OriGene and Santa Cruz Biotechnology, respectively. Control siRNA, HK2 siRNA, control mimic, miR-145 mimic, miR-148a mimic, miR-497 mimic, control inhibitor, miR-145 inhibitor, miR-148a inhibitor, or miR-497 inhibitor, was purchased from Invitrogen. When cells achieved 70% confluence, the above plasmid, shRNA, siRNA, or miRNA mimic (or inhibitor) was transfected into CC cells for 48 h. Finally, stable HK2-overexpressing or HK2 knockdown CC cell lines were established by selecting cells using G418 (Sigma-Aldrich) or Puromycin (Sigma-Aldrich) for 4 weeks.

## Dual-Luciferase Reporter Assay

The human HK2 wild-type 3'-UTR luciferase reporter vector was constructed from Genechem (Shanghai, China). Mutations of the miRNA binding sites in the HK2 3'-UTR sequence were made using site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). CC cells were seeded into 24-well plates, and then co-transfected with HK2 3'-UTR wild-type (or mutated) luciferase reporter vector, the pRL-CMV vector (Promega, Madison, WI), along with 30 nM of miRNA mimic, control mimic, miRNA inhibitor, or control inhibitor, using Lipofectamine 3000 (Invitrogen). After 48 h, luciferase activity was examined using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to that of the Renilla luciferase activity.

## Statistical Analysis

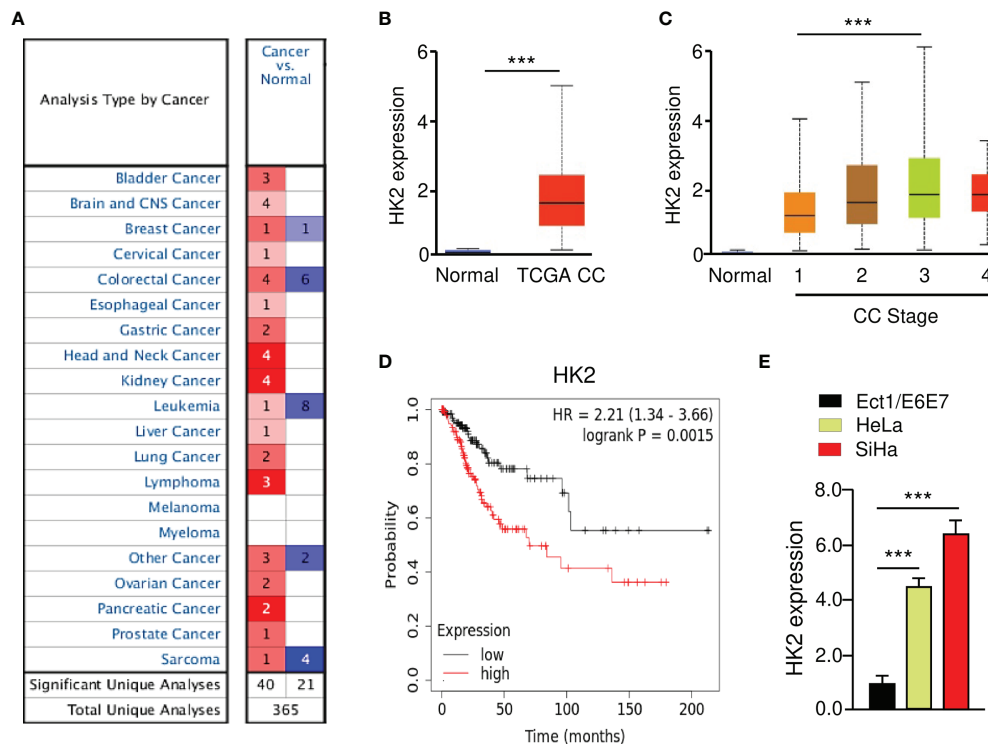
Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago). Results were presented as the mean ± standard error (SD) from three independent experiments. Statistical significance was determined using an unpaired two-tailed Student's *t*-test, one-way ANOVA test, or Mann-Whitney *U* test. Pearson's correlation test was used to measure the correlation coefficient between two variables. Differences were considered statistically significant if the *P*-value was < 0.05.

## RESULTS

### Overexpression of HK2 Correlates With Poor Prognosis in CC Patients

The Oncomine database (<https://www.oncomine.org/>) was utilized to investigate the expression of HK2 between tumors and normal tissues. The results revealed that HK2 expression was elevated in most cancer tissues (including CC) compared with normal tissues (Figure 1A). To verify these results, we explored the expression of HK2 in TCGA CC patients using the UALCAN database (<http://ualcan.path.uab.edu/index.html>). The levels of HK2 were significantly increased in CC tissues than in normal tissues (Figure 1B). We also analyzed the correlation between HK2 expression and tumor stage in CC patients using the





**FIGURE 1 |** Overexpression of HK2 Correlates with Poor Prognosis in CC Patients. **(A)** The expression of HK2 in different types of cancer (OncoPrint database). **(B)** The levels of HK2 in TCGA CC and normal tissues were examined using the UALCAN database. **(C)** Correlation between HK2 expression and tumor stage in CC patients (UALCAN database). **(D)** The prognostic value of HK2 in CC patients (KM plotter database). **(E)** The qRT-PCR analysis of HK2 expression in CC cells and normal ectocervical cell line Ect1/E6E7. \*\*\* $P < 0.001$ .

UALCAN database. The findings showed that those patients with advanced-stage CCs tended to exhibit higher levels of HK2 (**Figure 1C**). We evaluated the prognostic significance of HK2 in CC patients using the KM plotter database (<http://kmplot.com/analysis/>). Increased expression of HK2 was strongly associated with poor overall survival (**Figure 1D**). Using qRT-PCR analysis, we found an upregulation of *HK2* mRNA expression in CC cell lines (HeLa and SiHa), compared to the normal cervical epithelial cell line Ect1/E6E7 (**Figure 1E**). These results suggested that HK2 is upregulated in CC and might have a tumor-promoting function.

## HK2 Promotes Sphere Formation and CDDP Resistance in CC Cells

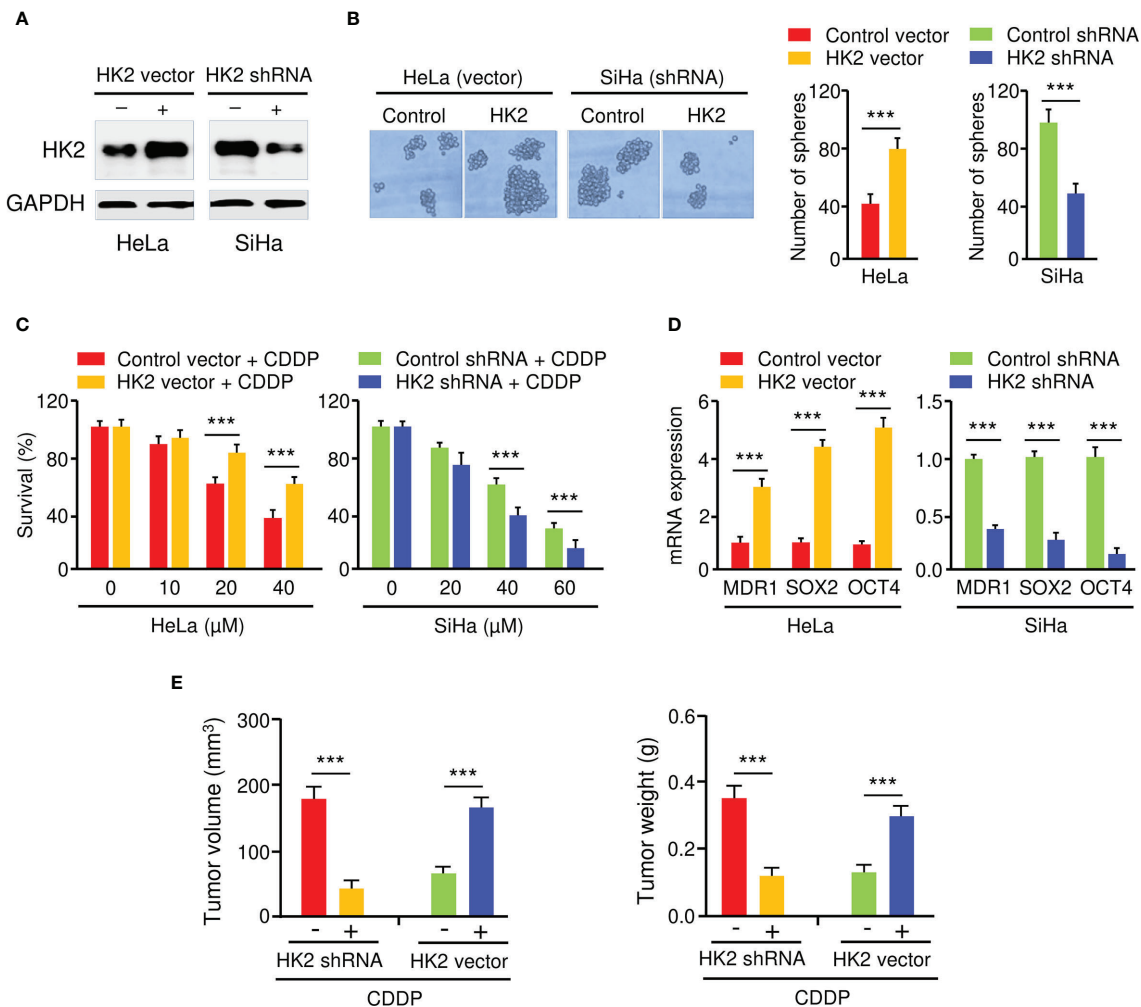
To elucidate the biological function of HK2 in CC cells, we generated HeLa cell lines with stable HK2 overexpression and established SiHa cell lines with silencing of HK2 using HK2 shRNA. The overexpression and knockdown of HK2 in CC cells were confirmed by western blotting analysis (**Figure 2A**). The overexpression of HK2 increased the sphere formation in HeLa cells, whereas the knockdown of HK2 decreased the sphere formation in SiHa cells (**Figure 2B**). Cell viability assay showed that the overexpression (or knockdown) of HK2 significantly increased (or decreased) the resistance of CC cells to CDDP in dose-dependent assays (**Figure 2C**). The expression

of *MDR1*, *SOX2*, and *OCT4* were increased after the overexpression of HK2 in HeLa cells, and an opposite effect was observed after HK2 shRNA transfection in SiHa cells (**Figure 2D**).

To analyze whether the inhibition of HK2 could sensitize CC cells to CDDP treatment *in vivo*, HK2-overexpressing HeLa cells, SiHa cells with HK2 silencing, as well as the respective control cells, were injected subcutaneously into nude mice and were treated with CDDP. Mice implanted with cells transfected with HK2 overexpression vector developed bigger tumor volumes than the control group (**Figure 2E**). Additionally, the weight of the tumors derived from the HK2-overexpressing HeLa cells was much heavier than those from the control cells (**Figure 2E**). Conversely, compared to the control mice, the mice implanted with the HK2-silenced SiHa cells had smaller tumor volumes and lighter tumor weight (**Figure 2E**). Collectively, these results suggested that HK2 overexpression facilitates the formation of spheroids and the development of CDDP resistance in CC cells.

## MiR-145, MiR-148a, and MiR-497 Are Upstream Regulators of HK2 in CC Cells

To investigate the upstream regulatory mechanism of HK2 in CC, we conducted *in silico* analysis to identify the potential of miRNAs to bind to *HK2* mRNA. Using TargetScan, miRDB, and miRSystem databases, a set of miRNAs (including miR-145,



**FIGURE 2 |** HK2 Promotes Sphere Formation and CDDP Resistance in CC Cells. **(A)** Western blot analysis of HK2 expression in CC cells transfected as indicated. **(B)** The effect of HK2 expression on the sphere formation was investigated using sphere formation assays. **(C)** Cell viability assay in response to CDDP treatment was determined using CCK-8 assay. **(D)** The expression of MDR1, SOX2, and OCT4 in CC cells transfected as indicated was detected by the qRT-PCR assay. **(E)** HK2 overexpression (or knockdown) decreased (or increased) the sensitivity of CC cells to CDDP *in vivo*. Tumor volume (left) and weight (right) were examined. \*\*\**P* < 0.001.

miR-148a, and miR-497) were found to have binding sites in the 3'-UTR of the *HK2* transcript (Figures 3A, B). Since the downregulation of miR-145, miR-148a, and miR-497, but not the remaining miRNAs, were correlated with poor overall survival in CC patients (Figure 3C), we decided to focus on these three miRNAs in our subsequent experiments.

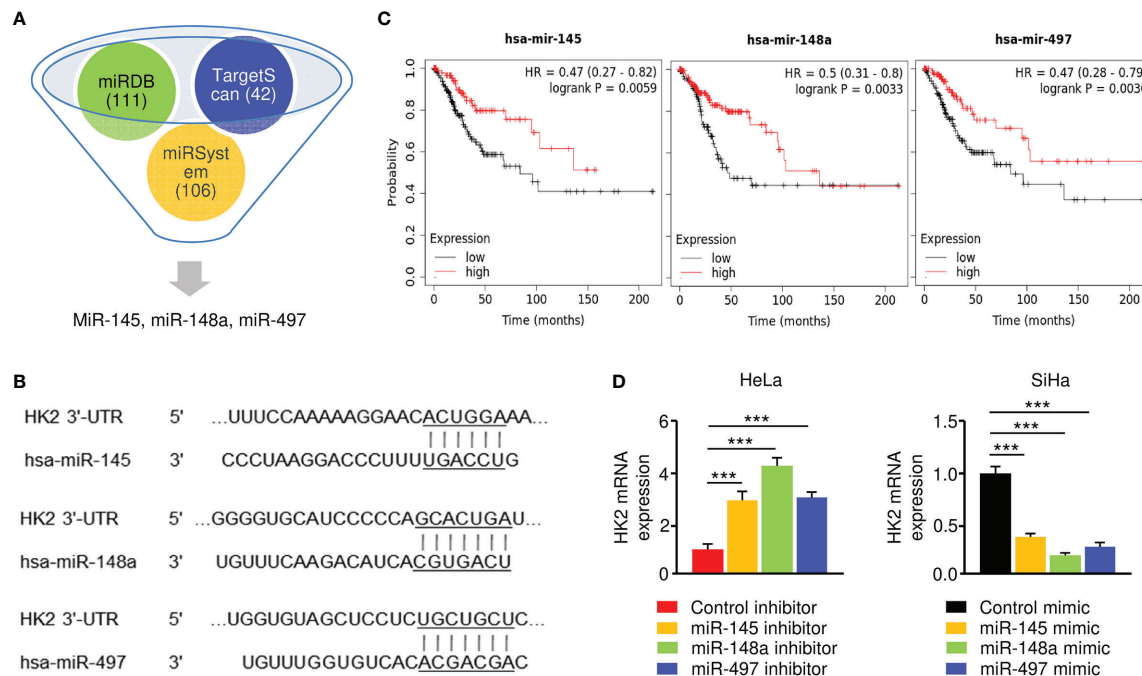
Our qRT-PCR experiments verified that the levels of miR-145, miR-148a, and miR-497 were significantly lower in SiHa cells than in HeLa cells (data not shown). To explore the regulatory relationship between these miRNAs and HK2, we performed the qRT-PCR assay to examine the mRNA expression of *HK2* in SiHa cells transfected with miRNA mimics and in HeLa cells transfected with miRNA inhibitors. The expression of *HK2* was significantly suppressed in miR-145/148a/497 mimic-expressing SiHa cells, while the inhibition of miR-145/148a/497 upregulated *HK2* expression in HeLa cells (Figure 3D). These

results demonstrated that HK2 is negatively regulated by miR-145/148a/497.

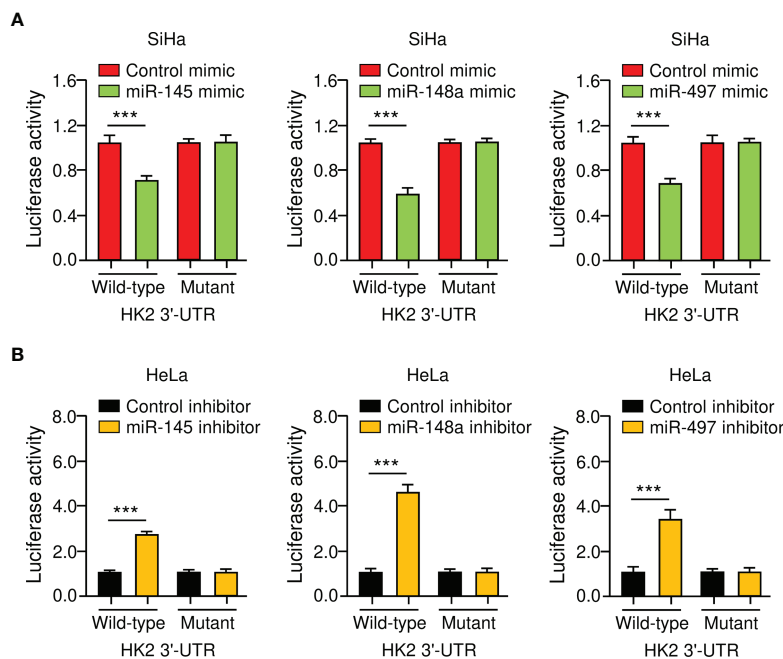
To verify the direct binding between miR-145/148a/497 and *HK2* 3'-UTR, we performed luciferase reporter assays with either a wild-type or a mutated *HK2* 3'-UTR. The luciferase activity of the wild-type *HK2* 3'-UTR was suppressed by miR-145/148a/497 mimic and was enhanced by the knockdown of miR-145/148a/497 (Figures 4A, B). Mutations of the 3'-UTR of *HK2* abolished the effects of miR-145/148a/497 (Figures 4A, B). All of these results indicated that miR-145, miR-148a, and miR-497 are upstream regulators of HK2 in CC cells.

## MiR-148a Inhibits Sphere Formation and CDDP Resistance in CC Cells

MiR-145 and miR-497 are known tumor suppressors in CC (16–19). However, little is known about the role of miR-148a in CC



**FIGURE 3 |** HK2 is negatively regulated by miR-145/148a/497 in CC Cells. **(A)** Prediction analysis revealed that several miRNAs might regulate HK2 expression. **(B)** The binding sites of miR-145/148a/497 in the wild-type *HK2* 3'-UTR were shown. **(C)** Survival analysis using the KM plotter database was conducted to assess the prognostic value of miR-145/148a/497 in CC patients. **(D)** The overexpression (or knockdown) of miR-145/148a/497 decreased (or increased) the mRNA expression of HK2 in CC cells. \*\*\* $P < 0.001$ .



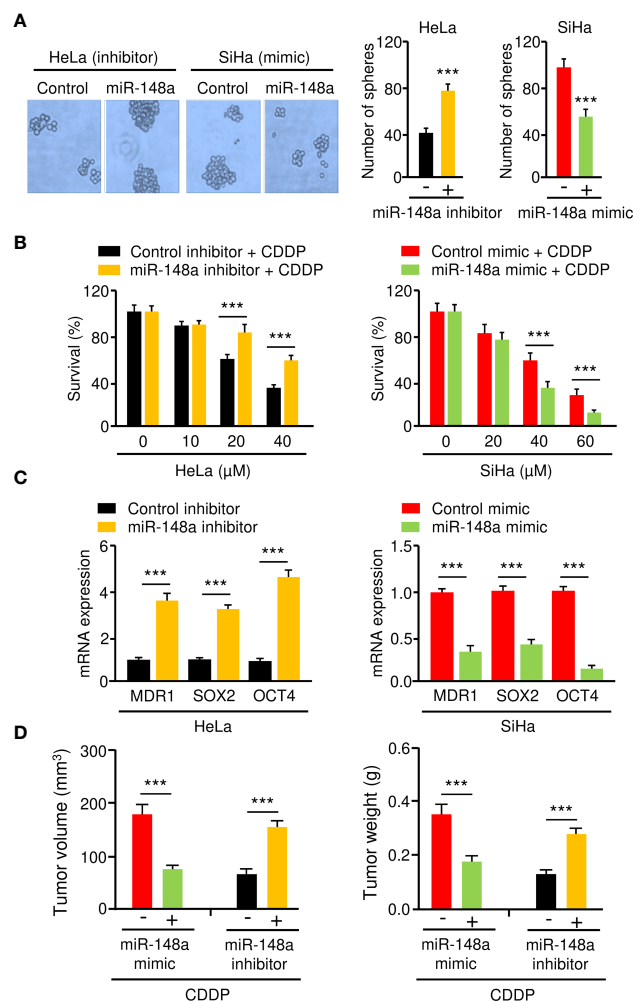
**FIGURE 4 |** *HK2* is a Direct Target Gene of MiR-145/148a/497 in CC Cells. **(A, B)** Luciferase reporter assays of CC cells that were co-transfected with indicated miRNA mimics **(A)** or miRNA inhibitors **(B)** and luciferase vector containing the wild-type or mutated *HK2* 3'-UTR. \*\*\* $P < 0.001$ .

CDDP resistance. Thus, we focused on miR-148a in this study. To analyze the effect of miR-148a overexpression or silencing on sphere formation and CDDP resistance in CC cells, we transfected miR-148a mimic into SiHa cells and introduced miR-148a inhibitor into HeLa cells. Sphere formation assays and cell viability assays showed that miR-148a overexpression significantly reduced the sphere-forming ability and attenuated CDDP resistance of SiHa cells (**Figures 5A, B**). In contrast, miR-148a inhibition significantly promoted sphere formation and increased CDDP resistance in HeLa cells (**Figures 5A, B**). The results of qRT-PCR assays confirmed the downregulation of *MDR1*, *SOX2*, and *OCT4* in miR-148a-overexpressing SiHa cells, as well as their upregulation in miR-148a-silenced HeLa cells (**Figure 5C**). To determine whether the above results were reproducible, miR-148a-overexpressing SiHa cells or miR-

148a-silenced HeLa cells were injected into nude mice and were treated with CDDP. We found that the tumors formed by miR-148a-silenced HeLa cells were larger and had more weight than control tumors (**Figure 5D**). However, tumors formed by miR-148a-overexpressing SiHa cells were smaller in both size and weight than the tumors formed by control cells (**Figure 5D**). Thus, we demonstrated that miR-148a serves as a tumor suppressor by antagonizing sphere formation and increasing the CDDP sensitivity of CC cells.

## The MiR-148a/HK2 Axis Mediates Sphere Formation and CDDP Resistance in CC Cells

To investigate whether miR-148a suppresses sphere-forming ability and CDDP resistance of CC cells by targeting HK2, we transfected SiHa cells with miR-148a mimic (or control mimic),



**FIGURE 5 |** MiR-148a Inhibits Sphere Formation and CDDP resistance in CC Cells. **(A)** The effect of miR-148a expression on the sphere formation was investigated using sphere formation assays. **(B)** After transfection with miR-148a mimic or miR-148a inhibitor, CC cells were treated with CDDP and cell viability was measured using cell viability assay. **(C)** The qRT-PCR analysis of *MDR1*, *SOX2*, and *OCT4* expression in CC cells transfected as indicated. **(D)** MiR-148a overexpression (or knockdown) increased (or decreased) the sensitivity of CC cells to CDDP *in vivo*. \*\*\**P* < 0.001.



along with HK2 overexpression vector (or control vector), and also transfected HeLa cells with miR-148a inhibitor (or control inhibitor), together with HK2 siRNA (or control siRNA). The protein expression of HK2 was examined by western blotting analysis. The expression of HK2 was decreased with the transfection with miR-148a mimic, and this reduction was reversed by the introduction of the HK2 expression vector (**Figure 6A**). In addition, the protein expression of HK2 was induced by miR-148a inhibition, and this increase was reversed by transfection with HK2 siRNA (**Figure 6A**). The results of sphere formation and cell viability assays showed that sphere formation and CDDP resistance were suppressed by transfection of miR-148a mimic, whereas this repression could be eliminated by forced expression of HK2 (**Figures 6B, C**). We also verified that miR-148a inhibition induced sphere formation and CDDP resistance, while knockdown of HK2 attenuated these malignant properties of CC cells (**Figures 6B, C**). Consequently, these results demonstrated that the miR-148a/HK2 axis functionally regulates sphere formation and CDDP resistance in CC cells.

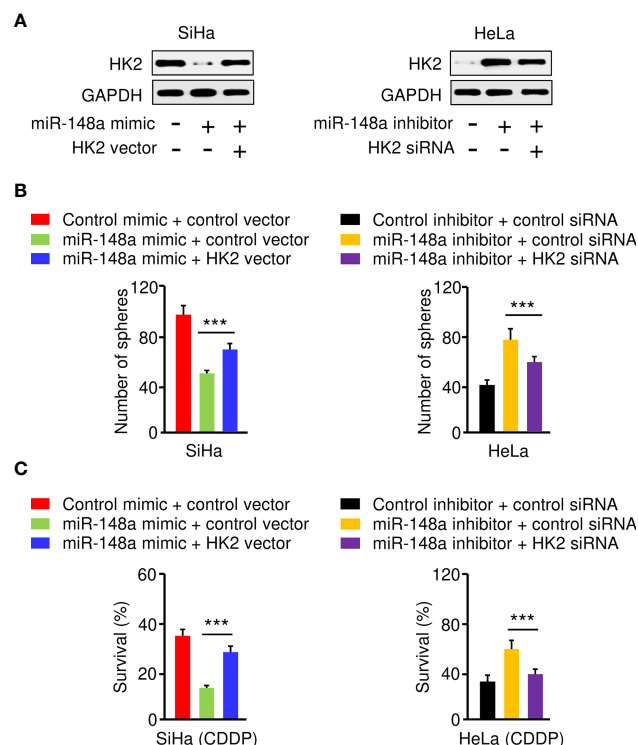
### Correlation Between MiR-145/148a/497 and HK2 Expression in CC Tissues

To further define the role of miR-145, miR-148a, miR-497, and HK2 in human CC, we measured their expression in 30 adjacent

normal tissues and 30 CC tissues using the qRT-PCR assay. We found that CC tissues exhibited lower expression of miR-145, miR-148a, and miR-497 (**Figure 7A**). Our results also showed increased expression of HK2 in human CC tissues (**Figure 7**). Overall, these results support the notion that HK2 is crucial for the maintenance of the sphere-forming property and CDDP resistance in CC cells, and upregulation of HK2 observed in CC tissues might be caused by the repression of multiple tumor suppressor miRNAs (including miR-145, miR-148a, and miR-497).

## DISCUSSION

Even though CDDP has been certified as a front-line drug for CC treatment, primary and acquired CDDP resistance leads to the failure of CDDP-based therapy and accounts for the recurrence of CC (2). Growing evidence has indicated that cancer stem cells could contribute to chemoresistance through various mechanisms, such as induction of metabolic enzyme aldehyde dehydrogenase (20), overexpression of ABC transporters (21), activation of Notch, Hedgehog, and Wnt pathways (22). In this work, we have demonstrated that HK2, a critical enzyme in glucose metabolism, is commonly



**FIGURE 6 |** The MiR-148a/HK2 Axis Mediates Sphere Formation and CDDP Resistance in CC Cells. **(A)** Western blot analysis of HK2 expression in CC cells transfected as indicated. **(B)** Sphere formation of CC cells transfected as indicated. **(C)** Cell viability of CC cells transfected as indicated in the presence of CDDP was detected by the cell viability assay. \*\*\* $P < 0.001$ .

overexpressed in human CC, and knocking down HK2 sensitizes CC cells to CDDP. Besides, overexpression of HK2 could be partially due to the downregulation of miRNAs (especially miR-148a). To our knowledge, this study is the first to elucidate the oncogenic role of HK2 in promoting CDDP resistance in CC, and to reveal the biological relevance of the miR-14a/HK2 axis in the sphere formation ability and CDDP sensitivity of CC cells.

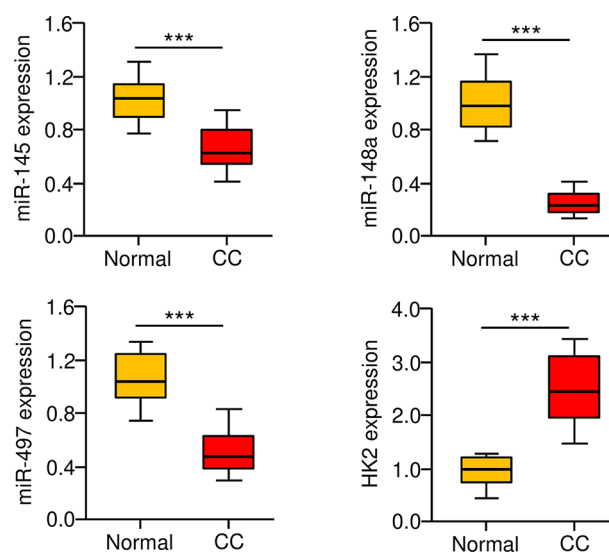
Aberrant expression of HK2 in CC has been previously reported (8, 9). The results from our meta-analysis and qRT-PCR assays have confirmed the elevated expression of HK2 in CC tissues and cell lines, and the upregulation of HK2 is closely associated with worse survival rates of CC patients. Although the silencing of HK2 was known to inhibit the growth and migration of CC cells (8, 9), whether HK2 could influence the sphere-forming properties and the development of CDDP resistance of CC cells remains unknown. Our gain-of-function and loss-of-function assays have uncovered the promoting role of HK2 in maintaining cancer stem cell-like ability and the acquisition of CDDP resistance. Importantly, knockdown of HK2 combined with CDDP treatment reduces CC proliferation *in vivo*. These results together suggested that targeting HK2 may abrogate cancer stem cell populations, as demonstrated by reduced sphere formation capacity, and regain the sensitivity of CC patients to CDDP.

In addition to regulating glucose metabolism, HK2 promotes cancer stem cell self-renewal in CC cells, elucidating that HK2 is an upstream activator of cancer stem cell expansion (23). In ovarian cancer, overexpression of HK2 enhances stemness properties by upregulating the expression of cancer stemness-related genes, such as OCT4 (24). Moreover, HK2 is required for the maintenance of esophageal cancer stem cell phenotypes (25). The chemoresistance to 5-FU and CDDP

and the tumorigenesis of esophageal cancer stem cells are reduced after HK2 knockdown (25). Overexpression of HK2 effectively promoted epithelial-mesenchymal transition (EMT) phenotypes and enhanced aerobic glycolysis in EC cells (26). Consistent with these previous studies, the current demonstrated that silencing HK2 decreases the expression of cancer stem cell markers OCT4 and SOX2, suggesting that therapeutic approaches targeting HK2 might be an effective strategy for suppressing the expansion and maintenance of the cancer stem cell population in CC cells.

Targeting HK2 using HK2 inhibitors has been a hopeful strategy for cancer treatment. To date, different types of HK2 inhibitors have been developed (27). For instance, the selective HK2 inhibitor Benitrobenrazide binds directly to HK2, blocks glycolysis, and induces apoptosis in HK2-overexpressing cancer cells (28). Glycolysis is activated in radioresistant CC cells, and inhibiting glycolysis with HK2 inhibitor 2-DG improves the sensitivity of radioresistant CC cells to irradiation (29). These results support the possibility that inhibition of glycolysis *via* specific HK2 inhibitors, in combination with other therapies, would be an effective strategy for the treatment of CC.

Having established the role of HK2 in sphere formation and CDDP resistance of CC cells, we sought mechanistic evidence for activation of HK2. According to our *in silico* analysis, qRT-PCR assays, and luciferase reporter assays, three miRNAs (miR-145, miR-148a, and miR-497) were determined as upstream regulators of HK2 in CC cells. MiR-145 and miR-497 suppress the aggressive phenotypes of CC cells (16–19). MiR-145 negatively modulates proliferation, EMT, migration, and invasion of CC cells (16). Interestingly, miR-145 directly binds to HK2 mRNA and reduces its expression in renal cell carcinoma (30). In our work, we have found that miR-145, miR-148a, and



**FIGURE 7 |** Correlation between MiR-145/148a/497 and HK2 Expression in CC Tissues. The expression of miR-145, miR-148a, and miR-497 in CC and adjacent normal tissues was measured using the qRT-PCR assays. \*\*\* $P < 0.001$ .

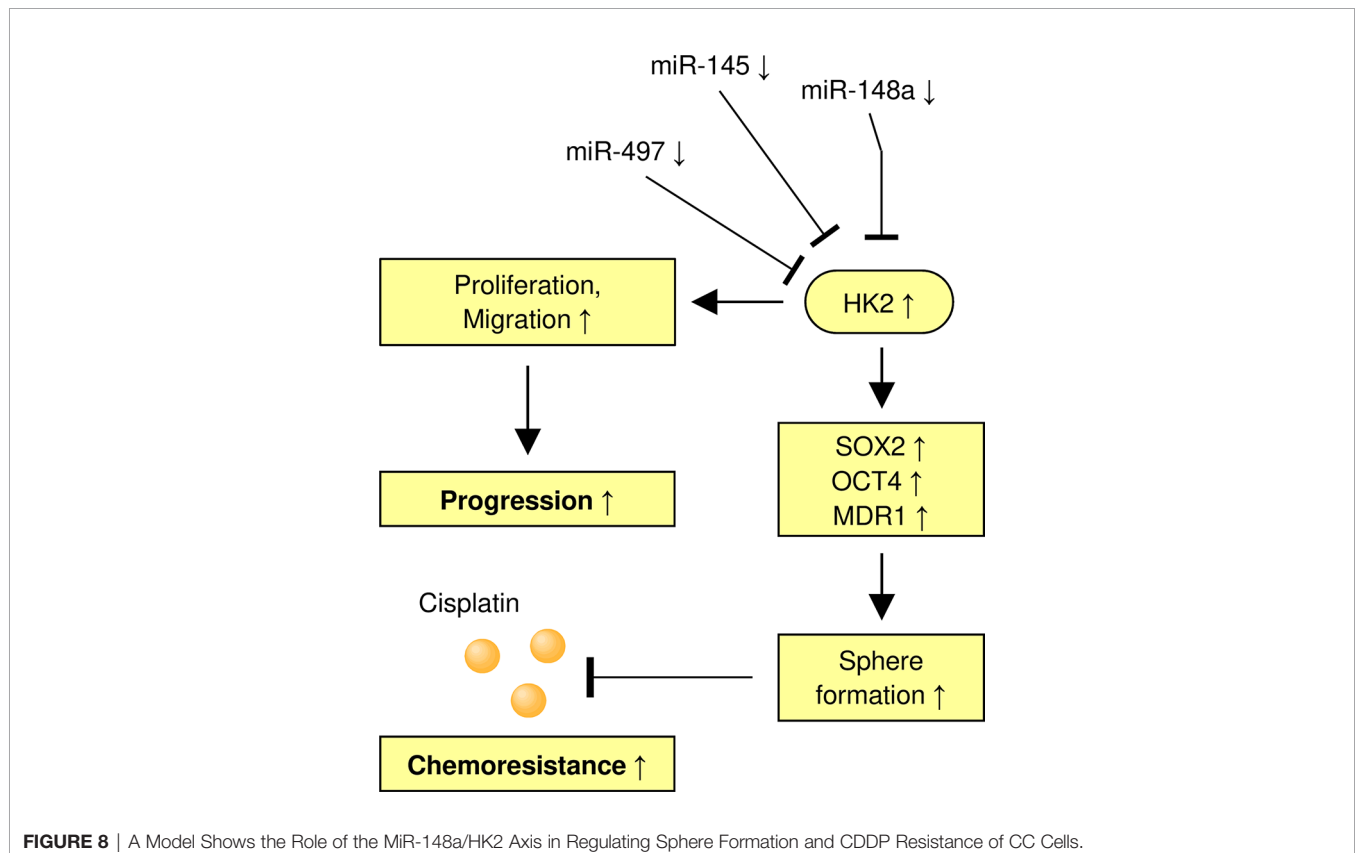
miR-497 inhibit endogenous HK2 expression by binding to its 3'-UTR. Consequently, silencing of these miRNAs in CC tissues stimulates the expression of HK2, facilitating the development of cancer stem cell-like properties and CDDP resistance. Collectively, we propose a complex regulatory relationship between these miRNAs and HK2 in CC. Of note, HK2 levels are regulated at the transcriptional, post-translational, and translational levels (31). Different genetic and epigenetic mechanisms may be involved in the regulation of HK2 in CC, the exact mechanisms for the upregulation of HK2 are deserving of future studies.

Aberrant expression of miR-148a is a common event in human tumors (32). For instance, the downregulation of miR-148a has been detected in various cancers, including gastric, colon, pancreatic, liver, esophageal, breast, and lung cancer (32). It is known that miR-148a could suppress the proliferation, migration, invasion, and metastasis of cancer cells by targeting a large number of downstream genes (32). Of note, in esophageal cancer cells, re-expression of miR-148a significantly improves the sensitivity of cells to CDDP and 5-FU (33). In cervical cancer, a previous study using miRNA array and qRT-PCR assay has suggested that miR-148a levels were significantly lower in CC tissues compared to normal tissues (34). Overexpression of miR-148a could markedly suppress the proliferation of CC cells (35). Moreover, lncRNA SNHG4 promotes the proliferation of CC cells through regulating c-Met *via* targeting miR-148a (36).

LncRNA SNHG12 sponges miR-148a to increase CDK1 expression, thereby attenuating the radiation-induced apoptosis in CC cells (37). In line with this research, we have shown that miR-148a attenuates CDDP resistance in CC cells, at least in part, by targeting HK2. Therefore, our results elucidated a new mechanism for miR-148a in chemoresistance. Cancer cells frequently overexpress some proteins implicated in glucose metabolism, such as GLUT1, the main glucose transporter (38). Since a recent study has reported that GLUT1 is a direct target of miR-148a in human intrahepatic cholangiocarcinoma (39), miR-148a may also regulate glucose metabolism by inhibiting GLUT1 in CC. Future studies to identify the interactions between miR-148a and its targets would strengthen our understanding of the function of miR-148a, and yield key insights into the metabolic landscape of CC.

## CONCLUSION

In summary, our findings demonstrate the involvement of HK2 in the maintenance of the sphere-forming property and CDDP resistance in CC cells. In addition, we have identified miR-148a as an upstream suppressor of HK2 to inhibit the ability of sphere formation and increase the sensitivity of CDDP in CC cells (**Figure 8**). We have uncovered a previously unknown miR-



148a/HK2 axis that modulates cancer stem cell-like features and CDDP resistance in CC.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University.

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## AUTHOR CONTRIBUTIONS

HZ and TY designed the experiments. HY and HH conducted the experiments. TY, YCH, YH, and JG analyzed the data. All authors contributed to the article and approved the submitted version.

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# Novel Implications of MicroRNAs, Long Non-coding RNAs and Circular RNAs in Drug Resistance of Esophageal Cancer

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Esophageal cancer is the eighth most common malignancy and the sixth leading cause of cancer-related deaths worldwide. Chemotherapy based on platinum drugs, 5-fluorouracil, adriamycin, paclitaxel, gemcitabine, and vinorelbine, as well as targeted treatment and immunotherapy with immune checkpoint inhibitors improved the prognosis in a portion of patients with advanced esophageal cancer. Unfortunately, a number of esophageal cancer patients develop drug resistance, resulting in poor outcomes. Multiple mechanisms contributing to drug resistance of esophageal cancer have been reported. Notably, non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), have been identified to play crucial roles in modulating esophageal cancer drug resistance. In the present review, we highlight the underlying mechanisms how miRNAs, lncRNAs, and circRNAs impact the drug resistance of esophageal cancer. Several miRNAs, lncRNAs, and circRNAs may have potential clinical implications as novel biomarkers and therapeutic targets for esophageal cancer.

**Keywords:** esophageal cancer, drug resistance, microRNA, long non-coding RNA, circular RNA

## INTRODUCTION

Esophageal cancer is a complex malignancy and the sixth leading cause of cancer death worldwide. A total of 572,034 esophageal cancer cases were diagnosed worldwide and 508,585 cases dead in 2018 (Bray et al., 2018). There are two major histological subtypes of esophageal cancer, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). EAC is prevalent in Western countries including North America and Western Europe; while ESCC is the major histologic type of esophageal cancer in Eastern Asia and Africa (Bray et al., 2018). In the past 15 years, the 5-year survival rate for patients with esophageal cancer for all stages combined is only 20%. However, patients with metastatic esophageal cancer have a poor prognosis, with the 5-year survival rate of approximately 5% (Bray et al., 2018; Siegel et al., 2020).

Although treatment options are limited for patients with unresectable, locally advanced, or metastatic esophageal cancer, a portion of patients could benefit from the comprehensive treatment

of chemotherapy, targeted therapy and immunotherapy with immune checkpoint inhibitors (Eltweri et al., 2019; Kojima et al., 2020; Lopez et al., 2020; Stroes et al., 2020). The commonly used chemotherapeutic agents in clinics include platinum drugs, 5-fluorouracil (5-FU), adriamycin (ADM), paclitaxel (PTX), irinotecan, gemcitabine (GEM), and vinorelbine (Liu S. L. et al., 2015; Wang Y. S. et al., 2016; Tin et al., 2018; van Zweeden et al., 2018; Ni et al., 2020). Molecular targeted therapy drugs include anti-HER2 monoclonal antibodies (trastuzumab and pertuzumab), oral tyrosine kinase inhibitors (TKIs) targeting HER-1/HER-2 (lapatinib), anti-vascular endothelial growth factor receptor 2 (VEGFR-2) antibody (ramucirumab) and anti-EGFR monoclonal antibody (panitumumab) (Press et al., 2017; Shepard et al., 2017; Chakrabarti et al., 2018; Guo et al., 2018; Yamaguchi et al., 2018; De Vita et al., 2019; Hassan et al., 2019; Rogers et al., 2019; Wagner et al., 2019; Stroes et al., 2020). Immune-checkpoint blockade agents are also used, such as anti-programmed cell death protein 1 (PD-1) monoclonal antibodies (nivolumab and pembrolizumab) (Herbst et al., 2019; Shah et al., 2019; Kato et al., 2020; Rogers et al., 2020).

Unfortunately, esophageal cancer cells frequently develop multi-drug resistance (MDR) which seriously impaired the efficacy of drugs and subsequently led to poor prognosis. The underlying complicated mechanisms involved in drug resistance of esophageal cancer have been reported, such as the enhanced DNA damage repair capability, the up-regulated expression of drug efflux transporters to pump out chemo-agents from cells, the accelerated cell growth and autophagy flux, dysregulation of cell cycle, epithelial-mesenchymal transition (EMT), apoptosis inactivation as well as activation of cancer stem cells (CSCs) (Liu D. S. et al., 2015; Cheng et al., 2017; Zhou et al., 2017, 2020; Huang et al., 2018; Qiao et al., 2018; Guo et al., 2019; Lin C. H. et al., 2019).

Non-coding RNAs (ncRNAs) are a class of RNA transcripts without protein-coding ability, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). Multiple miRNAs, lncRNAs, and circRNAs have been reported to be involved in controlling various cellular functions, such as apoptosis, cell growth, autophagy, EMT, and cell cycle regulation (Wang et al., 2015c; Bhan et al., 2017; Peng et al., 2017; Ren et al., 2017a,b; Rupaimoole and Slack, 2017; Pan et al., 2018; Vo et al., 2019; Chen et al., 2020; Yuan et al., 2020; Zhang et al., 2020a). Among these ncRNAs, several miRNAs, lncRNAs, and circRNAs are dysregulated in esophageal cancer and have been shown to be associated with tumorigenesis, metastasis, prognosis, as well as treatment resistance to radiotherapy and drugs (Song et al., 2014; Wang et al., 2015b; Wen et al., 2016; Zhang E. et al., 2017; Zhang et al., 2018; Sang et al., 2018). So far, there have been few reports of ncRNAs involvement in the resistance to targeted therapy and immunotherapy. Interestingly, serum miRNAs, including miR-1233-5p, miR-6885-5p, miR-4698, and miR-128-2-5p, have been identified to predict the response to nivolumab, a PD-1 inhibitor, in advanced ESCC patients (Sudo et al., 2020). Notably, a number of miRNAs, lncRNAs, and circRNAs have been shown to play crucial roles in esophageal cancer chemoresistance (Jin et al., 2016; Li et al., 2018; Lin K. et al., 2019; Wu et al.,

2020; Zou et al., 2020). Considering the importance of ncRNAs in the development of drug resistance of esophageal cancer, we systematically summarized the underlined mechanisms of these miRNAs, lncRNAs, and circRNAs in the current review (Figure 1).

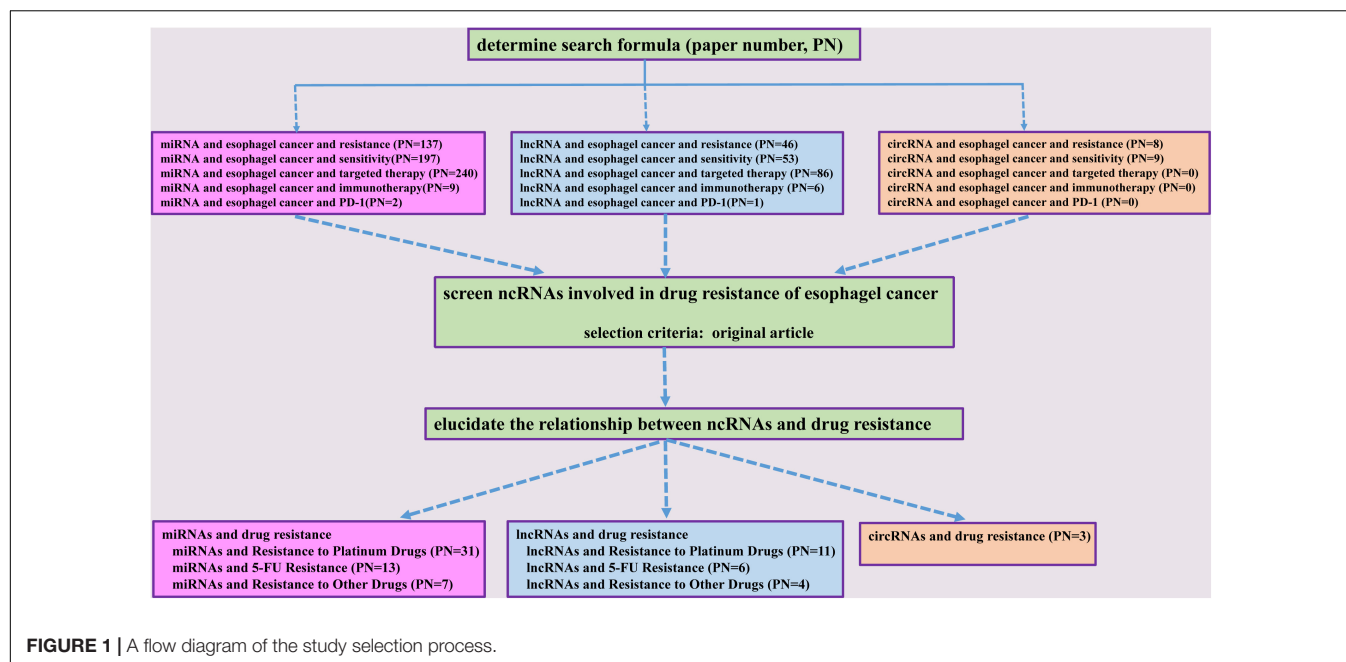
## MicroRNAs AND DRUG RESISTANCE

MicroRNAs are small, endogenous, single-stranded ncRNAs and function as crucial regulators of gene expression at the post-transcriptional level. MiRNAs down-regulate expression levels of target genes through binding to the 3'-untranslated region (3'-UTR) of target mRNA and leading to target mRNA degradation or blocking translation. It has been found that miRNAs can act as oncogenes or tumor suppressors to regulate cell differentiation, proliferation, apoptosis, metabolic reprogramming and angiogenesis (Bracken et al., 2016; Rupaimoole and Slack, 2017). Additionally, miRNAs could be released from esophageal cancer cells via exosomes and affect neighboring or distant cells. The exchange of the genetic information and/or regulation of target gene expression of miRNAs may change biological behaviors of recipient cells (Tanaka Y. et al., 2013; Luo et al., 2019; Gao et al., 2020). Multiple aberrantly expressed miRNAs have been identified in esophageal cancer, especially in the development of drug resistance (Hamano et al., 2011; Wang Y. et al., 2016; Liu et al., 2017; Zhang J. X. et al., 2017). Here, we summarized the roles of miRNAs in the resistance to platinum drugs, 5-FU and other agents in esophageal cancer.

### MicroRNAs and Resistance to Platinum Drugs

Platinum drugs are the most commonly used antitumor drugs in clinic. In cells, platinum binds to genomic DNA to form platinum-DNA adducts, resulting in DNA replication and transcription disorders, and subsequently tumor cell death. Multiple platinum drugs have been applied in clinical managements of esophageal cancer, such as cisplatin (DDP, the first-generation platinum agent), carboplatin (the second-generation platinum agent), oxaliplatin, and lopolatin (the third-generation platinum agents). However, response rates to platinum drugs are low in some esophageal cancer patients. Several miRNAs have been reported to participate in development of resistance to platinum drugs in esophageal cancer (Table 1).

There are many oncogenic miRNAs promoting the resistance of esophageal cancer to platinum drugs (Table 1). MiR-10b could enhance DDP resistance through silencing peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and activating the AKT/mTOR/p70S6K signaling pathway (Wu et al., 2020). MiR-432-3p has been found to promote the resistance to DDP by directly suppressing expression of Kelch-like ECH-associated protein 1 (KEAP1) and stabilizing NF-E2-related factor 2 (NRF2). On the contrary, miR-432-3p knocking-off through the CRISPR/Cas9 technology could reverse DDP resistance of ESCC cells (Akdemir et al., 2017). As the most highly expressed



miRNA in DDP-resistant ESCC cells, miR-141 could potentiate the resistance to DDP by directly silencing Yes-associated protein 1 (YAP1) (Imanaka et al., 2011). In addition, the expression of miR-200c was also found to be significantly up-regulated in DDP-resistant esophageal cancer cells compared to their parent cells. Mechanistically, miR-200c increases DDP resistance through modulating activity of the AKT pathway (Hamano et al., 2011). Among esophageal cancer patients, miR-200c levels were markedly correlated with response to chemotherapy. That is, high levels of miR-200c in patient serum were significantly correlated with poor response to neoadjuvant chemotherapy treated with DDP, 5-FU and adriamycin (ACF) (Tanaka K. et al., 2013). Ectopic miR-21 in ESCC cells has been found to promote DDP resistance (Komatsu et al., 2016a). Moreover, the levels of miR-21 and miR-23a in pre-operative plasma of ESCC patients might be used to predict the resistance to pre-operative chemotherapy regimens with DDP plus 5-FU (Komatsu et al., 2016a,b). Consistently, exosome-derived oncogenic miR-21 has also been shown to weaken DDP sensitivity of esophageal cancer cells by silencing programmed cell death 4 (PDCD4) (Yang et al., 2019). Interestingly, miR-27a/b may confer DDP resistance through transforming normal fibroblast into cancer-associated fibroblasts (CAF). Although ectopic miR-27a/b could not significantly impair chemosensitivity of esophageal cancer cells, the supernatant originating from miR-27a/b-transfected CAFs has been shown to promote DDP resistance in esophageal cancer cells, compared with supernatant deriving from normal fibroblast. Moreover, the resistance to DDP could be overcome after adding neutralized antibody against transforming growth factor- $\beta$  (TGF- $\beta$ ) to the supernatant (Tanaka et al., 2015). MiR-483 and miR-214 were dramatically up-regulated in ESCC tissues compared with those in normal tissues and could confer DDP resistance in ESCC cells (Zhou and Hong, 2013). Oncogenic miR-223 has been found to diminish DNA repair and apoptosis

potentials of esophageal cancer cells and increase the resistance to DDP via targeting and down-regulating Poly (ADP-ribose) polymerase 1 (PARP1) (Streppel et al., 2013). MiR-196a and miR-296 could promote DDP resistance via promoting the expression of cell membrane transporter ATP binding cassette subfamily G member 2 (ABCG2) and P-glycoprotein (P-gp) (Hong et al., 2010; Ma et al., 2016). Oncogenic miR-455-3p could increase the subpopulations of CD90<sup>+</sup> and CD271<sup>+</sup> CSCs/tumor-initiating cells (T-ICs) through activating the Wnt/ $\beta$ -catenin signaling and the TGF- $\beta$  signaling, which leads to resistance of ESCC cells to DDP (Liu et al., 2017). MiR-193, a highly expressed miRNA in DDP-resistant esophageal cancer cell exosomes (TE-1/DDP/exo), has been shown to promote DDP resistance by targeting transcription factor AP-2 gamma (TFAP2C). Moreover, level of high miR-193 or low TFAP2C could suppress apoptosis and abate cell cycle inhibition (Shi et al., 2020). Most recently, miR-106b-3p, an overexpressed miRNA in ESCC tissues, has also been demonstrated to confer the resistance to DDP by targeting glutamine  $\gamma$ -glutamyltransferase E (TGM3) in esophageal cancer cells (Zhu Y. et al., 2021). In addition, oncogenic miR-141-3p is highly expressed in oxaliplatin-resistant esophageal cancer cells and has been found to enhance resistance by silencing phosphatase and tensin homolog (PTEN), *in vitro* and *in vivo* (Jin et al., 2016).

By contrast, several tumor suppressor miRNAs can reverse resistance of esophageal cancer to platinum drugs (Table 1). Ectopic miR-544 and miR-338-5p could overcome DDP resistance of esophageal cancer via targeting and down-regulating oncogene E2F transcription factor 5 (E2F5) and fermitin family homolog 2 (FERMT2) (Lin W. C. et al., 2019; Sun et al., 2019). Similarly, ectopic miR-125a-5p could potentiate the cytotoxic and apoptotic effects of DDP on esophageal cancer cells through modulating the signal transducer and activator of transcription 3 (STAT3) signaling pathway (Zhao et al.,



**TABLE 1 |** MicroRNAs (miRNAs) and platinum drugs resistance in esophageal cancer.

MiRNAs	Expression <sup>a</sup>	Genes and pathways	Drug	References
miR-10b	↑	PPAR $\gamma$ /AKT/mTOR/P70S6K	Cisplatin	Wu et al., 2020
miR-432-3p	↑	KEAP1/NRF2	Cisplatin	Akdemir et al., 2017
miR-141	↑	YAP1	Cisplatin	Imanaka et al., 2011
miR-200c	↑	PPP2R1B/AKT	Cisplatin	Hamano et al., 2011
miR-21	↑	–	Cisplatin	Komatsu et al., 2016a
		PDCD4	Cisplatin	Yang et al., 2019
miR-27a/b	↑	CAF	Cisplatin	Tanaka et al., 2015
miR-483	↑	–	Cisplatin	Zhou and Hong, 2013
miR-214	↑	–	Cisplatin	Zhou and Hong, 2013
miR-223	↑	PARP	Cisplatin	Streppel et al., 2013
miR-196a	↑	ABCG2	Cisplatin	Ma et al., 2016
miR-296	↑	P-gp, Bcl-2, Bax, cyclin D1, P27	Cisplatin	Hong et al., 2010
miR-455-3p	↑	Wnt/ $\beta$ -catenin	Cisplatin	Liu et al., 2017
miR-193	↑	TFAP2C, cyclin D1, bax, caspase 3	Cisplatin	Shi et al., 2020
miR-106b-3p	↑	TGM3	Cisplatin	Zhu Y. et al., 2021
miR-141-3p	↑	PTEN	Oxaliplatin	Jin et al., 2016
miR-544	↓	E2F5	Cisplatin	Sun et al., 2019
miR-338-5p	↓	FERMT2	Cisplatin	Lin W. C. et al., 2019
miR-125a-5p	↓	STAT3	Cisplatin	Zhao et al., 2018
miR-218	↓	Survivin	Cisplatin	Jingjing et al., 2016
		PI3K/AKT/mTOR	Cisplatin	Tian et al., 2015
miR-214-3p	↓	CUG-BP1, survivin	Cisplatin	Phatak et al., 2016
miR-499	↓	pol $\beta$	Cisplatin	Wang et al., 2015d
let-7c	↓	IL-6/STAT3	Cisplatin	Sugimura et al., 2012
let-7g/i	↓	ABCC10	Oxaliplatin	Wu et al., 2016
miR-634	↓	OPA1, TFAM, LAMP2, AIP1, XIAP, BIRC5, NRF2	Cisplatin	Fujiwara et al., 2015
miR-187	↓	C3	Cisplatin	Winther et al., 2016
miR-130a-3p	↓	Bcl-2	Cisplatin	Lindner et al., 2018
miR-145	↓	PI3K/AKT, MRP1, P-gp	Cisplatin	Zheng et al., 2019
miR-181a-5p	↓	CBLB	Cisplatin	Yang S. et al., 2020
miR-153-3p	↓	Nrf-2	Cisplatin	Zuo et al., 2020

<sup>a</sup>miRNAs either up-regulated (↑) or down-regulated (↓) in platinum drugs resistant esophageal cancer cells. This table shows 29 miRNAs whose expression levels and potential targets in platinum drugs resistance of esophageal cancer.

2018). Tumor suppressor miR-218 could reverse DDP resistance and promote apoptosis of esophageal cancer cells by silencing oncogene survivin (Jingjing et al., 2016). Interestingly, miR-218 could inhibit cell proliferation, promote cell apoptosis, induce cell cycle arrested in G<sub>0</sub>/G<sub>1</sub> phase, as well as increase DDP sensitivity of esophageal cancer cells through suppressing phosphorylation of PI3K, AKT, and mTOR (Tian et al., 2015). MiR-214-3p, a highly down-regulated miRNA in ESCC cells, could weaken DDP resistance by targeting and down-regulating both survivin and RNA-binding protein (RBP) CUG-BP1 (Phatak et al., 2016). Tumor suppressor miR-499 have also been found to reverse the DDP resistance of esophageal cancer cells by silencing DNA polymerase  $\beta$  (pol $\beta$ ) (Wang et al., 2015d). Through suppressing the IL-6/STAT3 pathway and drug transporter ABCC10, let-7, and let-7g/i could restore the sensitivity to DDP and oxaliplatin and promote apoptosis of esophageal cancer cells (Sugimura et al., 2012; Wu et al., 2016). Tumor suppressor miR-634 could enhance the cytotoxicity induced by DDP via concurrently targeting multiple genes which were linked with anti-apoptosis, mitochondrial homeostasis, autophagy and antioxidant ability.

Specifically, anti-apoptotic genes include APAF1 interacting protein (AIP1), baculoviral IAP repeat containing 5 (BIRC5), and E3 ubiquitin protein ligase X-linked inhibitor of apoptosis (XIAP); mitochondrial homeostasis genes involve transcription factor A, mitochondrial (TFAM) and optic atrophy 1 (OPA1); autophagy and antioxidant genes refer to lysosomal-associated membrane protein 2 (LAMP2) and NRF2 (NFE2L2; nuclear factor, erythroid 2-like 2) (Fujiwara et al., 2015). MiR-187 was significantly down-regulated in pre-treatment tumors of EAC patients with worse response to neoadjuvant chemoradiation therapy. Mechanistically, miR-187 could reverse the resistance to DDP and X-ray irradiation in EAC cells by modulating multiple signaling pathways, including the complement component 3 (C3) signaling (Winther et al., 2016). Through silencing Bcl-2, miR-130a-3p could sensitize esophageal cancer cells to DDP (Lindner et al., 2018). By suppressing the PI3K/AKT pathway and expression of MDR-associated proteins MRP1 and P-gp, tumor suppressor miR-145 could sensitize ESCC to DDP and promote DDP-induced apoptosis and cell cycle arrest (Zheng et al., 2019). Most recently, miR-181a-5p, a down-regulated

miRNA in DDP-resistant EAC cell line (OE19/DDP), has also been demonstrated to reverse the resistance to DDP in EAC by modulating CBLB. Moreover, ectopic expression of miR-181a-5p could potentiate the *in vivo* sensitivity to DDP in EAC (Yang S. et al., 2020). Additionally, tumor suppressor miR-153-3p could also potentiate the sensitivity of EC cells to DDP via Nrf-2 (Zuo et al., 2020).

## MicroRNAs and 5-Fluorouracil Resistance

5-fluorouracil is a heterocyclic aromatic chemotherapeutic agent which is broadly utilized in esophageal cancer treatments. 5-FU inhibits thymidylate synthase (TS), hampers DNA replication, and subsequently resulting in arrested cell cycle and apoptosis (Longley et al., 2003; Subbarayan et al., 2010). It has been reported that several oncogenic or tumor suppressive miRNAs are involved in 5-FU resistance (Table 2).

Multiple oncogenic miRNAs could promote 5-FU resistance of esophageal cancer cells. Oncogenic miR-141-3p can confer 5-FU resistance by silencing PTEN and the elevated levels of miR-141-3p was associated with TNM stage and differentiation status of ESCC patients (Jin et al., 2016). MiR-221 was overexpressed in 5-FU resistant esophageal cancer cells and EAC tissue and could potentiate 5-FU resistance by directly down-regulating the expression of dickkopf Wnt signaling pathway inhibitor 2 (DKK2) and activating the Wnt/ $\beta$ -catenin-EMT pathways (Wang Y. et al., 2016). In 5-FU resistant esophageal cancer cells, miR-27b-3p and miR-193b-3p have been found to be significantly up-regulated. Ectopic miR-27b-3p and miR-193b-3p could promote 5-FU resistance through silencing expression of their target gene KRAS (Hummel et al., 2014). MiR-296 has been found to contribute to 5-FU resistance in esophageal cancer cells through modulating the expression of P-gp, Bcl-2, Bax, cyclin D1 and P27 (Hong et al., 2010). In addition, oncogenic miR-21, miR-214, and miR-483 could also promote 5-FU resistance of ESCC cells (Zhou and Hong, 2013; Komatsu et al., 2016a). By targeting presenilin-1 (PSEN1), miR-193a-3p could also confer 5-FU resistance of esophageal cancer cells (Meng et al., 2016).

On the contrary, a number of tumor suppressor miRNAs can reverse 5-FU resistance of esophageal cancer cells. MiR-193b was highly expressed in chemosensitive esophageal cancer cells. MiR-193b has been shown to significantly promote the sensitivity to 5-FU in KYSE450 cells by silencing stathmin 1, which leads to activation of the autophagic flux and non-apoptotic cell death (Nyhan et al., 2016). Additionally, tumor suppressor miR-634 have also been found to be involved in development of 5-FU resistance by directly targeting a number of mitochondrial apoptosis pathway genes, such as *OPA1*, *TFAM*, *LAMP2*, *APIP*, *XIAP*, *BIRC5*, and *NRF2* (Fujiwara et al., 2015). Several dysregulated miRNAs, including miR-192-5p, miR-378a-3p, miR-194-5p, miR-18a-3p, and miR-125a-5p, have been identified to be down-regulated in 5-FU resistant esophageal cancer cells. Ectopic miR-192-5p, miR-378a-3p, miR-194-5p, miR-18a-3p, and miR-125a-5p could reverse 5-FU resistance through silencing the expression of their target genes thymidylate synthase (TYMS), CBL-B, ABCC3, KRAS, and ERBB2 (Hummel

et al., 2014). In ESCC cells treated with 5-FU, miR-145 has been found to obviously enhance apoptosis and expression of Bax, Bcl-2, and caspase3, via down-regulating REV3L (Chen Q. et al., 2019). MiR-29c was down-regulated in tumor tissues and serum samples of ESCC patients and has also been found to reverse 5-FU resistance by silencing F-box only protein 31 (FBXO31) (Li et al., 2019). Tumor suppressor miR-338-5p was down-regulated in 5-FU resistant ESCC cells as well as sera and tumor tissue of ESCC patients. Low miR-338-5p levels in serum was associated with poor response to neoadjuvant chemoradiotherapy based on 5-FU/DDP and worse survival of ESCC patients. Mechanistically, miR-338-5p could restore 5-FU sensitivity of ESCC cells by silencing the gene expression of inhibitor of differentiation 1 (Id-1) *in vitro* and *in vivo* (Han et al., 2019).

## MicroRNAs and Resistance to Other Drugs

In clinics, adriamycin, vincristine, paclitaxel, gemcitabine, and vinorelbine are also commonly used in esophageal cancer therapy. Multiple miRNAs have been shown to participate in their resistance (Table 3). MiR-27a could confer adriamycin resistance and inhibit the apoptosis induced by adriamycin. It has been found that miR-27a could increase the expression of P-gp and Bcl-2, as well as reduce Bax expression in esophageal cancer cells (Zhang et al., 2010). Besides promoting the resistance to DDP and 5-FU, miR-483 and miR-214 could also potentiate adriamycin resistance and reduce intracellular accumulation of adriamycin in esophageal cancer cells (Zhou and Hong, 2013). MiR-223 has also been found to confer adriamycin resistance through inhibiting PARP levels (Streppel et al., 2013). Oncogenic miR-296 could confer the resistance to adriamycin and vincristine through silencing gene expression controlling apoptosis and cell cycle (Hong et al., 2010). For paclitaxel resistance, it has been reported that the combined miR-133a and miR-133b down-regulation could predict the sensitivity to paclitaxel-based chemotherapy in ESCC patients (Chen et al., 2014). Interestingly, esophageal cancer patients with low expression of miR-214 appeared to show higher sensitivity to the combination regimen of gemcitabine plus vinorelbine, indicating that miR-214 may predict esophageal cancer chemosensitivity (Wang Y. S. et al., 2016). In addition, miR-193a-3p has been found to potentiate the chemoresistance to docetaxel, paclitaxel and vinorelbine in esophageal cancer cells via silencing PSEN1 (Meng et al., 2016).

## LONG NON-CODING RNAs AND DRUG RESISTANCE

Long non-coding RNAs are a group of ncRNAs longer than 200 nt without protein-coding capacity. Accumulating evidences showed that lncRNAs play important roles in regulating various cellular processes (Frye et al., 2016; Kopp and Mendell, 2018). For instance, lncRNAs could regulate target gene expression at either the transcriptional level or the post-transcriptional level through interaction with various DNA, RNA or proteins. Abnormally expressed lncRNAs have been identified in almost all cancer types, including esophageal cancer (Li J. et al., 2014;

**TABLE 2 |** MicroRNAs (miRNAs) and 5-FU resistance in esophageal cancer.

MiRNAs	Expression <sup>a</sup>	Genes and pathways	References
miR-141-3p	↑	PTEN	Jin et al., 2016
miR-221	↑	DKK2	Wang Y. et al., 2016
miR-21	↑	–	Komatsu et al., 2016a
miR-214	↑	–	Zhou and Hong, 2013
miR-483	↑	–	Zhou and Hong, 2013
miR-193a-3p	↑	PSEN1	Meng et al., 2016
miR-193b-3p	↑	KRAS	Hummel et al., 2014
miR-27b-3p	↑	KRAS	Hummel et al., 2014
miR-296	↑	P-gp, Bcl-2, Bax, cyclin D1, P27	Hong et al., 2010
miR-193b	↓	Stathmin 1	Nyhan et al., 2016
miR-634	↓	OPA1, TFAM, LAMP2, APIP, XIAP, BIRC5, NRF2	Fujiwara et al., 2015
miR-192-5p	↓	TYMS	Hummel et al., 2014
miR-378a-3p	↓	CBL-B	Hummel et al., 2014
miR-194-5p	↓	ABCC3	Hummel et al., 2014
miR-18a-3p	↓	KRAS	Hummel et al., 2014
miR-125a-5p	↓	ERBB2	Hummel et al., 2014
miR-145	↓	REV3L	Chen Q. et al., 2019
miR-29c	↓	FBXO31	Li et al., 2019
miR-338-5p	↓	Id-1	Han et al., 2019

<sup>a</sup>miRNAs either up-regulated (↑) or down-regulated (↓) in 5-FU resistant esophageal cancer cells. This table shows 19 miRNAs whose expression levels and potential targets in 5-FU resistance of esophageal cancer.

**TABLE 3 |** MicroRNAs (miRNAs) and resistance to other drugs in esophageal cancer.

MiRNAs	Expression <sup>a</sup>	Genes and pathways	Drugs	References
miR-27a	↑	MDR1, Bcl-2, Bax	ADM	Zhang et al., 2010
miR-483	↑	–	ADM	Zhou and Hong, 2013
miR-214	↑	–	ADM	Zhou and Hong, 2013
miR-223	↑	PARP	ADM	Streppel et al., 2013
miR-296	↑	P-gp, Bcl-2, Bax, cyclinD1, P27	ADM, vincristine	Hong et al., 2010
miR-133a/b	↑	–	PTX	Chen et al., 2014
miR-214	↑	–	GEM, vinorelbine	Wang Y. S. et al., 2016
miR-193a-3p	↑	PSEN1	Docetaxel, PTX, vinorelbine	Meng et al., 2016

<sup>a</sup>miRNAs up-regulated (↑) in other drugs resistant esophageal cancer cells. This table shows eight miRNAs whose expression levels and potential targets in other drugs resistance of esophageal cancer. ADM, adriamycin; PTX, paclitaxel; GEM, gemcitabine.

Yang et al., 2014; Tan et al., 2017; Xu et al., 2019) and have been implicated in diagnosis, metastasis, prognosis, radioresistance, and chemoresistance of esophageal cancer (Li W. et al., 2014; Wang et al., 2015a, 2017; Lin et al., 2018; You et al., 2019; Zhang H. et al., 2019; Liu J. et al., 2020). Importantly, several lncRNAs have been found to contribute to development of drug resistance in esophageal cancer.

## Long Non-coding RNAs and Resistance to Platinum Drugs

It has been found that multiple lncRNAs were involved in the resistance to platinum drugs in esophageal cancer (Table 4), including oncogenic lncRNAs NSUN2 methylated lncRNA (NMR), colon cancer-associated transcript-1 (CCAT1), taurine up-regulated gene 1 (TUG1), TP73-AS1, prostate cancer

associated ncRNA transcript 1 (PCAT-1), AFAP1-AS1, FOXD2-AS1, POU3F3, LINC00337, LINC00152, and tumor suppressive lncRNA tumor suppressor candidate 7 (TUSC7).

Oncogenic lncRNA NMR (namely ENST00000432429.1 in GENCODE v13 or ENST00000432429.5 in Ensembl release 83), highly methylated by methyltransferase NSUN2 which can catalyze cytosine methylation to 5-methylcytosine (m5C) in tRNA and some poly(A) RNAs, has been found to play crucial roles in regulating DDP resistance and metastasis of ESCC cells (Li et al., 2018). lncRNA NMR was evidently up-regulated in ESCC and associated with poor overall survival (OS) of ESCC patients (Li et al., 2018). Functionally, ectopic expression of lncRNA NMR could not only suppress DDP-induced apoptosis, but also promote invasion and migration of ESCC cells. Mechanistically, it has been shown that lncRNA NMR could competitively suppress potential mRNAs m5C levels, such as procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3),

**TABLE 4 |** Long non-coding RNAs (lncRNAs) and platinum drugs resistance in esophageal cancer.

LncRNAs	Expression <sup>a</sup>	Genes and pathways	Drugs	References
NMR	↑	BPTF	Cisplatin	Li et al., 2018
CCAT1	↑	miR-143/PLK1/BUBR1	Cisplatin	Hu M. et al., 2019
TUG1	↑	Nrf2	Cisplatin	Zhang Z. et al., 2019
TP73-AS1	↑	–	Cisplatin	Zang et al., 2016
PCAT-1	↑	–	Cisplatin	Zhen et al., 2018
AFAP1-AS1	↑	–	Cisplatin	Zhou et al., 2016
FOXD2-AS1	↑	miR-195/Akt/mTOR	Cisplatin	Liu H. et al., 2020
POU3F3	↑	IL-6	Cisplatin	Tong et al., 2020
LINC00337	↑	TPX2, E2F4	Cisplatin	Yang C. et al., 2020
LINC00152	↑	ZEB1, EZH2	Oxaliplatin	Zhang et al., 2020b
TUSC7	↓	miR-224/DESC1/EGFR/AKT	Cisplatin	Chang et al., 2018

<sup>a</sup>lncRNAs either up-regulated (↑) or down-regulated (↓) in platinum drugs resistant esophageal cancer cells. This table shows 11 lncRNAs whose expression levels and underlying pathways in platinum drugs' resistance of esophageal cancer.

collagen type IV Alpha 5 (COL4A5), laminin beta 1 (LAMB1), and heparan sulfate proteoglycan 2 (HSPG2). Moreover, lncRNA NMR directly bond to chromatin regulator of bromodomain PHD finger transcription factor (BPTF), and regulated the expression of matrix metalloproteinase 3 (MMP3) and matrix metalloproteinase 10 (MMP10) through the ERK1/2 pathway (Li et al., 2018).

Long non-coding RNA CCAT1, which is highly expressed in esophageal cancer, has also been found to confer DDP resistance in ESCC cells through the miR-143/PLK1/BUBR1 signaling axis (Hu M. et al., 2019). Specifically, silencing of CCAT1 could dramatically potentiate miR-143 expression in a negative regulatory manner and inhibit both mRNA and protein expression of Polo-like kinase 1 (PLK1) and BUBR. Moreover, ectopic expression of miR-143 has been shown to suppress the expression of PLK1, BUBR1, and CCAT1. Functionally, silencing of lncRNA CCAT1 and ectopic miR-143 could reverse DDP drug resistance and inhibit ESCC cell proliferation. Inhibition of lncRNA CCAT1 has also been found to enhance sensitivity of ESCC xenografts in nude mice to DDP, indicating that lncRNA CCAT1 may act as a potential regulator of DDP chemoresistance in esophageal cancer (Hu M. et al., 2019).

Long non-coding RNA TUG1 has also been found to be abundantly expressed in TE-1-derived DDP-resistant esophageal cancer cells TE-1/DDP (Zhang Z. et al., 2019). Mechanistically, lncRNA TUG1 could confer DDP resistance of ESCC cells through elevating P-gp expression and inhibiting apoptosis. Conversely, silencing of lncRNA TUG1 reversed DDP resistance of ESCC cells (Zhang Z. et al., 2019). RNA immunoprecipitation and RNA pull-down assays verified that TUG1 could directly bind the protein of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and increase Nrf2 protein level. Moreover, Nrf2 antibody could relieve DDP resistance mediated by TUG1 overexpression in ESCC cell, indicating an involvement of TUG1/Nrf2 signaling pathway in DDP resistance (Zhang Z. et al., 2019).

Long non-coding RNAs TP73-AS1 also could promote DDP resistance of esophageal cancer cells (Zang et al., 2016). lncRNA PCAT-1 has been shown to accelerate DDP resistance and tumor growth of esophageal cancer cells (Zhen et al., 2018). Additionally, AFAP1-AS1, a dramatically up-regulated lncRNA

in esophageal cancer tissues and DDP-resistant esophageal cancer cells, has been found to be positively associated with not only advanced clinical stages and definitive chemoradiotherapy (dCRT) response, but also shorter OS and progression free survival (PFS) (Zhou et al., 2016). Oncogenic lncRNAs FOXD2-AS1, POU3F3, and LINC00337 were revealed to be involved in DDP resistance of esophageal cancer (Liu H. et al., 2020; Tong et al., 2020; Yang C. et al., 2020). Via the miR-195/Akt/mTOR axis, ectopic expression of FOXD2-AS1, an up-regulated lncRNA in ESCC patients and DDP resistant ESCC cells (TE-1/DDP), could contribute to DDP resistance in ESCC (Liu H. et al., 2020). lncRNA POU3F3 could confer DDP resistance of ESCC cells through exosome POU3F3 inducing normal fibroblasts (NFs) to differentiate into CAFs via secreting interleukin 6 (IL-6). In addition, higher expression of plasma exosome POU3F3 has been shown to predict bad complete response and survival of ESCC patients (Tong et al., 2020). By increasing ESCC cell autophagy, exogenous expression of LINC00337 has been demonstrated to potentially promote DDP resistance through TPX2 up-regulation via recruiting E2F4 (Yang C. et al., 2020). Additionally, through interacting with EZH2, oncogenic LINC00152 has been found to increase ZEB1 expression and accelerate EMT and oxaliplatin resistance in esophageal cancer (Zhang et al., 2020b).

On the contrary, tumor suppressor lncRNA may reverse the resistance of cancer cells to platinum drugs. For instance, lncRNA TUSC7 could overcome the resistance to DDP and promote apoptosis of ESCC cells, via inhibiting miR-224 to modulate differentially expressed in squamous cell carcinoma 1 (DESC1)/EGFR/AKT signaling pathway. Overexpression of DESC1 could reverse the resistance to DDP through EGFR/AKT pathway in ESCC EC9706 and KYSE30 cells. Moreover, esophageal cancer patients with lower lncRNA TUSC7 expression had short OS (Chang et al., 2018).

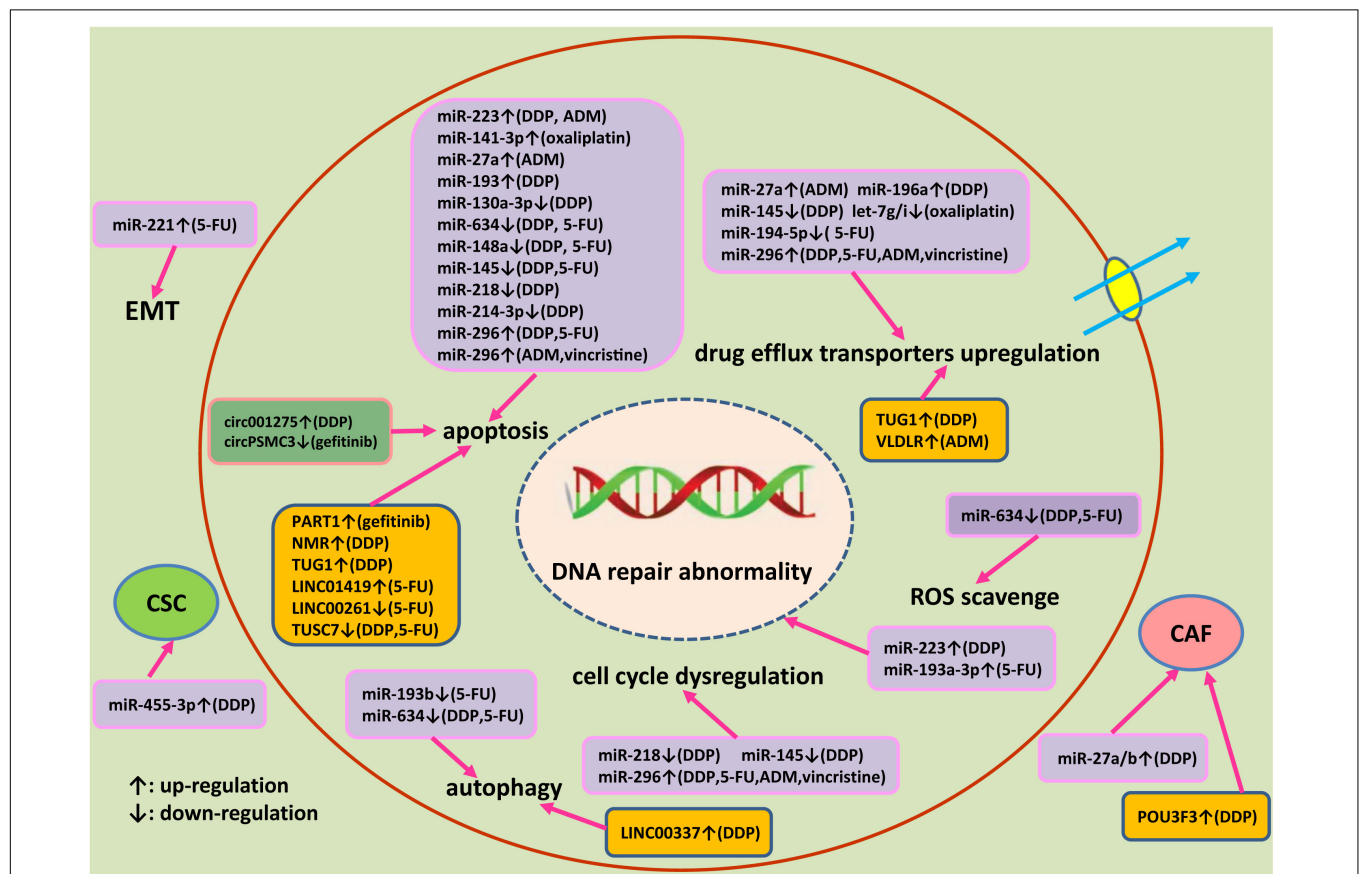
## Long Non-coding RNAs and 5-Fluorouracil Resistance

Several oncogenic or tumor suppressive lncRNAs are associated with the resistance to 5-FU in esophageal cancer (Table 5). Oncogenic LINC01419 has been found to promote 5-FU



LncRNAs	Expression <sup>a</sup>	Genes and Pathways	Drugs	References
LINC01419	↑	GSTP1	5-FU	Chen J. L. et al., 2019
HOTAIR	↑	MTHFR	5-FU	Zhang et al., 2020c
LINC01270	↑	DNMT3A, DNMT3B, DNMT1	5-FU	Li et al., 2021
TP73-AS1	↑	–	5-FU	Zang et al., 2016
TUSC7	↓	miR-224/DESC1/EGFR/AKT	5-FU	Chang et al., 2018
LINC00261	↓	DPYD	5-FU	Lin K. et al., 2019

LncRNAs	Expression <sup>a</sup>	Genes and pathways	Drugs	References
DDX11-AS1	↑	TAF1/TOP2A	Paclitaxel	Zhang S. et al., 2019
VLDLR	↑	ABCG2	Adriamycin	Chen Y. et al., 2019
PART1	↑	miR-129/Bcl-2	Gefitinib	Kang et al., 2018
Linc01014	↑	PI3K-AKT-mTOR	Gefitinib	Fu et al., 2020



resistance and inhibit apoptosis of ESCC cells (Chen J. L. et al., 2019). LINC01419 could bind to the promoter region of *glutathione S-transferase pi 1* (*GSTP1*) gene, increase DNA methylation levels of the region through recruiting DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3A (DNMT3A) and DNA methyltransferase 3B (DNMT3B) into *GSTP1* promoter region and diminish *GSTP1* expression in ESCC cells (Chen J. L. et al., 2019). On the contrary, the demethylation of *GSTP1* via DNA methyltransferase inhibitor 5-Aza-CdR could weaken 5-FU resistance in LINC01419 overexpressed ESCC cells, demonstrating that LINC01419 functions as a modulator of 5-FU-based chemotherapy sensitivity in ESCC (Chen J. L. et al., 2019). Recently, oncogenic lncRNA HOTAIR has also been found to accelerate 5-FU resistance in esophageal cancer cells by promoting the promoter hypermethylation of methylene tetrahydrofolate reductase (*MTHFR*) gene. Silencing of HOTAIR could promote the apoptosis induced by 5-FU and alleviate cell proliferation and *MTHFR* promoter methylation of esophageal cancer cells. Moreover, overexpression of *MTHFR* has been shown to reverse 5-FU resistance caused by HOTAIR overexpression. Meanwhile, xenografts from HOTAIR-silenced esophageal cancer cells in nude mice also demonstrated the diminished 5-FU resistance, indicating that HOTAIR may represent a novel potential target for conquering 5-FU resistance of esophageal cancer (Zhang et al., 2020c). In addition, oncogenic lncRNA LINC01270 has also been shown to promote the resistance to 5-FU through regulating *GSTP1* promoter methylation via recruiting three important DNA methyltransferases, including DNMT3A, DNMT3B, and DNMT1 (Li et al., 2021).

By contrast, tumor suppressor lncRNAs play opposite role in development of 5-FU resistance of esophageal cancer. For instance, through suppressing miR-224 and regulating the ESC1/EGFR/AKT signaling, lncRNA TUSC7 has been demonstrated to conquer 5-FU resistance and increase the apoptosis of ESCC cells. Moreover, exogenous expression of *DESC1* could enhance the sensitivity to 5-FU in ESCC cells (Chang et al., 2018). In addition, tumor suppressor lncRNA LINC00261 could also reverse the chemoresistance to 5-FU in human esophageal cancer cells through regulating DNA methylation-dependent expression inhibition of dihydropyrimidine dehydrogenase (*DYPD*). Exogenous expression of LINC00261 could significantly suppress cell growth and potentiate apoptosis sensitivity to 5-FU in ESCC cells. On the contrary, inhibition of LINC00261 has been shown to promote proliferation and apoptosis resistance of ESCC cells. Moreover, 5-aza-2'-deoxycytidine, a demethylation reagent, could reverse DNA methylation of *DYPD* promoter and *DYPD* activity in 5-FU resistant ESCC cells (Lin K. et al., 2019).

## Long Non-coding RNAs and Resistance to Other Drugs

Paclitaxel, adriamycin, and gefitinib are also used in esophageal cancer treatments. It has been found that multiple oncogenic lncRNAs participate in the resistance to these anti-cancer agents (Table 6). lncRNA DDX11-AS1, a highly expressed

lncRNA in esophageal cancer tissues, has been found to increase paclitaxel resistance of esophageal cancer cells. Through binding to transcription factor TATA-box binding protein-associated factor 1 (TAF1) and up-regulating TAF1 expression, lncRNA DDX11-AS1 could promote the transcription of topoisomerase alpha 2 (*TOP2A*) and subsequently, increase *TOP2A* expression levels (Zhang S. et al., 2019). Silencing of lncRNA DDX11-AS1 could potentiate the inhibitory effects of paclitaxel on esophageal cancer xenografts in nude mice and suppress *TOP2A* expression, suggesting that lncRNA DDX11-AS1 may be a promising potential target for overcoming paclitaxel resistance of esophageal cancer (Zhang S. et al., 2019). lncRNA *VLDLR* was up-regulated in ESCC tissue and could promote adriamycin resistance of esophageal cancer cells via increasing *ABCG2* expression (Chen Y. et al., 2019). lncRNA prostate androgen-regulated transcript 1 (*PART1*) has been found to confer the resistance to gefitinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), through regulating miR-129/Bcl-2 pathway in ESCC cells. Interestingly, extracellular lncRNA *PART1* could be secreted with exosomes, transferred to the sensitive ESCC cells, and promoted gefitinib resistance of ESCC cells. In addition, high expression of serum lncRNA *PART1* in exosome was also associated with unfavorable response to gefitinib in ESCC patients (Kang et al., 2018). Through modulating PI3K-AKT-mTOR signaling, lncRNA *LINC01014* overexpression could also dramatically suppress the apoptosis of esophagus cancer cells and promote gefitinib resistance (Fu et al., 2020).

## CIRCULAR RNAs AND DRUG RESISTANCE IN ESOPHAGEAL CANCER

Circular RNAs, a special type of endogenous circular ncRNAs, are generated through the process called back-splicing of linear precursor mRNA (pre-mRNA) transcripts and lack 3' poly (A) tail and 5' cap (Chen, 2016). CircRNAs could regulate gene expression through sponge adsorption of miRNA, modification of parental genes, and regulation of transcription and splicing of target genes. Mounting evidence has demonstrated that circRNAs are involved in multiple cellular processes and several malignancies, including esophageal cancer (Hu X. et al., 2019; Kristensen et al., 2019). Interestingly, circRNAs could not only serve as diagnostic and prognosis markers of esophageal cancer, but also participate in drug resistance (Zhang et al., 2020d; Zou et al., 2020). For instance, circRNA\_001275, an up-regulated circRNA in DDP-resistant esophageal cancer cells and tissues, has been shown to accelerate cell growth and reduce the apoptosis of DDP-resistant cells. On the contrary, knockdown of circRNA\_001275 inhibited the proliferation of DDP-resistant cells. It has been found that circRNA\_001275 could contribute to DDP resistance in esophageal cancer through directly binding to and competitively sponging miR-370-3p to up-regulate Wnt family member 7A (*Wnt7a*) expression (Zou et al., 2020). In addition, via regulating miR-194-5p/JMJD1C axis, oncogenic circ\_0006168 has been shown to potentiate Taxol resistance in ESCC (Qu et al., 2021). Recently, tumor suppressive circPSMC3,

down-regulated in ESCC tissues and gefitinib-resistant (GR) ESCC cells, its overexpression could conquer gefitinib resistance, increase apoptosis rate and cleaved caspase-3 level in GR ESCC cells through modulating the miR-10a-5p/PTEN axis, which provide a promising therapeutic strategy for overcoming gefitinib resistance in ESCC (Zhu H. et al., 2021).

## CONCLUSION

Accumulating evidences have shown that ncRNAs significantly contribute to drug resistance of esophageal cancer. The corresponding mechanisms of miRNAs, lncRNAs, and circRNAs involved in drug resistance of esophageal cancer are illustrated in **Figure 2**. Multiple mechanisms including abnormal histone and DNA modifications, genomic amplification/loss and post-transcriptional regulations are involved in the dysregulation of 3 kinds of ncRNAs in esophageal cancer. Personalized therapy according to abnormally expressed miRNAs, lncRNAs, and circRNAs may be a promising way to overcome drug resistance. Silencing of oncogenic ncRNAs using small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) have been reported to be effective in restoring the therapeutic sensitivity of esophageal cancer (Hu X. et al., 2019). Alternatively, ectopic expression of tumor suppressor ncRNAs have been demonstrated to be beneficial to conquer therapeutic resistance in esophageal cancer. Locked nucleic acid (LNA) modifications of ncRNAs can enhance *in vivo* stability and affinity. However, drug safety, immune-related toxicities or other adverse effects remain important issues to be solved for ncRNAs-based therapeutics. After searching the <http://clinicaltrials.gov> database, we found that there are still

no therapeutic clinical trials based on ncRNAs in esophageal cancer currently. Due to the complexities of cancer signaling pathways, the inhibition of a single target signaling or ncRNA may show minor effects. The combination based on modulation of ncRNAs expression and classical chemotherapy, novel targeted therapy or immunotherapy may be a promising choice to treat advanced or metastatic esophageal cancer patients. However, selecting key target ncRNA from numerous candidate ncRNAs for the intervention remains a difficult issue.

## AUTHOR CONTRIBUTIONS

LW and MY conceived the review, acquired data, provided project funding, and drafted the manuscript. MY and ZL reviewed and supervised the manuscript. JS, NZ, YS, and TW undertook the initial research. All authors read and approved the submitted version.

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# A Novel miR-98 Negatively Regulates the Resistance of Endometrial Cancer Cells to Paclitaxel by Suppressing ABCC10/MRP-7

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Endometrial cancer (EC) is one of the most frequent gynecological tumors, and chemoresistance is a major obstacle to improving the prognosis of EC patients. MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have recently emerged as crucial chemoresistance regulators that alter the levels of downstream target genes. Multidrug Resistance Protein 7 (MRP-7/ABCC10) is an ATP-binding cassette transporter that causes the resistance to anti-cancer drugs. The purpose of this research is to determine whether MRP-7 has a role in mediating the sensitivity of EC cells to paclitaxel and whether the expression of MRP-7 is regulated by miR-98 and lncRNA NEAT1. We reported that the levels of MRP-7 were significantly increased in EC tissues and associated with an unfavorable prognosis. Downregulation of MRP-7 in EC cells sensitized these cells to paclitaxel and reduced cell invasion. PLAUR serves as a downstream molecule of MRP-7 and facilitates paclitaxel resistance and EC cell invasiveness. Moreover, miR-98 serves as a tumor suppressor to inhibit MRP-7 expression, leading to the repression of paclitaxel resistance. Furthermore, a novel lncRNA, NEAT1, was identified as a suppressor of miR-98, and NEAT1 could upregulate MRP-7 levels by reducing the expression of miR-98. Taken together, these findings demonstrate that upregulation of MRP-7 and NEAT1, and downregulation of miR-98 have important roles in conferring paclitaxel resistance to EC cells. The modulation of these molecules may help overcome the chemoresistance against paclitaxel in EC cells.

**Keywords:** MRP-7, miR-98, NEAT1, paclitaxel resistance, endometrial cancer



## INTRODUCTION

Endometrial cancer (EC) is the most prevalent type of gynecological cancer, with 382,000 new cases and approximately 90,000 deaths worldwide (1). Globally, the incidence of EC is rising (2). The majority of EC are detected at an early stage and treated with surgery or a combination of treatments (including surgery, chemotherapy, radiation therapy, and potentially targeted therapy) (3). Advanced and recurrent ECs, on the other hand, are difficult to cure. Resistance to treatment by EC cells is closely connected with poor survival of advanced and recurring ECs (4). Elucidating the signaling pathways involved in EC chemoresistance is crucial for finding valuable therapeutic targets for EC.

Multidrug resistance (MDR) is still a prominent factor that results in the failure of chemotherapy in EC patients (5). In general, cancer cells may develop resistance to medicinal medications by overexpressing ATP-binding cassette (ABC) proteins (5). ABC transporters are thought to reduce intracellular concentrations of anti-tumor agents, thereby resulting in MDR (6). Multidrug resistance protein 7 (MRP-7, ATP-binding cassette subfamily C member 10, ABCC10) is one of the ABC transporters that allows cancer cells to become resistant to cytotoxic medicines (like paclitaxel) (7, 8). In patients with gastric cancer and lung cancer, increased MRP-7 expression levels have been linked to a worse prognosis in several studies (9, 10). Overexpression of MRP-7 inhibits apoptosis and promotes cell proliferation in human leukemia cells (11). Interestingly, MRP-7 has been shown to promote ovarian cancer cell motility and cause epithelial-mesenchymal transition (EMT) (12). It is unclear whether MRP-7 contributes to paclitaxel resistance in EC cells and mediates their invasive abilities.

Dysregulation of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) has recently been discovered to have a key role in carcinogenesis, tumor development, and chemoresistance in a variety of malignancies, including EC (13, 14). MiR-98, for example, has been discovered to suppress the malignant phenotypes in glioma and lung cancer (15, 16).

MiR-98 reduces cancer cell resistance to cisplatin therapy in lung cancer cells (17). MiR-98 expression was found to be lowly expressed in EC tissues compared to normal tissues (18, 19). Several studies have found that lncRNA NEAT1 promotes tumor growth in a variety of malignancies (20). NEAT1 has been shown to promote EC cell invasion and confer paclitaxel resistance (21). It is unknown whether miR-98 or NEAT1 influences MRP-7 expression and paclitaxel resistance in EC.

In this study, we investigated the role of MRP-7 in mediating EC cell paclitaxel sensitivity, as well as whether dysregulation of miR-98 and NEAT1 could regulate MRP-7 expression. Our findings showed that MRP-7 promotes paclitaxel resistance in EC cells, and its expression is regulated by the NEAT1/miR-98 pathway. Mechanistic studies confirmed that MRP-7 is a direct target of tumor suppressor miR-98, and NEAT1 sponges miR-98 to increase MRP-7 levels in EC cells. Thus, focusing on this signaling pathway could help overcome paclitaxel resistance and

have implications for the treatment of chemoresistant EC patients in the future.

## MATERIALS AND METHODS

### EC Tissue Samples

Fresh EC samples ( $n = 30$ ) and paired normal tissue samples ( $n = 30$ ) were obtained with informed consent from 30 EC patients undergoing surgery at the Department of Gynecology and Oncology, Hubei Cancer Hospital of Tongji Medical College of Huazhong University of Science and Technology. Before being diagnosed, none of these patients had received either chemotherapy or radiotherapy. All specimens were promptly snap-frozen using liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The use of all human tissue samples was approved by the Research Ethics Committee of Hubei Cancer Hospital of Tongji Medical College of Huazhong University of Science and Technology.

### Cell Culture and Reagents

Human EC cell lines RL95 (CRL-1671) and HEC-1 (HTB-112), human normal endometrial fibroblast cell line HESC (CRL-4003), and HEK293 (CRL-1573) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Paclitaxel-resistant HEC-1 cell lines, namely HEC-1-TX, were established by culturing HEC-1 cells with increasing concentrations of paclitaxel. These cells were maintained in DMEM/F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Gaithersburg, MD, USA) at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

Stable knockdown of MRP-7 in HEC-1 cells was achieved by transfection with MRP-7 shRNA plasmid (MRP-7 shRNA, sc-62641-SH, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or control plasmid (control shRNA, sc-108060, Santa Cruz Biotechnology). Stable colonies were selected with 400  $\mu\text{g}/\text{ml}$  of G418 (Sigma-Aldrich).

To establish the stably transfected RL95 cells overexpressing MRP-7, RL95 cells were transfected with pCMV6-MRP-7 (RC221247, OriGene, Rockville, MD, USA) or control vector (PS100001, OriGene) using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). 400  $\mu\text{g}/\text{ml}$  of G418 (Sigma-Aldrich) was used to select stable transfectants.

The mimic and inhibitor of miR-98, their negative controls (control mimic and control inhibitor), the siRNA against human NEAT1, and the control siRNA were synthesized by Invitrogen. The control and overexpression vectors for PLAUR were purchased from (OriGene). These miRNA mimics, miRNA inhibitors, siRNAs, and vectors were transiently transfected into EC cells using Lipofectamine 3000 reagent (Invitrogen).

### RNA Extraction and Quantitative Reverse Transcription PCR

TRIzol reagent (Invitrogen) was used to isolate total RNA from tissues and cultured cells, and the cDNA Reverse transcription Kit (TOYOBO, Japan) was used to reverse transcribe the RNA into cDNA. The expression of mRNA and lncRNA was

quantified using a 7500 Fast Real-time PCR System (Applied Biosystems, USA).

The primers were synthesized as follows: *MRP-7*-Forward: 5'-GTCCAGATTACATCCTACCCTGC-3' and *MRP-7*-Reverse: 5'-GCCAACACCTCTAGCCCTATG-3'; *PLAUR*-Forward: 5'-TGTAAGACCAACGGGGATTGC-3' and *PLAUR*-Reverse: 5'-AGCCAGTCCGATAGCTCAGG-3'; *GAPDH*-Forward: 5'-AATCCCATCACCATCTTC-3'; and *GAPDH*-Reverse: 5'-AGGCTGTTGTCATACTTC-3'.

The primers used to amplify NEAT1 were as follows: NEAT1-Forward: 5'-TTCACCTGCTCTGGCTCTTG-3' and NEAT1-Reverse: 5'-GCCAGGCACCGTGTACTACT-3', respectively (22, 23).

The levels of miR-98 were examined with the NCode SYBR GreenER miRNA qRT-PCR analysis kit (Invitrogen). The forward primer for miR-98 was 5'-TGAGGTAGTAAGTTGTATTGTT-3', and the reverse primer was supplied by Invitrogen. The U6 primer sequences were as follows: U6-Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and U6-Reverse: 5'-CGCTTCACGAATTTGCGTGCAT-3'. Fold changes in the relative gene, lncRNA, and miRNA expression were calculated and normalized to GAPDH or U6 expression.

### In Vitro Drug Sensitivity Assay and Cell Proliferation Assay

Cell viability was assessed using a Cell Counting Kit-8 assay (CCK-8, Dojindo, Japan) after EC cells were treated with different doses of paclitaxel for 24 hours. The dose-response curves were used to establish the half-maximal inhibitory concentration (IC<sub>50</sub>) of paclitaxel. Cell proliferation was evaluated using a CCK-8 assay. Briefly, 2000 cells were seeded to 96-well plates and incubated for 3 days. The OD values at 450 nm were recorded using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell Invasion Assay

Transwell chambers (Corning Costar, Cambridge, MA, USA) were used for the cell invasion assay. A total of 50000 cells were seeded into the top chambers after being suspended in 500 µl of serum-free media. The lower chambers were filled with 750 µl of medium containing 10% FBS. The invaded cells were fixed, stained, and counted using a microscope after 24 hours of incubation.

### Tumor Xenograft Assay

All animal protocols were approved by the Ethics Committee of Hubei Cancer Hospital of Tongji Medical College of Huazhong University of Science and Technology. In brief, female BALB/c nude mice (4–6 weeks old) were purchased from Shanghai SLAC Laboratory Animal (Shanghai, China). HEC-1 cells with *MRP-7* knockdown (or control HEC-1 cells), and RL95 cells overexpressing *MRP-7* (or control RL95 cells) were subcutaneously injected into the flanks of mice ( $n = 5$  per group). Tumor volume was determined using the following method:  $V$  (volume) = (length  $\times$  width<sup>2</sup>)/2. Mice were sacrificed on day 24, and tumors were dissected and weighted.

### Western Blotting

RIPA lysis buffer (Cell Signaling Technology, MA) was used to lyse EC cells for Western blotting. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were separated by electrophoresis on a 12% SDS-polyacrylamide gel, transferred to a PVDF membrane (GE Healthcare Life Sciences, Piscataway, NJ), blocked for 1 h in 5% non-fat milk, and probed overnight at 4°C with anti-*MRP-7* (1:1000, ab69296, Abcam, Cambridge, MA, USA) and anti-*GAPDH* antibody (1:5000, #2118, Cell Signaling, Danvers, MA, USA). The immunoreactive bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare, UK) after 1 h of incubation with secondary antibodies at room temperature.

### Luciferase Reporter Assay

Three hundred and one base pairs of the *MRP-7* 3'-UTR sequence was amplified by PCR using the following primers (Forward: 5'-TGCAGAGTTCTCCCCTCTCT-3'; Reverse: 5'-TTTTAATACACAGAATGTAAGATGGA-3') and cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA), namely WT *MRP-7* 3'-UTR vector. Using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), the mutant *MRP-7* 3'-UTR with mutations in the miR-98 binding site was created, specifically MUT *MRP-7* 3'-UTR vector. HEC-1 or RL95 cells were co-transfected with 100 ng of WT *MRP-7* 3'-UTR (or MUT *MRP-7* 3'-UTR) reporter vector, 10 ng of pRL-CMV vector (Promega), miR-98 mimic (30 nM), control mimic (30 nM), miR-98 inhibitor (30 nM), or control inhibitor (30 nM) using Lipofectamine 3000 reagent (Invitrogen). The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) after 48 h. The activity of firefly luciferase was normalized to the activity of renilla luciferase.

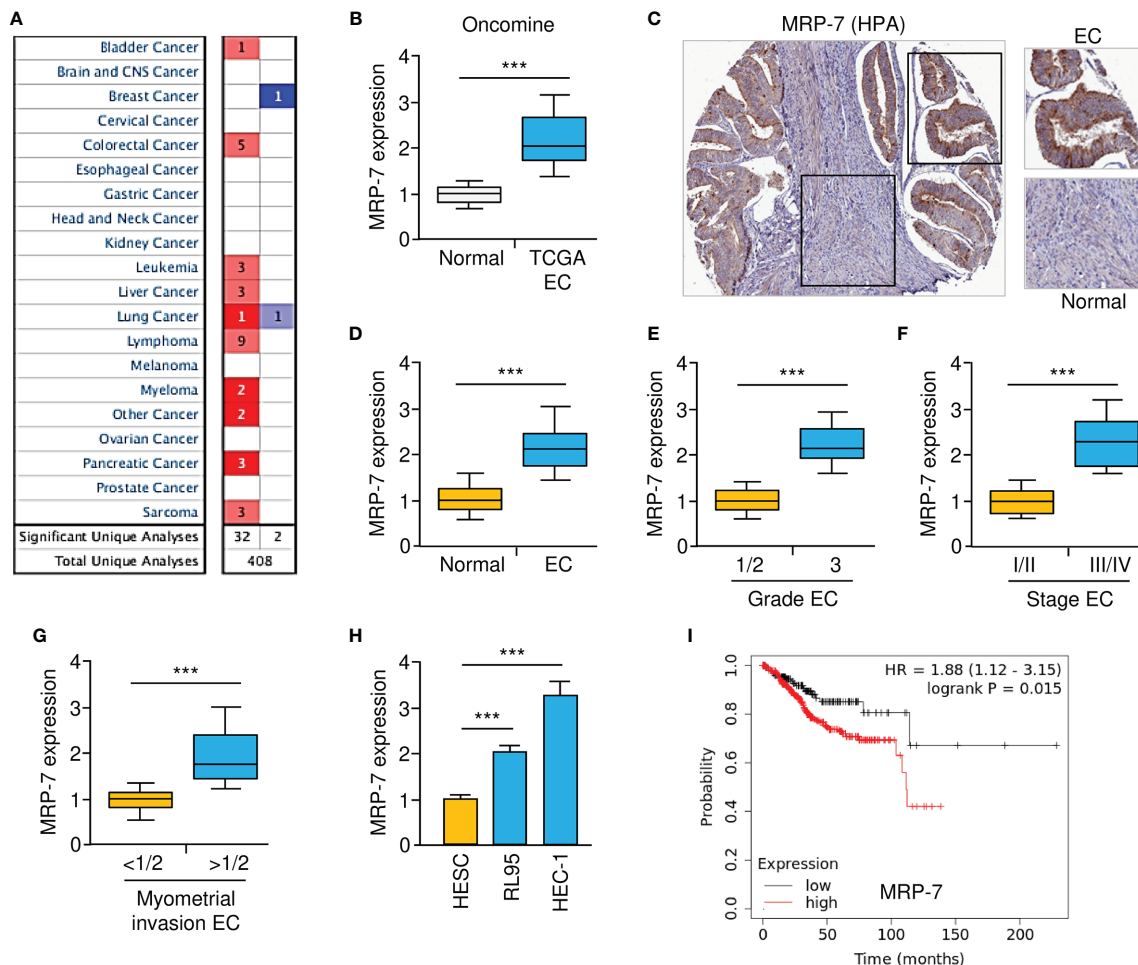
### Statistics

The results were expressed as mean  $\pm$  standard error (SD) from at least three independent experiments. Statistical analysis was conducted using Student's *t*-test, one-way ANOVA test, or Mann-Whitney *U* test with SPSS 19.0 software (SPSS, Chicago). A *P*-value of less than 0.05 was used to determine statistical significance.

## RESULTS

### Gain of *MRP-7* Expression Correlates With EC Progression

We initially used the Oncomine database (www.oncomine.org) to evaluate the mRNA expression of *MRP-7* in human tumors and normal tissues. A total of 408 publicly available datasets were retrieved from Oncomine, of which 34 published studies showed significant changes in *MRP-7* expression between tumor and the respective normal tissues (Figure 1A). Compared to normal tissues, most tumor tissues have elevated *MRP-7* levels when compared to normal tissues (Figure 1A). The mRNA expression



**FIGURE 1 |** Gain of MRP-7 Expression Correlates with EC Progression. **(A)** The Oncomine database was used to compare *MRP-7* mRNA expression between different tumor and normal tissues. Red denotes an increase in *MRP-7* expression, while blue denotes a reduction in *MRP-7* expression. **(B)** *MRP-7* mRNA expression in TCGA EC tissues compared to normal tissues (Oncomine database). **(C)** Immunohistochemistry images of MRP-7 staining using tissue microarray tissue sections (HPA database). **(D)** The mRNA levels of *MRP-7* in EC and normal tissues were examined using qRT-PCR experiments. **(E–G)** The expression of *MRP-7* mRNA was considerably higher in EC patients with a high tumor grade **(E)**, late-stage disease **(F)**, or deeper myometrial invasion **(G)**. **(H)** *MRP-7* mRNA expression is substantially higher in EC cells than in normal HESC cells. **(I)** KM plotter database was used to analyze the association between *MRP-7* expression and the overall survival of EC patients. \*\*\* $P < 0.001$ .

of *MRP-7* was highly expressed in EC tissues compared to normal tissues (**Figure 1B**). In addition, analysis of *MRP-7* protein expression in EC tissues using the Human Protein Atlas database (HPA, <https://www.proteinatlas.org/>) revealed that the protein levels of *MRP-7* were relatively higher in EC in comparison with the surrounding normal tissues (**Figure 1C**). Furthermore, real-time quantitative qPCR analysis showed that *MRP-7* expression was considerably higher in EC samples than in paired non-malignant endometrial tissues at the mRNA level (**Figure 1D**). Notably, *MRP-7* overexpression was frequently observed in patients with advanced EC and late-stage disease (**Figures 1E, F**). Increased expression of *MRP-7* was found to be correlated with deeper myometrial invasion (**Figure 1G**). In line with these results, we discovered that *MRP-7* mRNA expression was significantly higher in EC cells than in HESC cells

(**Figure 1H**). According to the results from the KM plotter database, *MRP-7* levels were negatively associated with the overall survival of patients with EC (**Figure 1I**). Taken together, the above results suggest that *MRP-7* is overexpressed in EC and might have oncogenic functions in this disease.

## MRP-7 Modulates Paclitaxel Resistance and Invasion of EC Cells

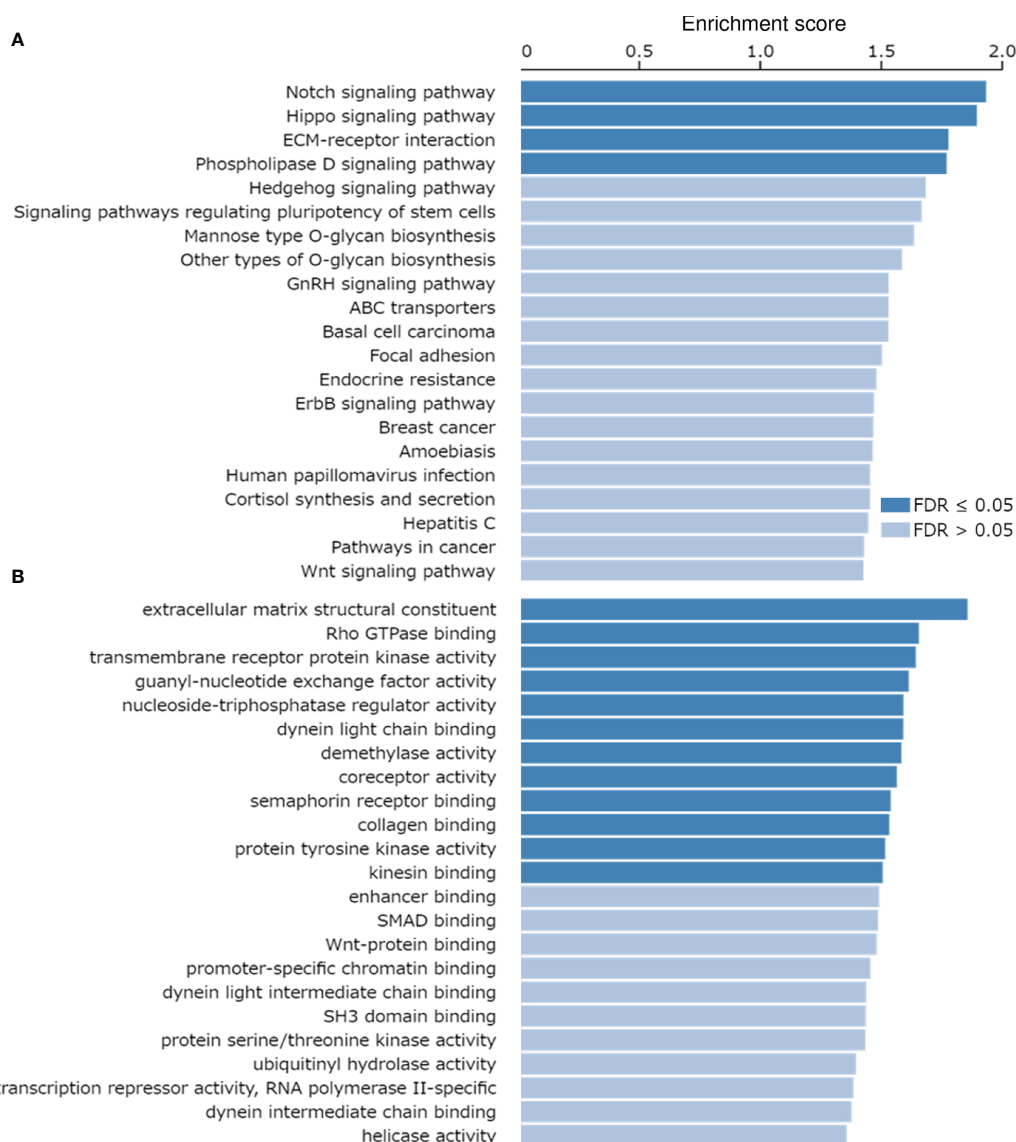
The correlation of *MRP-7* expression with other genes in EC samples was analyzed using the LinkedOmics database (<http://linkedomics.org/login.php>). 19898 genes showed correlation with *MRP-7* in EC tissues (including 11783 genes that were positively correlated and 8115 genes that were negatively correlated), indicating a wide-range impact of *MRP-7* on the transcriptome. Those genes showing positive correlation with

MRP-7 expression were enriched in ABC transporters, and multiple cancer-associated KEGG pathways, including Notch signaling pathway, Hippo signaling pathway, Pathways in cancer and Wnt signaling pathway (Figure 2A). MRP-7 co-expressed genes in EC samples were mostly involved in the regulation of Rho GTPase binding, SMAD binding, and Wnt-protein binding, according to gene ontology (GO) enrichment analysis (Figure 2B). Thus, these results support that MRP-7 may play a unique role in modulating EC cell proliferation and invasion, as well as stem cell-like activities, beyond its function in chemoresistance.

Firstly, we used *in vitro* drug sensitivity experiments to calculate the IC<sub>50</sub> values for paclitaxel in the parental EC cell

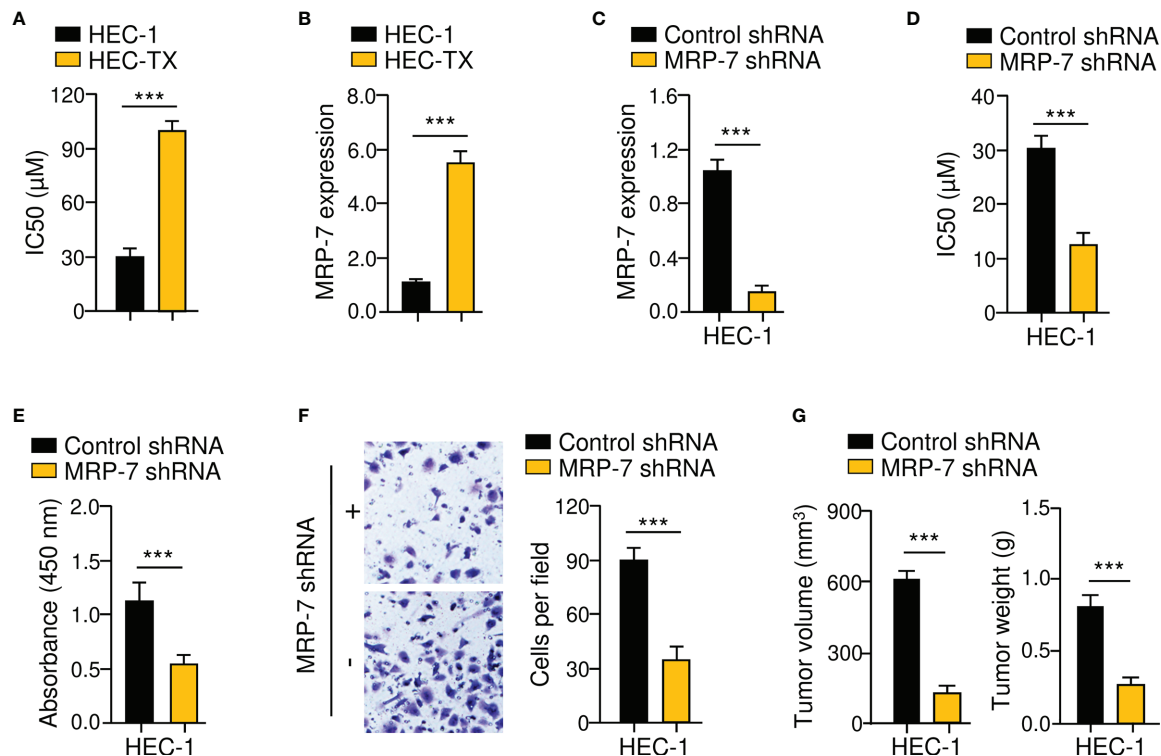
line HEC-1 and the paclitaxel-resistant EC cell line HEC-1-TX. As expected, HEC-1-TX cells had much higher IC<sub>50</sub> values for paclitaxel than the parental HEC-1 cells (Figure 3A). Then, the expression of *MRP-7* mRNA in these cell lines was compared using qRT-PCR analysis. Relative to the parental HEC-1 cells, those paclitaxel-resistant HEC-1-TX cells had increased *MRP-7* expression (Figure 3B), suggesting that MRP-7 expression might be involved in the development of chemoresistance in EC cells.

Given that *MRP-7* levels were higher in HEC-1 cells (Figure 1H), we investigated the possible effects of MRP-7 knockdown on chemoresistance. After shRNA-mediated suppression of MRP-7 in HEC-1 cells, cell viability was investigated (Figure 3C). Compared with the corresponding



**FIGURE 2 |** KEGG Pathways Analysis and GO Functional Annotation of the Co-expressed Genes of MRP-7 in EC Tissues. **(A, B)** KEGG pathway enrichment analysis **(A)** and GO functional annotation (cellular function) **(B)** of the co-expressed genes of MRP-7 in EC samples were investigated using the LinkedOmics database.





**FIGURE 3 |** MRP-7 Modulates Paclitaxel Resistance and EC Cell Invasion. **(A)** The cytotoxic effects of paclitaxel on paclitaxel-resistant HEC-1-TX cells and their parental cells. **(B)** The mRNA expression of *MRP-7* in HEC-1-TX cells compared to their parental cells. **(C)** The qRT-PCR analysis of *MRP-7* expression in HEC-1 *MRP-7* shRNA cells and control cells. **(D)** *MRP-7* knockdown increased the paclitaxel sensitivity of HEC-1 cells. **(E, F)** The proliferation **(E)** and invasion **(F)** of HEC-1 cells was attenuated after *MRP-7* silencing. **(G)** HEC-1 cells transfected with *MRP-7* shRNA (or control shRNA) were subcutaneously injected into nude mice. Tumor volume (left) and tumor weight (right) were displayed. \*\*\* $P < 0.001$ .

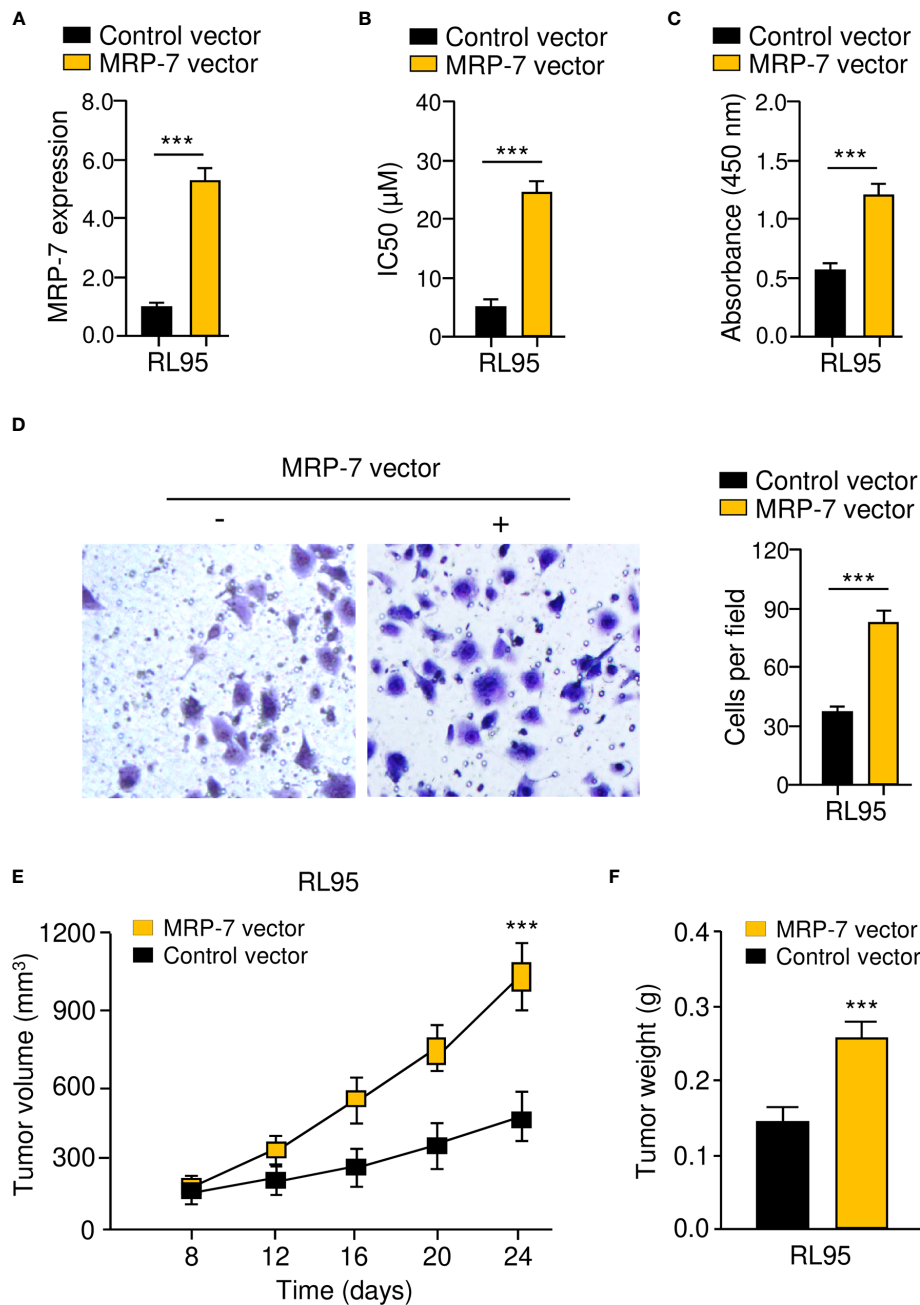
control cells, *MRP-7* shRNA-transfected HEC-1 cells had greater sensitivity to paclitaxel treatment (**Figure 3D**). Furthermore, cell proliferation and invasion assays suggested that the growth and invasion abilities of HEC-1 cells were significantly impaired following *MRP-7* knockdown (**Figures 3E, F**). We compared the abilities of HEC-1 *MRP-7* shRNA cells or control cells to produce tumors in nude mice. The tumorigenic potential of HEC-1 cells was considerably reduced when *MRP-7* expression was knocked down (**Figure 3G**). These data indicated that increased *MRP-7* expression promotes the paclitaxel-resistant and invasive phenotypes of EC cells.

To verify the above data, we generated *MRP-7*-overexpressing cell lines with RL95 cells (**Figure 4A**). *In vitro* drug sensitivity assays, cell proliferation assays, and invasion assays collectively showed that *MRP-7* overexpression dramatically enhanced the resistance of RL95 cells to paclitaxel, and increased the proliferation and invasion capacities of RL95 cells (**Figures 4B–D**). The effects of the overexpression of *MRP-7* on tumor growth *in vivo* were further examined. Interestingly, the tumors formed by *MRP-7*-overexpressing RL95 cells were significantly larger and heavier than control tumors (**Figure 4E, F**). Together, these results support the idea that *MRP-7* enhances paclitaxel resistance and aggressive phenotypes of EC cells.

## MRP-7 Is a Direct Target Gene of MiR-98

To reveal the upstream mechanisms that drive the upregulation of *MRP-7* in EC cells, we conducted the bioinformatic analysis using the TargetScan database. In the 3'-UTR of the *MRP-7* mRNA, a putative miR-98 binding region was found (**Figure 5A**). As shown in **Figure 5B**, we found that the expression of miR-98 was lower in EC than in normal tissues. In addition, there was a correlation between decreased expression of miR-98 and higher pathological grade, advanced clinical stages, or deeper myometrial invasion (**Figures 5C–E**). Relative to normal HESC cells, miR-98 levels were significantly downregulated in EC cells (**Figure 5F**). Survival analysis using the KM plotter database suggested that lower miR-98 expression was correlated with poorer overall survival in EC patients (**Figure 5G**). These findings indicated that reduced expression of miR-98 is possibly associated with increased *MRP-7* expression as well as a worse outcome in EC.

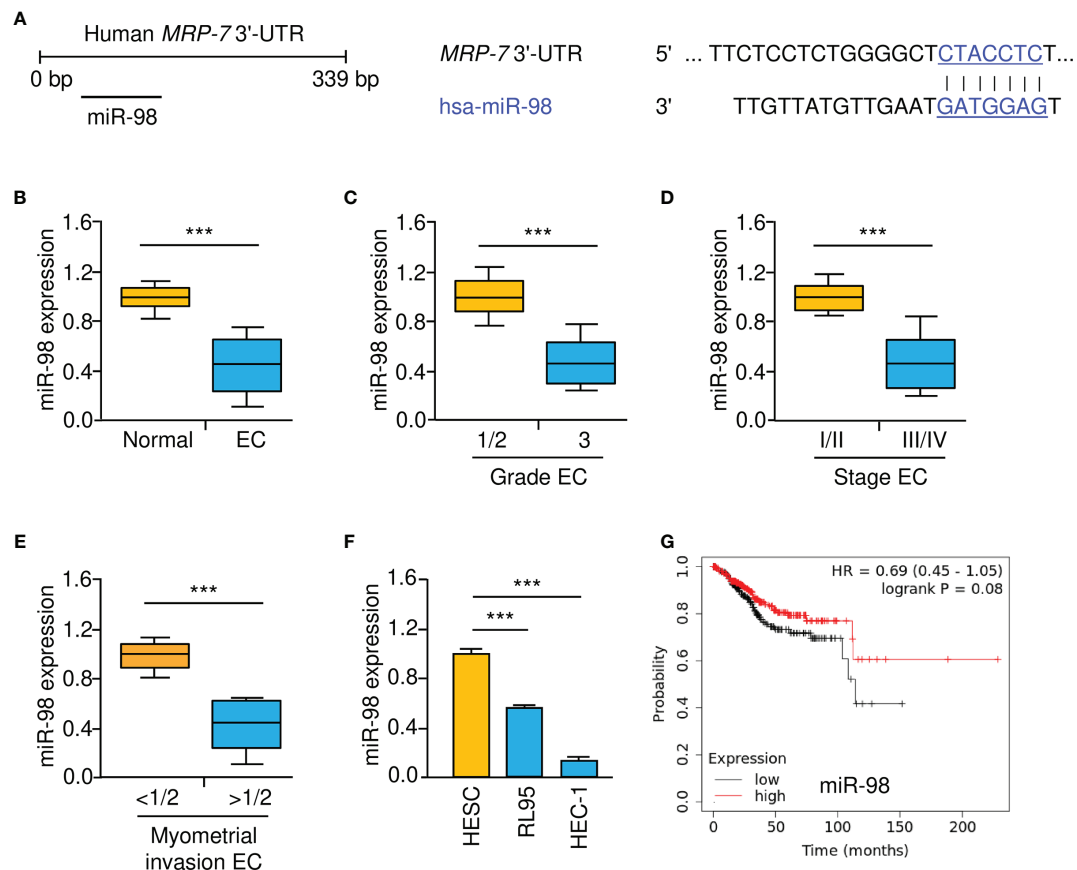
Given that miR-98 might interact with the 3'-UTR of *MRP-7* mRNA, we speculated that miR-98 may reduce *MRP-7* protein expression in EC cells. As expected, the protein levels of *MRP-7* in HEC-1 cells were negatively modulated by miR-98 overexpression (**Figures 6A, B**). Consistently, miR-98 silencing significantly increased *MRP-7* expression on the protein level in



**FIGURE 4 |** MRP-7 Enhances Paclitaxel Resistance and Aggressive Phenotypes of EC Cells. **(A)** *MRP-7* expression in *MRP-7*-expressing RL95 cells and control cells. **(B–D)** The IC50 values for paclitaxel **(B)**, cell proliferation **(C)**, and invasion **(D)** were examined in *MRP-7*-expressing RL95 cells and control cells. **(E, F)** Nude mice were injected subcutaneously with *MRP-7*-expressing RL95 cells or control cells. The tumor volume (left) and tumor weight (right) were displayed. \*\*\* $P < 0.001$ .

RL95 cells (**Figures 6A, B**). We further validate the integration between miR-98 and *MRP-7* mRNA by performing the luciferase reporter assays. Our data showed that miR-98 mimic remarkably downregulated the luciferase activity of WT *MRP-7* 3'-UTR (**Figure 6C**). The miR-98 inhibitor, on the other hand, greatly increased the luciferase activity of the WT *MRP-7* 3'-UTR (**Figure 6C**). However, neither overexpression nor silencing of

miR-98 had a significant effect in EC cells transfected with MUT *MRP-7* 3'-UTR (**Figure 6C**). The qRT-PCR analysis in EC tissues has demonstrated that there was a negative and significant correlation between the expression of miR-98 and *MRP-7* (**Figure 6D**). As a result, these findings show that miR-98 binds directly to *MRP-7* mRNA and suppresses its expression in EC cells.



**FIGURE 5 |** Lower MiR-98 Levels are Correlated with Worse Prognosis in EC. **(A)** Schematic representation of the predicted binding between *MRP-7* 3'-UTR sequence and miR-98 (TargetScan database). **(B)** The expression of miR-98 in EC and normal tissues were examined using qRT-PCR assays. **(C–E)** MiR-98 levels were significantly decreased in EC patients with advanced tumors **(C)**, late-stage disease **(D)**, or deeper myometrial invasion **(E)**. **(F)** EC cells have a much lower expression of miR-98 than normal HESC cells. **(G)** The relationship between miR-98 expression and the overall survival of EC patients (KM plotter database). \*\*\**P* < 0.001.

## Knockdown of MiR-98 Induces Paclitaxel Resistance and Aggressive Properties of EC Cells

To confirm whether downregulation of miR-98 was involved in chemoresistance and EC progression, we assessed the effects of either miR-98-overexpression or miR-98-knockdown on paclitaxel resistance, cell proliferation, and cell invasion using cellular functional assays. As shown in **Figures 7A–C**, the resistance of RL95 cells to paclitaxel, as well as cell proliferation and invasion, was significantly promoted in the miR-98-silencing group compared with the control group. Consistent with these results, miR-98 overexpression could significantly attenuate paclitaxel resistance, proliferation, and invasion of HEC-1 cells (**Figures 7D–F**). These results suggest that knocking down miR-98 is enough to increase paclitaxel resistance and aggressiveness in EC cells.

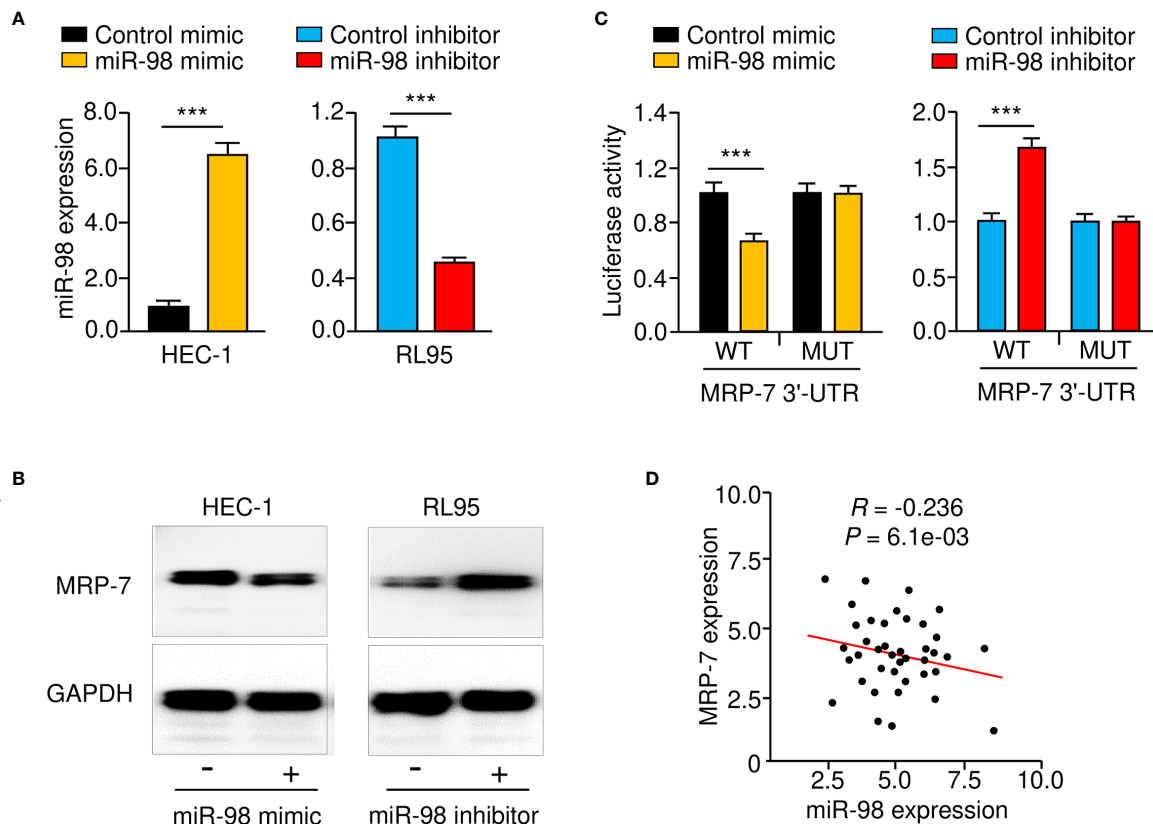
## LncRNA NEAT1 Functions as a Suppressor of MiR-98 in EC Cells

Accumulating reports indicate that lncRNA NEAT1 works as a sponge for miR-98, downregulating its levels in lung cancer and colon cancer cells (15, 24–26). Therefore, we postulated that NEAT1

would sponge miR-98 in EC cells. Using the ENCORI database (<https://starbase.sysu.edu.cn/index.php>), we confirmed an association between NEAT1 and miR-98 (**Figure 8A**). NEAT1 levels were significantly elevated in EC cell lines compared to normal HESC cells, according to qRT-PCR tests (**Figure 8B**). Furthermore, knockdown of NEAT1 with specific siRNA led to a significant increase in miR-98 expression in EC cells (**Figures 8C, D**). The results from western blotting assays further suggested that NEAT1 knockdown resulted in a reduction in MRP-7 protein expression (**Figure 8E**). The qRT-PCR analysis revealed a substantial positive connection between NEAT1 and MRP-7 expression (**Figure 8F**). Therefore, these findings demonstrate that lncRNA NEAT1 might inhibit miR-98 expression in EC cells.

## Identification of PLAUR as a Downstream Effector of the NEAT1/miR-98/MRP-7 Pathway

MRP-7 has been demonstrated to enhance the expression of PLAUR (uPAR), which facilitates the migration and invasion of EC cells in previous investigations (27, 28). Importantly, PLAUR has also been demonstrated to assist cancer cells in decreasing the



**FIGURE 6 |** MRP-7 is a Downstream Target of MiR-98. **(A)** The expression of miR-98 in EC cells transfected with miR-98 mimic, miR-98 inhibitor, or their controls. **(B)** MRP-7 expression in EC cells transfected as indicated as determined by Western blotting. **(C)** The luciferase activity of reporter vectors carrying WT *MRP-7* 3'-UTR or MUT *MRP-7* 3'-UTR in EC cells transfected with miR-98 mimic, miR-98 inhibitor, or the corresponding negative controls. **(D)** The correlation for the expression of miR-98 and *MRP-7* mRNA in EC specimens. \*\*\* $P < 0.001$ .

cytotoxic effects of anti-cancer drugs (29). Based on these findings, we tried to determine whether lncRNA NEAT increases PLAUR expression in EC cells by sponging miR-98 and upregulating MRP-7 expression. The results from qRT-PCR assays suggested that the knocking down NEAT1 or MRP-7, as well as overexpression of miR-98, dramatically reduced the levels of PLAUR, whereas upregulation of MRP-7 or inhibition of miR-98 significantly increased PLAUR expression in EC cells (Figure 9A–C). Analysis of TCGA EC data using the Wanderer database (<http://maplab.imppc.org/wanderer/>) and UALCAN database (<http://ualcan.path.uab.edu/index.html>) showed that EC samples had much greater mRNA (Figure 9D) and protein (Figure 9E) levels of PLAUR in comparison with normal tissues. Collectively, the above results suggest that lncRNA NEAT1 regulates the miR-98/MRP-7 pathway to upregulate PLAUR expression in EC cells.

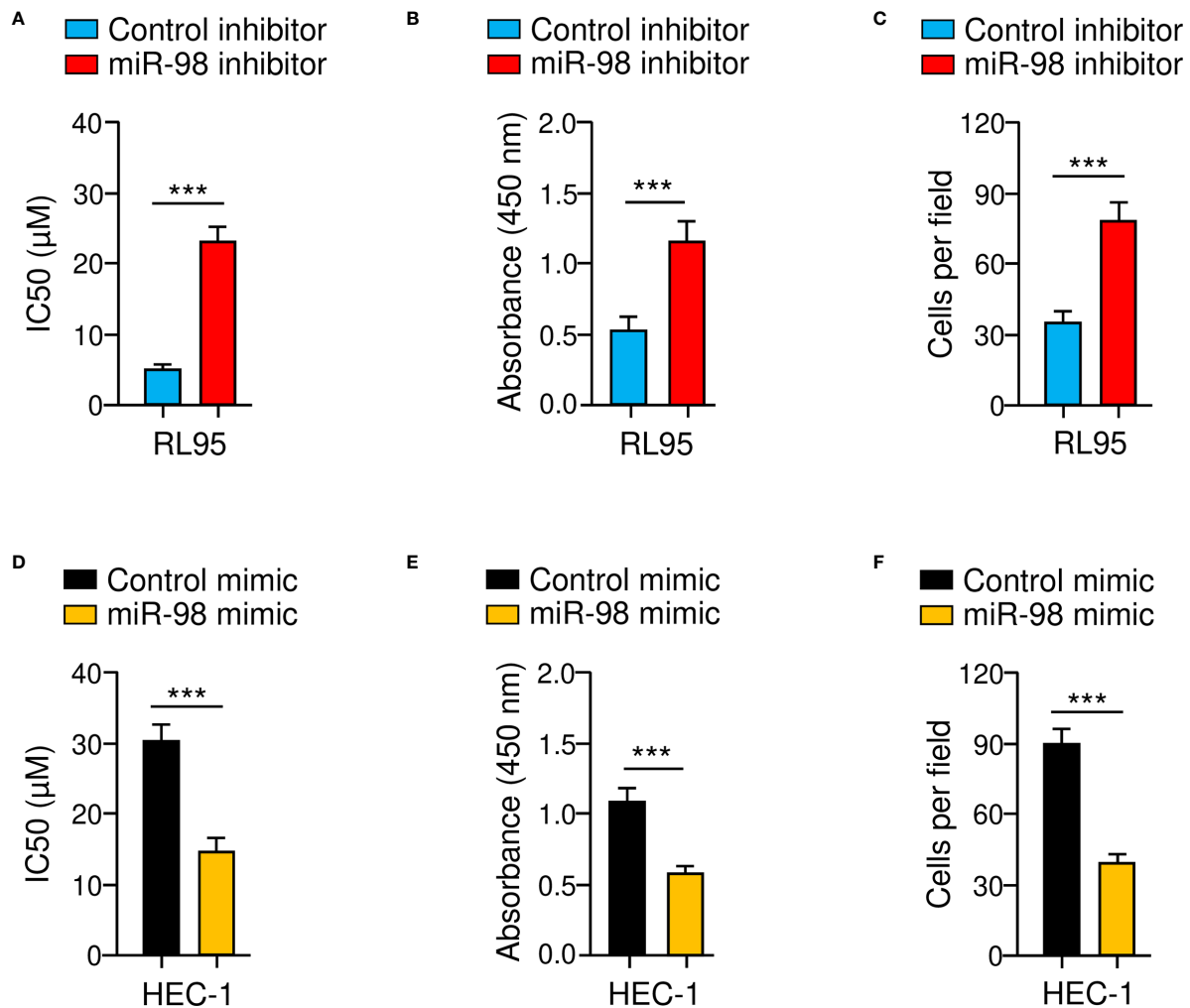
### PLAUR Silencing Reduces Paclitaxel Resistance and Invasion of EC Cells

To investigate whether PLAUR is functionally important for the chemoresistant and invasive properties of EC cells, either PLAUR overexpression or PLAUR knockdown experiments were conducted. Since HEC-1 cells exhibited relatively high

PLAUR levels (Figure 10A), the siRNA against PLAUR or control siRNA was transfected into this cell line (Figure 10B). We observed that PLAUR-knockdown increased the sensitivity of HEC-1 cells to paclitaxel and impaired cell invasion (Figures 10C, D). Also, overexpression of PLAUR in RL95 cells significantly increased paclitaxel resistance and invasion (Figures 10E–H). The above data indicated that increased PLAUR expression contributes to paclitaxel resistance and EC cell invasion.

The genetic information of NEAT1, MRP-7, and PLAUR in patients with EC was explored with the cBioPortal database (<http://www.cbioportal.org>). Analysis of TCGA EC data suggested that NEAT1, MRP-7, and PLAUR exhibited gene amplification and mRNA upregulation in most EC tissues (Figure 11A). Then, the expression profile of miR-98 in diverse tumor and normal samples was analyzed using the BioXpress database (<https://hive.biochemistry.gwu.edu/bioexpress/>), which has RNASeqV2 miRNA expression data from the TCGA datasets. Comparison of miR-98 expression in tumor and matching normal tissues using the BioXpress database revealed under-expression of miR-98 in numerous tumor types and showed that miR-98 expression is





**FIGURE 7 |** Knockdown of MiR-98 Induces Paclitaxel Resistance and Aggressive Properties of EC cells. (A–C) Drug sensitivity assays (A), cell proliferation assays (B), and cell invasion assays (C) were carried out in RL95 cells transfected with miR-98 inhibitor or control inhibitor. (D–F) Drug sensitivity assays (D), cell proliferation assays (E), and cell invasion assays (F) were performed in HEC-1 cells transfected with miR-98 mimic or control mimic. \*\*\* $P < 0.001$ .

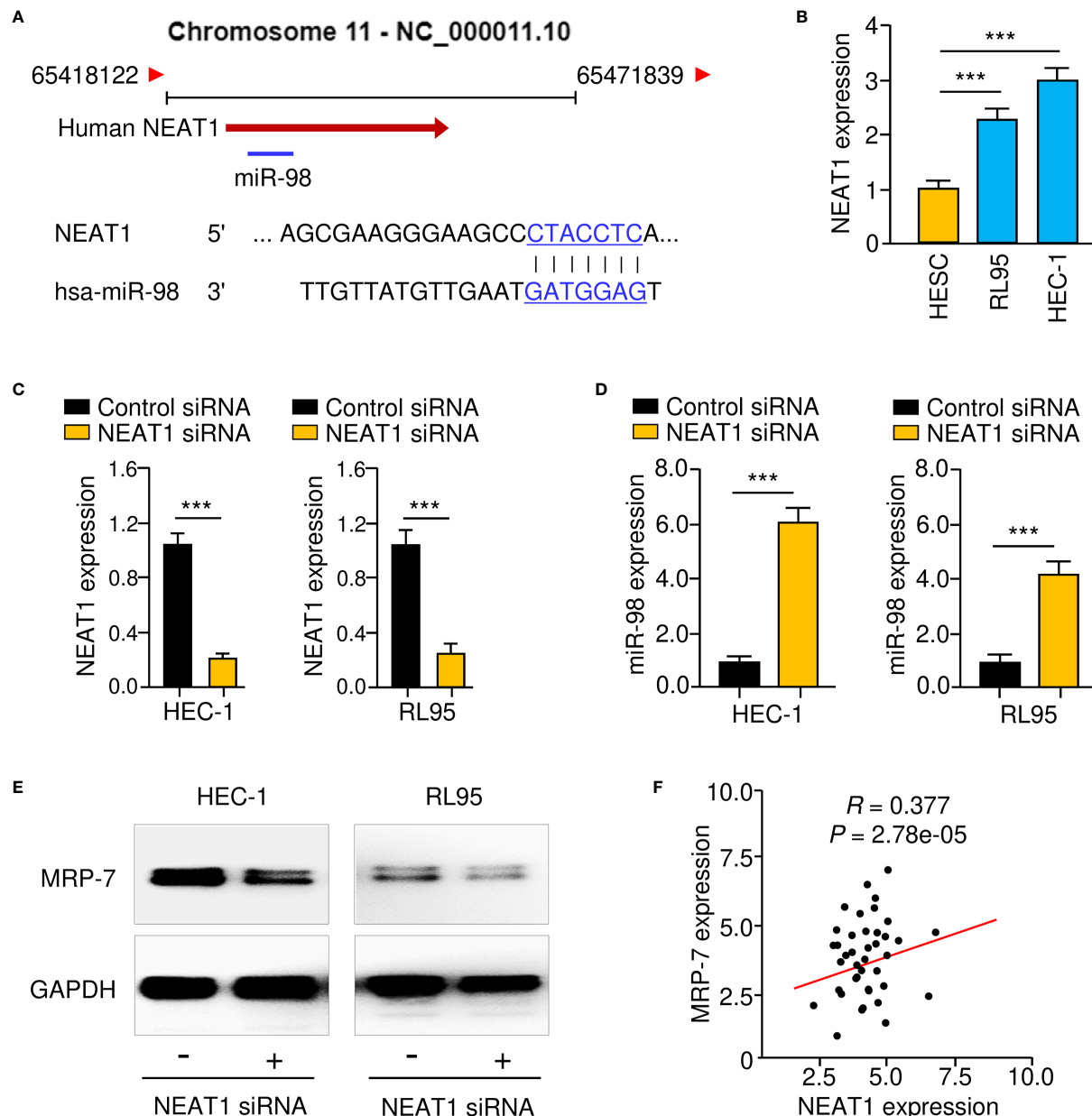
downregulated in 83% of EC tissues relative to paired normal tissues (**Figure 11B**). Taken together, the above results show that NEAT1 suppresses miR-98 expression to upregulate the protein expression of MRP-7 and subsequent overexpression of PLAUR, thus facilitating paclitaxel resistance in EC cells (**Figure 11C**).

## DISCUSSION

Cancer cells can acquire resistance to multiple anti-cancer drugs, eventually leading to treatment failure. ABC transporters-induced drug efflux, accelerated DNA repair, autophagy, EMT, and cancer stem cell-like characteristics are some of the molecular processes that might lead to MDR (30). ABC transporters are important mediators of MDR in human cancer (5). MRP-7 (a member of the ABCC subfamily) was discovered in 2001 (31). Previous studies

have shown that MRP-7 contributes to the development of drug resistance to various anti-tumor drugs, including paclitaxel, vincristine, and vinorelbine, and gemcitabine (7, 32, 33). However, it is unclear whether MRP-7 expression plays a role in EC cell paclitaxel resistance. Here, our results showed that overexpression of MRP-7 could significantly promote paclitaxel resistance. In addition, inhibiting efflux activity directly is a strategy for preventing efflux-mediated resistance (34). A third-generation inhibitor of P-glycoprotein Tariquidar effectively reverses MRP7-mediated MDR by sensitizing MRP7-expressing cells to a number of chemotherapeutic agents (including paclitaxel, docetaxel, vincristine, vinblastine, and vinorelbine) (35). Therefore, modifying MRP-7 expression or activity could be a possible treatment option for EC patients who are resistant to paclitaxel.

Despite the link between MRP-7 expression and chemoresistance, earlier investigations have also suggested that

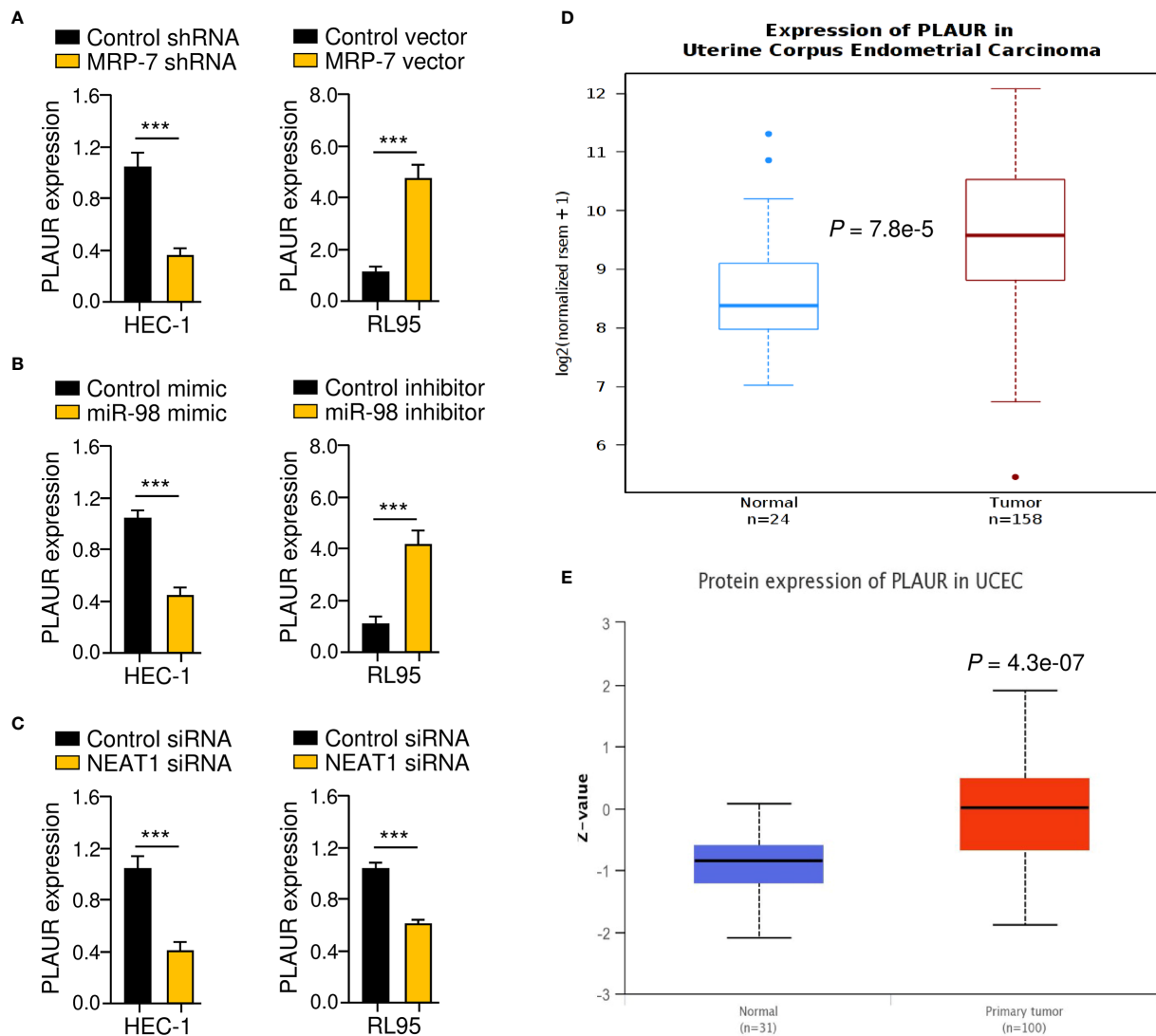


**FIGURE 8 |** LncRNA NEAT1 Functions as a Suppressor of MiR-98 in EC Cells. **(A)** In the lncRNA NEAT1 sequence, the anticipated miR-98 binding site was found. **(B)** The levels of NEAT1 in EC cell lines and HESC cells. **(C, D)** The expression of NEAT1 **(C)** and miR-98 **(D)** in EC cells transfected with NEAT1 siRNA or control siRNA. **(E)** Western blotting was used to assess MRP-7 protein expression in EC cells transfected with NEAT1 siRNA or control siRNA was examined using western blotting analysis. **(F)** The relationship between NEAT1 and MRP-7 expression in EC specimens. \*\*\* $P < 0.001$ .

MRP-7 may play other biological roles during carcinogenesis and cancer progression (9–12, 36). MRP-7 expression has been found to be high in lung cancer tissues, while it was rarely detected in normal lung tissues (36). Studies have shown that high MRP-7 expression is associated with a worse prognosis in patients with gastric cancer and lung cancer (9, 10). Other research has recently revealed that MRP-7 stimulates cell proliferation and attenuates apoptosis in human leukemia cells (11). In ovarian cancer cells, MRP-7 has also been

shown to trigger EMT-related signaling and promote cell migration (12). Our *in vitro* and *in vivo* experiments have confirmed that increased MRP-7 expression is a negative prognostic factor in EC patients, and MRP-7 can boost cell proliferation and invasion in EC cells. Hence, MRP-7 could be a useful diagnostic and prognostic biomarker, as well as a therapeutic target for EC.

The tumor suppressor capability of miR-98 has been demonstrated in numerous human malignancies (15, 16),

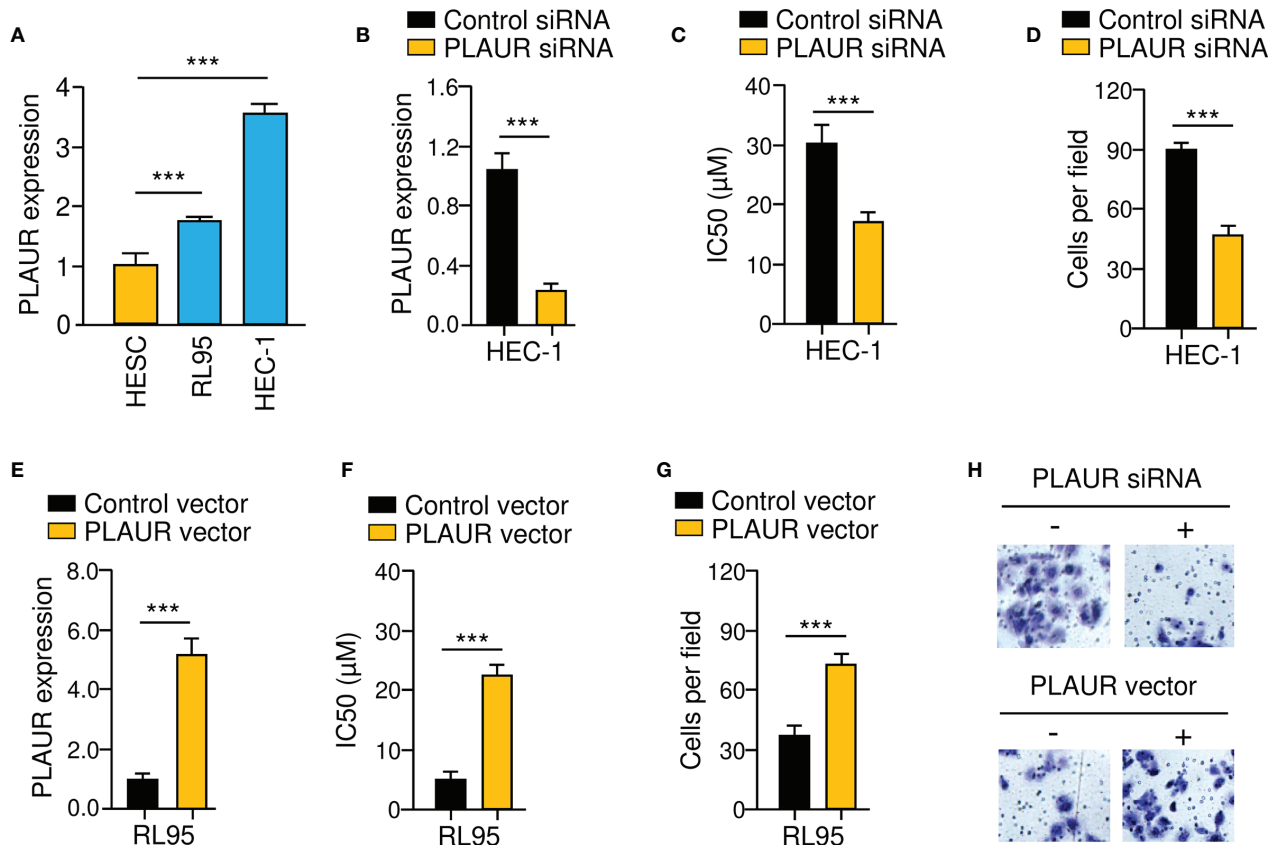


**FIGURE 9 |** Identification of PLAUR as a Downstream Effector of the NEAT1/miR-98/MRP-7 Pathway. **(A–C)** The qRT-PCR analysis of *PLAUR* expression in EC cells transfected with MRP-7 shRNA or MRP-7 expression vector **(A)**, in EC cells transfected with miR-98 mimic or miR-98 inhibitor **(B)**, and in EC cells transfected with NEAT1 siRNA or control siRNA **(C)**. **(D, E)** The Wanderer database **(D)** and the UALCAN database **(E)** were used to examine PLAUR levels in TGA EC and normal tissues. \*\*\* $P < 0.001$ .

where it negatively regulates cancer cell growth, migration, and invasion. The downregulation of miR-98 has been linked to cisplatin resistance in lung cancer (17). Although reduced expression of miR-98 in EC tissues has been described (18, 19), the function of miR-98 in EC cells has not been explored. In the current research, we predicted possible miRNAs that might influence the expression of MRP-7, and reported for the first time that restoring miR-98 re-sensitized EC cells to paclitaxel, and inhibited EC cell proliferation and invasion by directly binding to the 3'-UTR region of *MRP-7* mRNA. Additionally, the expression of MRP-7 in tumor cells was mediated by some other miRNAs (including let-7a, let-7e, let-7g, let-7i, and let-7f) (11, 37–39). Taken together, it is possible that chemoresistance

and aggressiveness associated with MRP-7 may be induced by the downregulation of miR-98 and other miRNAs. Therefore, a more comprehensive understanding of the mechanisms that control MRP-7 expression would be crucial for developing innovative therapies that increase the survival of EC patients who have high MRP-7 levels.

In tumor cells, interactions between miRNAs and lncRNAs are critical mechanisms that lead to miRNA dysregulation (13). Increasing reports have indicated that lncRNA NEAT1 shows tumor-promoting functions in a variety of malignancies, including EC (20, 21). The link between NEAT1 and miR-98 in lung cancer and prostate cancer has been clarified in previous investigations (24–26). NEAT1 has been found to directly bind



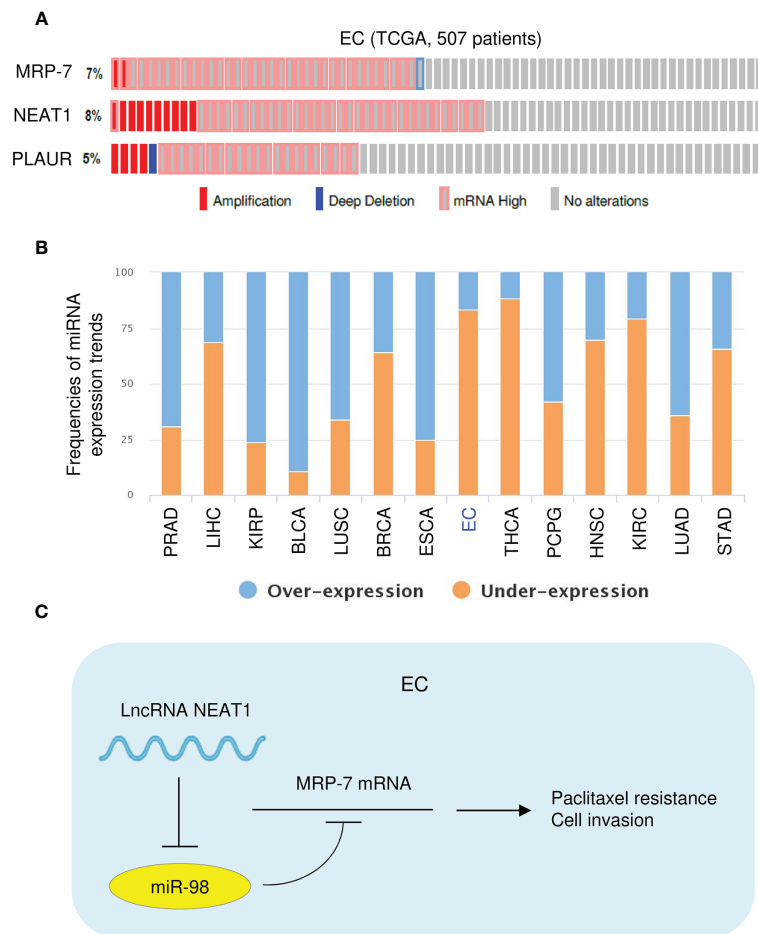
**FIGURE 10 |** PLAUR Silencing Reduces Paclitaxel Resistance and EC Cell Invasion. **(A)** *PLAUR* mRNA levels in EC cells and HESC cells. **(B)** Expression of *PLAUR* in HEC-1 cells transfected with *PLAUR* siRNA or control siRNA. **(C)** Paclitaxel cytotoxicity in HEC-1 cells transfected with *PLAUR* siRNA or control siRNA. **(D)** The invasion of HEC-1 cells transfected with *PLAUR* siRNA or control siRNA was measured using cell invasion assays. **(E)** Expression of *PLAUR* in RL95 cells transfected with *PLAUR* expression vector or control vector. **(F)** Paclitaxel cytotoxicity in RL95 cells transfected with *PLAUR* expression vector or control vector. **(G)** The invasion of RL95 cells transfected with *PLAUR* expression vector or control vector was measured using cell invasion assays. **(H)** Representative images of cell invasion assays. \*\*\* $P < 0.001$ .

to miR-98 and lower its levels (24–26). However, it is yet to be determined whether NEAT1 regulates miR-98 expression in EC cells. In the present study, we demonstrated that NEAT1 is a critical suppressor of miR-98, suggesting that the tumor suppressor roles of miR-98 might be rescued by NEAT1 inhibition. Our results implied that targeting the NEAT1/miR-98 signaling might be an alternative approach to inhibit paclitaxel resistance and EC progression.

PLAUR has been discovered as a drug resistance and carcinogenic factor (29). High levels of uPAR expression are linked to aggressive phenotypes and a worse prognosis (29). PLAUR has been found to have a key role in tumor cell motility, invasion, metastasis, EMT, cancer stemness, survival, and treatment resistance in recent research (29). Notably, in benign endometrium, UPAR protein expression was undetectable, whereas it was highly elevated in EC tissues (40). UPAR protein expression was correlated with advanced stage,

high tumor grade, recurrence, and mortality rates in patients with EC (40, 41). However, little is known about its role in EC. In agreement with these previous reports (40, 41), our investigation confirmed that PLAUR is upregulated in EC, and further showed for the first time that higher PLAUR levels contribute to paclitaxel resistance and EC cell invasiveness. Moreover, overexpression of PLAUR was shown to increase the activity of the WNT signaling to promote cancer stemness in medulloblastoma cells (42). Consistent with this previous research, our KEGG pathway and GO term annotation analysis showed that MRP-7 co-expressed genes were enriched in the WNT pathway in EC tissues. Therefore, we propose that MRP-7-mediated upregulation of PLAUR may facilitate paclitaxel resistance and cancer stem cell-like features of EC by activating the WNT signaling pathway. Future experiments are needed to verify this possibility. Taken together, PLAUR could be used as a novel biomarker in human EC to predict aggressive disease and chemoresistance.





**FIGURE 11** | High NEAT, *MRP-7*, and *PLAUR* expression, and Low MiR-98 Expression in TCGA EC Tissues. **(A)** Genomic alteration frequencies of NEAT1, *MRP-7*, and *PLAUR* were derived from cBioPortal using TCGA EC data. **(B)** The BioXpress database was used to determine the expression of miR-98 in tumor and normal tissues. **(C)** The schematic diagram of this study: lncRNA NEAT1 suppresses miR-98 expression to facilitate paclitaxel resistance in EC cells via upregulating *MRP-7* and *PLAUR* expression.

## CONCLUSION

In conclusion, we discovered that *MRP-7* shows a significant role in promoting paclitaxel resistance and EC cell invasion, and the expression of *MRP-7* in EC cells is regulated by the upstream NEAT1/miR-98 pathway. Our findings have exciting clinical implications for the development of future medicines that reduce *MRP-7* expression to overcome EC chemoresistance and metastasis.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Hubei Cancer Hospital of Tongji Medical College of Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of Hubei Cancer Hospital of Tongji Medical College of Huazhong University of Science and Technology.

## AUTHOR CONTRIBUTIONS

LS and XL designed the study. WH, JZ, and BD carried out the experiments. HC analyzed the results. All authors read and approved the final manuscript.

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# The Molecular Roles and Clinical Implications of Non-Coding RNAs in Gastric Cancer

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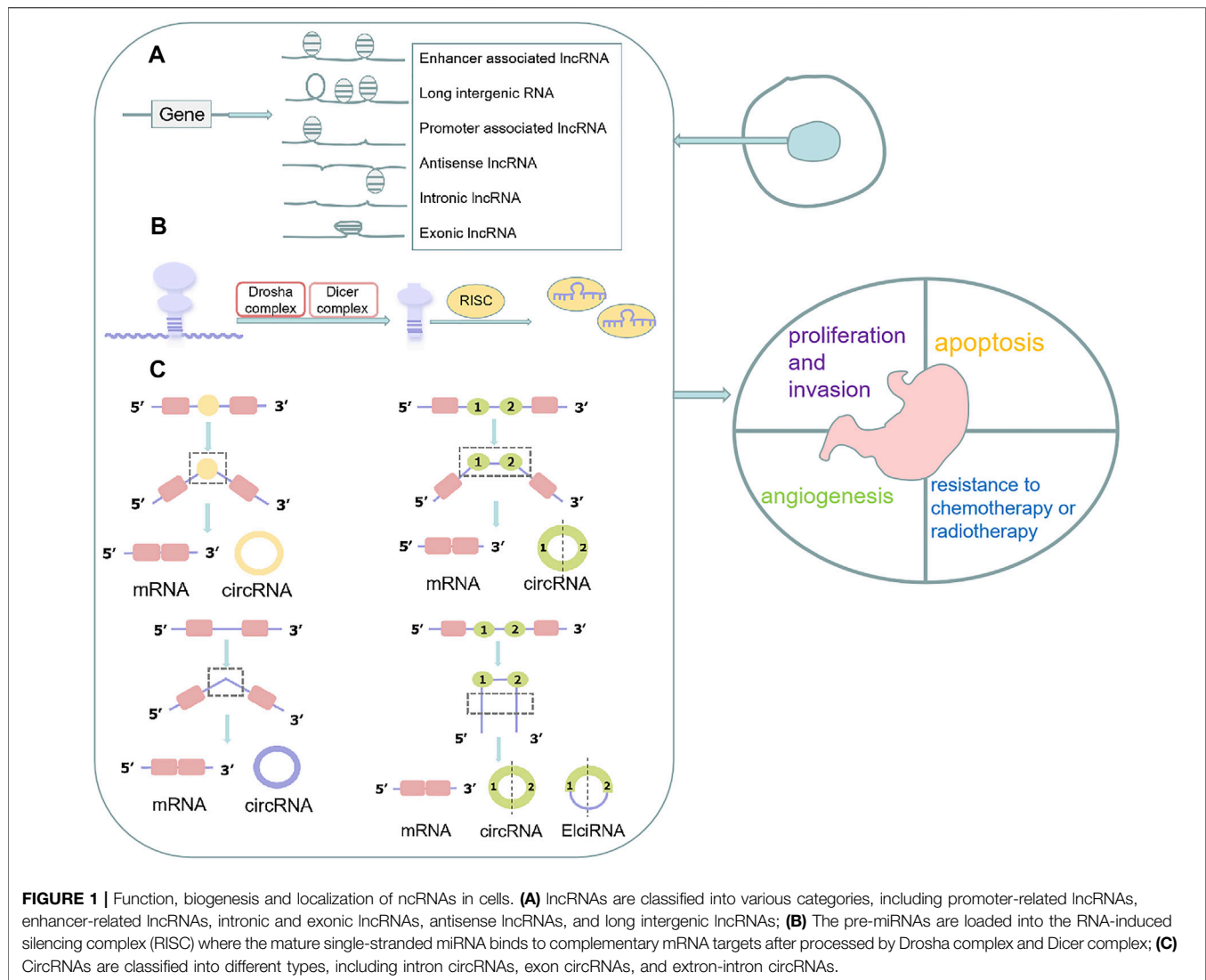
Gastric cancer (GC) is one of the most common malignancies in the world. It is also the fifth most common cancer in China. In recent years, a large number of studies have proved that non-coding RNAs (ncRNAs) can regulate cell proliferation, invasion, metastasis, apoptosis, and angiogenesis. ncRNAs also influence the therapeutic resistance of gastric cancer. ncRNAs mainly consist of miRNAs, lncRNAs and circRNAs. In this paper, we summarized ncRNAs as biomarkers and therapeutic targets for gastric cancer, and also reviewed their role in clinical trials and diagnosis. We sum up different ncRNAs and related moleculars and signaling pathway in gastric cancer, like Bcl-2, PTEN, Wnt signaling. In addition, the potential clinical application of ncRNAs in overcoming chemotherapy and radiotherapy resistance in GC in the future were also focused on.

**Keywords:** gastric cancer, non-coding RNA, therapy resistance, microRNA, lncRNA

## 1 INTRODUCTION

Gastric cancer (GC) is the fifth most common type of cancer and the third most common cause of cancer-related deaths worldwide after lung and colorectal cancer (Bray et al., 2018). In 2018, the global age-standardized incidence and mortality rates for GC were 11.1 and 8.2 per 100,000 persons, respectively (Thrift and El-Serag, 2020). The Global Burden of Disease Study (GBD) 2017 showed that the global age-standardized incidence decreased by 28.0% in 2017 compared with that in 1990, and the age-standardized mortality decreased by 48.7% (collaborators, 2020). The uniform declines in incidence have been observed in many parts of the world for decades, including China (Luo et al., 2017). Early detection of gastric cancer is considered to have contributed to favorable survival (Sekiguchi et al., 2021). In 2004, an early detection and treatment program was initiated in China with special funds from the Ministry of Health (Zheng et al., 2015). Therefore, one of the main reasons for the improvement of gastric cancer is the popularity of endoscopic screening (Zhang et al., 2018a). Endoscopic resection is the preferred treatment for early GC, whereas traditional surgery, including D2 lymphadenectomy, consisting of lymph node stations in the perigastric mesentery and along the celiac arterial branches, is the main treatment for operable GC (Hiki et al., 2013; Pimentel-Nunes et al., 2015). The first line of treatment for patients with advanced GC consists of sequential chemotherapy with platinum and fluoropyrimidine (Smyth et al., 2016). Targeted therapies currently approved to treat GC include trastuzumab as first line therapy for HER2-positive patients, ramucirumab as an anti-angiogenic agent for second line treatment, and nivolumab or pembrolizumab as an anti-PD-1 agent for third line treatment (Group et al., 2010; Fuchs et al., 2014; Kang et al., 2017). Some patients, however, have multiple drug resistance (MDR) to these agents, leading to a poor prognosis. Understanding the mechanisms underlying resistance to these drugs is needed to develop new methods for accurate early detection and effective treatment of GC.





Non-coding RNAs (ncRNAs) are unique transcripts that do not encode proteins (Esteller and Pandolfi, 2017). According to the length and shape, ncRNAs can be subdivided into the following types: tiny/short ncRNAs, long ncRNAs (lncRNAs) and circular RNA (circRNAs). There are various small ncRNAs, such as microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) (Wei et al., 2019). The most widely studied class of ncRNAs are miRNAs, which are small ncRNAs of ~22 nucleotides (nt) that, in animals, mediate post-transcriptional gene silencing by controlling the translation of mRNA into proteins (He and Hannon, 2004). lncRNAs are a heterogeneous group of non-coding transcripts more than 200 nt long that are involved in many biological processes (Esteller, 2011). PiRNAs are ncRNAs of 24–30 nt in length, are Dicer-independent and bind the PIWI subfamily of Argonaute family proteins that are involved in maintaining genome stability in germline cells (Aravin et al., 2007).

SnoRNAs are intermediate-sized ncRNAs (60–300 bp). They are components of small nucleolar ribonucleoproteins (snoRNPs), which are complexes that are responsible for sequence-specific 2'-O-methylation and pseudouridylation of ribosomal RNA (rRNA) (Ni et al., 1997). Several of these ncRNAs have been found to act as key regulators of various cell functions in GC, including cell proliferation, apoptosis, the cell cycle, and cellular metabolism (Chun-Zhi et al., 2010; Liu et al., 2019a; Zhang et al., 2019a). Moreover, some ncRNAs were reported closely related to the development of chemoresistance in GCs (Liu et al., 2019a; Xi et al., 2019) (Figure 1).

This review systematically summarizes current knowledge about the mechanisms by which ncRNAs regulate cell proliferation, invasion, and apoptosis, as well as their impact on drug resistance. Elucidation of these mechanisms may provide insight into the future use of ncRNAs as hypothetical biomarkers and/or therapeutic targets of GC.

**TABLE 1 |** ncRNAs and oncogenes and suppressor genes in gastric cancer behavior.

NcRNA	Targeted genes	Genetic properties	References	behavior
lncRNA HOXA11-AS;lncRNA MNX1-AS1;lncRNA FOXP4-AS1	EZH2	oncogene	Liu et al. (2017); Shuai et al. (2020); Chen et al. (2019a)	proliferation
lncRNA LINC00673;lncRNA FOXD2-AS1;lncRNA UCA1			Huang et al. (2017a); Xu et al. (2018); Wang et al. (2017)	
lncRNA SNHG6;lncRNA SPRY4-IT1;lncRNA PART1			Li et al. (2018a); Zhou et al. (2017a); Han et al. (2020)	
lncRNA AFAP1-AS1;lncRNA SNHG17;lncRNA AGAP2-AS1			Yuan et al. (2020); Zhang et al. (2019b); Qi et al. (2017a)	
lncRNA OIP5-AS1;lncRNA CASC15;lncRNA HOTAIR			Bai and Li (2020); Wu et al. (2018a); Song et al. (2019)	
miR-101;miR-31;miR-26b			Carvalho et al. (2012); Sun et al. (2019); Rossi et al. (2019)	
miR-370;miR-138-5p;miR-204-5p;miR-146a	EGFR	oncogene	Ning et al. (2017); Wang et al. (2018a); Kogo et al. (2011)	
miR-873	STRA6	oncogene	Lin et al. (2019)	
miR-133b	FSCN1	oncogene	Guo et al. (2014)	
miR-511	TRIM24	oncogene	Fang et al. (2017)	
miR-216a;miR-18a	RUNX1	suppressor gene	Wu et al. (2018b)	
			Qi et al. (2020)	
miR-5683	PDK4	oncogene	Miao et al. (2020)	
miR-885	YPEL1	suppressor gene	Li et al. (2019a)	
miR-122	LYN	oncogene	Meng et al. (2020)	
miR-665	PPP2R2A	oncogene	Zhang et al. (2020a)	
LINC00152;miR-383;miR-1915-3p;miR-24	Bcl-2	oncogene	Tao et al. (2021); Cui et al. (2019); Mao et al. (2019); Zhang et al. (2016a)	apoptosis
miR-23a/b;miR-499-5p;miR-183; miR-93	PDCD4	suppressor gene	Hu et al. (2017); Yang et al. (2020); Li et al. (2016); Liang et al. (2016)	
miR-200a;lncRNA XIAP-AS1	TRAIL	suppressor gene	Guo et al. (2018); Cai et al. (2017)	
LncRNA PVT1;miR-125a;miR-1	VEGF	oncogene	Dai et al. (2015); Xie et al. (2018); Zhao et al. (2018); Xie et al. (2020a)	angiogenesis
X26nt;lncRNA MALAT1	VE-cadherin	oncogene	Chen et al. (2021a); Li et al. (2017a)	

## 2 MECHANISMS OF NCRNAS THAT REGULATE GASTRIC CANCER BEHAVIOR

Multiple ncRNAs dysregulate the behavior of GC by post-transcriptionally binding to the 3'UTR of downstream gene mRNAs, including mRNAs encoded by various oncogenes, such as *EZH2*, *EGFR*, *STRA6*, *FSCN1*, *TRIM24*, *PDK4*, *LYN*, and *PPP2R2A*, and suppressor genes, such as *PTEN*, *RUNX1*, and *YPEL1* (Table 1) (Markopoulos et al., 2017). In addition, complex interactions among internal members play crucial roles in proliferation, apoptosis, angiogenesis, and other behaviors by affecting classic signaling pathways.

### 2.1 Proliferation and Invasion of ncRNAs

*EZH2*, which plays a central role in all aspects of GC pathogenesis, is more highly expressed in GC tissues than in non-tumor epithelium, with increased expression associated with more aggressive biological behavior and poor prognosis of GC (Gan et al., 2018). The lncRNAs *HOXA11-AS*, *MNX1-AS1*, and *FOXP4-AS1*, and the microRNAs -101, -31, and -26b, all have an impact on the progression of GC by altering the level of expression of *EZH2* (Carvalho et al., 2012; Huang et al., 2017a; Qi et al., 2017a; Zhou et al., 2017a; Liu et al., 2017; Wang et al., 2017; Li et al., 2018a; Wu et al., 2018a; Xu et al., 2018; Chen et al., 2019a; Zhang et al., 2019b; Rossi et al., 2019; Song et al., 2019; Sun et al., 2019; Bai and Li, 2020; Han et al., 2020; Shuai et al., 2020; Yuan et al., 2020). Epidermal growth

factor receptor (EGFR) is an oncogenic transmembrane receptor that is overexpressed in many cancers, including GC (Zhen et al., 2014). MiRs-370 (Ning et al., 2017), -138-5p, and -204-5p (Wang et al., 2018a), and -146a (Kogo et al., 2011) target EGFR, thereby altering the migration and proliferation of GC cells. MiR-873, which down-regulates *STRA6* mRNA in GC, plays a suppressor role (Lin et al., 2019). MiR-133b directly targets and inhibits *FSCN1*, which acts as an oncogene in GC cells, whereas miR-511 inhibits the tumor suppressor gene *TRIM24* in GC (Guo et al., 2014; Fang et al., 2017). MiR-216a and miR-18a directly target and downregulate *RUNX1* (Wu et al., 2018b; Qi et al., 2020); and miR-5683 promotes glycolysis and progression by up-regulating *PDK4* expression (Miao et al., 2020). In addition, miR-885, which targets *YPEL1*, enhances GC cell proliferation, colony formation, and invasion; and miR-122 and miR-665 inhibit proliferation, invasion, and EMT by reducing the expression of *LYN* and *PPP2R2A*, respectively, in GC (Li et al., 2019a; Zhang et al., 2020a; Meng et al., 2020).

In addition, many other lncRNAs and circRNAs act as competitive endogenous RNAs (ceRNAs) of miRNAs, regulating the expression of oncogenes or tumor suppressor genes in GC. For example, the lncRNA *LINC0130*, which inhibits miR-101-3p activity, enhances the expression of *EZH2*, thereby promoting GC progression (Cao et al., 2019). The lncRNA *MT1JP* has been shown to act as a ceRNA for miR-92a-3p, up-regulating the expression of *FBXW7* and reducing GC cell proliferation (Zhang et al., 2018b). The lncRNA

LINC00240 promotes GC through the LINC00240/miR-124-3p/DNMT3B axis as an oncogene (Li et al., 2020a). By binding to and neutralizing miR-286-5p, CircPSMC3 enhances the expression of *PTEN* (Rong et al., 2019). CircGRAMD1B plays a negative role in GC progression by affecting the miR-130A-3P-*PTEN*/P21 axis (Dai et al., 2019). CircFAT1 binds to and neutralizes miR-548g, increasing the expression of the tumor suppressor gene *RUNX1* in GC cells (Fang et al., 2019). Hsa-circ-0017639 promotes GC proliferation and metastasis by binding to and neutralizing miR-224-5p, thereby up-regulating *USP3* expression (Li et al., 2020b).

NcRNAs are involved in many pathways, including the *PTEN*/PI3K/AKT/mTOR, Hippo, and Wnt/ $\beta$ -catenin signaling pathways, adding a new dimension to the understanding of GC progression. The lncRNA BX357664, which acts as a ceRNA of miR-183-3p to target *PTEN*, affects the PI3K/AKT/mTOR pathway and inhibits GC proliferation and metastasis (Liang et al., 2021). LncRNA HORAI1M1 acts as a ceRNA of miR-17-5p to up-regulate the expression of *PTEN* and mediate the PI3K/AKT pathway (Lu et al., 2019). By binding to and neutralizing miR-149-5p, circNRIP1 affects the level of expression of the AKT/mTOR axis and acts as a tumor promoter in GC (Zhang et al., 2019c). Overexpression of the circRNA ciRS-7 inhibits the tumor suppressor effect of miR-7 through the *PTEN*/PI3K/AKT signaling pathway (Pan et al., 2018). The lncRNA HCG18, which inhibits the expression of miR-141-3p; the lncRNA LINC00649, which targets miR-16-5p; and circLARP4, which adsorbs miR-424 are all directly implicated in the proliferation and invasion of GC cells by influencing the Hippo signaling pathway (Zhang et al., 2017a; Liu et al., 2020). The lncRNAs LINC01133, OIP5-AS1, and LINC01355, which bind to and neutralize miR-106a-3p, miR-367-3p, and miR-431-5p, respectively, all activate the Wnt/ $\beta$ -catenin signaling pathway and induce cell proliferation (Yang et al., 2018a; Tao et al., 2020; Piao et al., 2021).

## 2.2 Apoptosis of ncRNAs

At present, the known apoptosis-related genes in GC include those encoding the anti-apoptosis-related factors genes B-cell lymphoma-2 (*Bcl-2*), programmed cell death 4 (*PDCD4*), and tumor necrosis factor- $\alpha$ -related apoptosis-inducing ligand (*TRAIL*) (Carthy et al., 2003; Wu et al., 2004; Wang et al., 2010; Zhu et al., 2016; Lim et al., 2017). In addition, gastric cancer cells may undergo apoptosis through the MAPK/ERK, PI3K/Akt/mTOR, Wnt/ $\beta$ -catenin and other signaling pathways (Fattahi et al., 2020; Su et al., 2020). Many studies have found that ncRNAs related to these genes and signaling pathways play important roles in the GC cell apoptosis.

The anti-apoptotic protein *Bcl-2* has been shown to play an important role in GC. MiR-383 and miR-1915-3p have been shown to reduce *Bcl-2* expression in GC, whereas LINC00152 has the opposite effect (Cui et al., 2019; Mao et al., 2019; Tao et al., 2021). The expression of *BCL2L11*, a pro-apoptotic member of the *Bcl-2* family, is reduced by miR-24, thereby inhibiting apoptosis (Zhang et al., 2016a). The roles of tumor suppressor *PDCD4* in GC mainly include promoting cell apoptosis. *PDCD4* may be a key downstream protein in the development of GC

(Zhao et al., 2020). MiR-23a/b, miR-499-5p, miR-183, miR-93 all target and negatively regulate *PDCD4* to promote GC development (Li et al., 2016; Liang et al., 2016; Hu et al., 2017; Yang et al., 2020). *TRAIL* is a protein that promotes apoptosis in cancer cells by inducing the formation of the death-inducing signal complex (DISC). Over-expression of miR-200a and knockdown of the lncRNA *XIAP-AS1* enhance *TRAIL*-induced apoptosis in GC cells (Cai et al., 2017; Guo et al., 2018).

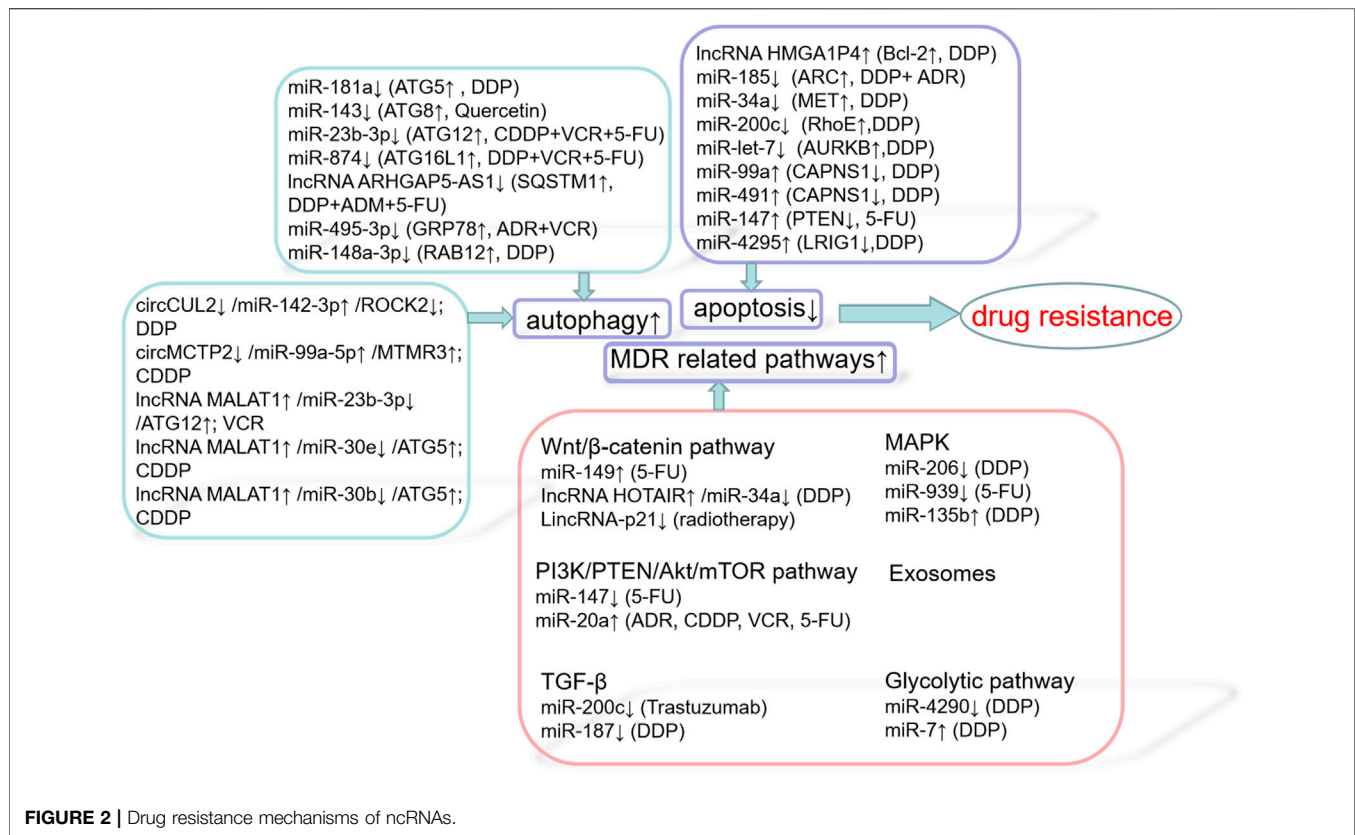
MiR-206 targets and suppresses *MAPK3* mRNA, whereas miR-135b activates MAPK/ERK signaling, thereby affecting GC cell apoptosis (Chen et al., 2019b; Zhou and Chen, 2019). The lncRNA *BCAR4* facilitates MAPK/ERK signaling, whereas the lncRNA LINC00858 reduces the methylation of the *WNK2* gene promoter and its downstream MAPK signaling pathway (Zhou et al., 2020; Du et al., 2021). As such, ncRNAs function as master regulators of apoptosis in GC by controlling two other signaling pathways, the PI3K/Akt/mTOR and Wnt/ $\beta$ -catenin pathways. Some of these regulatory molecules, including miR-495 (Wang et al., 2018b), miR-193b (Tian et al., 2020a), miR-139-5p (Zhang et al., 2020b), lncRNA *SLC25A5-AS1* (Li et al., 2019b), and miR-195-5p (Zhao and Wu, 2019), inactivate both pathways; whereas others, including miR-194 (Peng et al., 2018), miR-324-3p (Sun et al., 2018), and LINC00355 (Luan et al., 2020), activate both pathways to inhibit apoptosis of GC cells.

## 2.3 Angiogenesis of ncRNAs

Tumor expansion depends on angiogenesis, as blood vessels provide oxygen and nutrients to tumors (Carmeliet and Jain, 2011). Angiogenesis is therefore essential for tumor occurrence, progression, invasion, migration, and metastasis (Hanahan and Weinberg, 2000). Tumors and stromal cells secrete abnormal levels of growth factors that cause tumor vascular dysplasia, including vascular disorders, immaturity, and permeability (Viallard and Larrivée, 2017). The important role of angiogenesis in gastric cancer and other tumors has been extensively studied. Inhibition of the angiogenesis signaling pathway will help prevent tumor growth and prolong the survival time of cancer patients. For gastric cancer, the REGARD trial, RAINBOW trial, and several ongoing RCTs have shown that ramucirumab that targeting VEGF signals help GC patients to achieve better survival (Li et al., 2021).

Continuous expression of vascular endothelial growth factor (VEGF) can stimulate angiogenesis (Maeda et al., 1996). Low expression of miR-125a maintains the secretion of VEGF-A in GC, thereby modulating tumor angiogenesis (Dai et al., 2015). Overexpression of miR-1 inhibits the growth of blood vessels by reducing the expression of VEGF-A and endothelin 1 (*EDN1*) (Xie et al., 2018). The lncRNA *PVT1* plays an important role in GC angiogenesis by activating the *STAT3*/VEGFA axis (Zhao et al., 2018). The adsorption of circSHKBP1 on exosomes by miR-582-3p enhances the stability of VEGF mRNA (Xie et al., 2020a).

Vascular endothelial cadherin (VE-cadherin) has been shown to enhance vascular permeability, angiogenesis and tumor growth (Viallard and Larrivée, 2017). The exosomal 26-nt-long ncRNA (X26 nt) secreted by GCs can target VE-cadherin to increase angiogenesis and vascular permeability (Chen et al., 2021a). The long-chain non-coding RNA *MALAT1* was shown to enhance the



**FIGURE 2 |** Drug resistance mechanisms of ncRNAs.

expression of classic markers such as VE-cadherin and components of related signaling pathways to promote blood vessel growth and angiogenesis (Li et al., 2017a).

Exosomes are nano-scale membrane vesicles containing proteins, lipids, mRNA, and miRNA that are important in cell-to-cell communications. Signaling pathways composed of exosomes, miRNAs and target genes can affect tumor angiogenesis. For example, miR-135b delivered by gastric tumor exosomes was found to promote angiogenesis by inhibiting FOXO1 expression in endothelial cells (Bai et al., 2019). Similarly, miR-130a activates GC angiogenesis by targeting C-MYB in vascular endothelial cells (Yang et al., 2018b).

### 3 THE MECHANISM OF RESISTANCE TO CHEMOTHERAPY OR RADIOTHERAPY OF NCRNAS FOR GASTRIC CANCER

The main cause of death in patients with solid tumors is their eventual development of resistance to one or more chemotherapy drugs, which may eventually lead to metastatic disease. The mechanisms involved in GC resistance to cancer drugs are complex, including decreased apoptosis, increased autophagy, lost cell cycle checkpoint control, accelerated cell proliferation, inactivated signaling pathways and targeted genes, as well as accelerated cancer stem cell (CSC) drug metabolism and activation (Figure 2) (Wei et al., 2020a). Chemotherapy is the main treatment for early and late tumors. However, drug

resistance is the main obstacle to cancer treatment, which seriously limits the role of traditional chemotherapy and new biological agents (Broxterman et al., 2009). In recent years, there are more and more studies on ncRNAs, target gene regulation, affecting drug function, pharmacogenomics or drug resistance. For example, according to previous studies, the down-regulation of miR-21 makes cancer cells sensitive to different chemotherapy *in vitro*, including cisplatin, etoposide and Adriamycin (Seca et al., 2013; Yang et al., 2015; Vanas et al., 2016). On the other hand, some drugs can induce alterations in miR-21 levels: soladosine can inhibit lung cancer cell invasion through miR-21 down-regulation. miR-34a was reported to be downstream of p53 and to function as a tumor suppressor (Welch et al., 2007). It is down-modulated in colorectal cancer (CRC) (Akao et al., 2010). In 5-Fluorouracil (5-FU)-resistant colon cancer cells ectopic expression of miR-34a inhibited cell growth and attenuated the resistance to 5-FU through downregulation of SIRT1 and E2F3, inhibition of LDHA and of c-Kit, thus reducing stem cell factor (SCF)-induced migration/invasion (Akao et al., 2011).

#### 3.1 Apoptosis

Several ncRNAs affect apoptosis by influencing effective and multifunctional inhibitors of apoptosis, including Bcl-2 (Voss et al., 2010), apoptosis repressor with a caspase recruitment domain (ARC) (Ludwig-Galezowska et al., 2011), MET (Montagne et al., 2015), RhoE (Ongusaha et al., 2006), and AURKB (He et al., 2016); or by influencing enhancers of



apoptosis, including calpain small subunit 1 (CAPNS1) (Bertoli et al., 2009), PTEN (Zhu et al., 2020), and LRIG1 (Chang et al., 2013). The lncRNA HMGA1P4 was shown to trigger DDP resistance in GC by adjusting the expression levels of genes associated with apoptosis, including those encoding the proteins Bcl-2, Bax, and caspase-3 (Qiao et al., 2020). Enhanced expression of miR-185 was found to increase the chemosensitivity of GC cells by preventing ARC (Li et al., 2014). Upregulation of miR-34a increased the sensitivity of GC cells to DDP by stopping MET, thereby affecting GC cell proliferation and apoptosis (Zhang et al., 2016b). MiR-200c, which targets RhoE, increases cisplatin-induced apoptosis and is therefore considered a potent factor in eliminating chemoresistance (Ghasabi et al., 2019). MiR-let-7, which targets AURKB, increases the cytotoxicity of DDP in SGC7901/DDP cells by inducing apoptosis and increasing sensitivity to chemotherapeutic agents (Han et al., 2018). Inhibition of miR-99a and miR-491 was found to enhance chemosensitivity to cisplatin in human GC cells by increasing CAPNS1 expression (Zhang et al., 2016c). Down-regulation of miR-147 had a positive effect on PTEN, increasing the sensitivity of GC cells to 5-FU by inducing apoptosis (Shen et al., 2018). MiR-4295 negatively regulates LRIG1 expression to activate the EGFR/PI3K/Akt signal pathway, thereby promoting GC cell proliferation and inhibiting DDP-induced GC cell apoptosis (Yan et al., 2018). Several other ncRNAs affect the apoptosis of GC cells through various pathways. For example, circAKT3, which acts as a ceRNA to miR-198, enhances the expression of *PIK3R1*, activates the PI3K/AKT signaling cascade, and promotes CDDP resistance (Huang et al., 2019). Up-regulating the expression of miR-206, which targets MAPK3 expression, was found to attenuate the proliferation of drug-resistant GC cells, promote apoptosis and reduce DDP resistance (Chen et al., 2019b). Low expression of miR-135b induced the apoptosis of GC cells through the MST1-mediated MAPK signaling pathway, thus enhancing cell sensitivity to cisplatin (Bai et al., 2019).

### 3.2 Autophagy

Autophagy exhibits different effects in different situations. Protective autophagy inhibition reduces previously activated cell defense mechanisms, increasing cell sensitivity to chemotherapeutic agents. Overactivated autophagy can lead to cell death through solute overactivation, activating another cell death pathway, in addition to apoptosis (Taylor et al., 2018). Autophagy is crucial to the mechanism by which GC cells become resistant to chemotherapy. Abnormal activation of autophagy induced by chemotherapeutic drugs can provide energy to support cancer cells, enhancing resistance to chemotherapy. ncRNAs influence autophagy through autophagy-related genes, including *ATG*, *SQSTM1*, *GRP78*, *GABARAPL1*, and *RAB12*. MiR-181a targets *ATG5* as a major autophagy-related modulator and reverses cisplatin resistance in GC cells (Zhao et al., 2016). MiR-143 potently inhibits autophagy by decreasing *GABARAPL1* (*ATG8*), thereby modulating chemosensitivity to quercetin (Du et al., 2015). The miR-23b-3p/*ATG12*/*HMGB2*/autophagy regulatory cycle plays a crucial part in multi-drug resistance (MDR) in GC cells. MiR-23b-3p has been shown to

inhibit autophagy mediated by *ATG12* and *HMGB2*, sensitizing GC cells to chemotherapy (An et al., 2015). By targeting *ATG16L1*, miR-874 inhibits autophagy and sensitizes GC cells to chemotherapy (Huang et al., 2018). Impaired autophagic degradation of the lncRNA ARHGAP5-AS1 recruited by SQSTM1 in cancer cells resistant to agents such as DDP, ADM, and 5-FU promotes chemoresistance (Zhu et al., 2019a). MiR-495-3p inhibits autophagy and MDR by down-regulating the target gene *GRP78* (Chen et al., 2018a). In addition, miR-148a-3p inhibits cellular protective autophagy in DDP-resistant GC cells by suppressing *RAB12* expression and mTOR1 activation (Li et al., 2017b).

Interactions among ncRNAs also play a crucial role in autophagy in GC. CircCUL2 decreases autophagy through miR-142-3p/ROCK2 to modulate malignant transformation and cisplatin resistance in GC cells (Peng et al., 2020). CircMCTP2 can reduce the autophagy of platinum-resistant GC cells and inhibit the expression of miR-99a-5p and MTMR3, thus inhibiting cisplatin resistance in these. Moreover, inhibition of miR-99a-5p can sensitize GC cells sensitive to CDDP (Sun et al., 2020). The lncRNA MALAT1, an endogenous competitor of miR-23b-3p RNA, attenuates its inhibition of *ATG12* expression, leading to chemotherapy-induced autophagy and resistance to chemotherapy in GC cells (YiRen et al., 2017). The lncRNA MALAT1, which binds to miR-30e to regulate *ATG5* expression, promotes autophagy and inhibits autophagy-related chemotherapy (Zhang et al., 2020c). In addition, the lncRNA MALAT1 has been found to enhance autophagy through the miR-30b/*ATG5* axis in HGC-27/CDDP cells, which has a potent effect on autophagy-related CDDP resistance (Xi et al., 2019).

### 3.3 MDR Related Pathways

MDR is the leading cause of chemotherapy failure in cancer treatment. Signaling pathways associated with MDR include the Wnt/ $\beta$ -catenin, PI3K/PTEN/Akt/mTOR, TGF- $\beta$ , MAPK, and exosome pathways.

#### 3.3.1 Wnt/ $\beta$ -Catenin Pathway

The Wnt/ $\beta$ -catenin pathway is one of the major signaling pathways involved in epithelial-neutral transition (EMT). The EMT-like morphology of cancer cells may be responsible for their chemoresistance and invasion (Chen et al., 2011). MiR-149 promotes 5-FU resistance in GC cells, mainly by targeting *TREM2* and activating the  $\beta$ -catenin pathway (Wang et al., 2020a). Knockdown of the lncRNA HOTAIR and upregulation of miR-34a inhibits DDP resistance in GC cells by inactivating the PI3K/Akt and Wnt/ $\beta$ -catenin signal pathways (Cheng et al., 2018a). LincRNA-p21 enhances the sensitivity of GC to radiotherapy by suppressing the  $\beta$ -catenin signaling pathway (Chen et al., 2019c).

#### 3.3.2 PI3K/PTEN/Akt/mTOR Pathway

The PI3K/PTEN/Akt/mTOR signaling pathway plays a prominent part in mediating drug resistance. Poor outcomes in patients with many types of malignancy has been associated with the loss of *PTEN*, resulting in resistance to new

chemotherapeutic agents (Keniry and Parsons, 2008). MiR-147 was found to inhibit the PI3K/AKT signaling pathway by directly increasing *PTEN* expression and enhancing the resistance of GC cells to 5-FU. The miR-20a/LRIG1 axis regulates GC cells through EGFR mediated PI3K/AKT and MAPK/ERK signaling pathways to modulate MDR in GC (Cheng et al., 2018a).

### 3.3.3 TGF- $\beta$

In normal cells and the early stages of cancer, this pathway has tumor-inhibiting functions, including cell cycle arrest and apoptosis. In advanced cancers, however, activation of this pathway can promote tumorigenesis, for example by promoting tumor metastasis (Colak and Ten Dijke, 2017). The TGF- $\beta$ /ZEB2 axis plays an important role in drug resistance of GC, whereas miR-200C overexpression inhibits ZEB1/ZEB2, leading to sensitizing GC cells to trastuzumab (Zhou et al., 2018). MiR-187 enhances the sensitivity of GC cells to cisplatin by inhibiting the transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad signaling pathway (Zhu et al., 2019b).

### 3.3.4 MAPK

Triple root-activated protein kinase 3 (MAPK3) plays a key role in the extracellular signal-regulated kinase (ERK)/MAPK pathway. Upregulation of miR-206 can inhibit the proliferation of drug-resistant GC cells, promote apoptosis and reduce DDP resistance by targeting *MAPK3* expression (Chen et al., 2019b). Down-regulation of miR-135b leads to inactivation of the MAPK signaling pathway and increases the expression of *MST1* and *Bax*, thus enhancing the sensitivity of GC cells to CDDP. Mir-939 inhibits the growth of GC cells, both *in vitro* and *in vivo*, primarily by inhibiting the activated SLC34A2/Raf/MEK/ERK pathway, and enhances GC cell sensitivity to 5-FU by inducing apoptosis (Zhang et al., 2017b).

### 3.3.5 Exosomes

Exosomes are biologically active nanosized extracellular vesicles that are released by cells into the extracellular space, and play a central role in the initiation and development of intercellular signaling networks (Rajagopal and Harikumar, 2018). Exosomes can also enhance resistance to chemotherapy by exporting drugs or sharing antiapoptotic drugs in cancer cells, thereby interfering with drug metabolism (Abak et al., 2018).

Cancer-associated fibroblasts (CAFs) support tumor progression and drug resistance by secreting various bioactive substances, including exosomes. MiR-522 secreted by CAFs inhibits iron-associated death in GC and ultimately reduces their sensitivity to chemotherapy (Zhang et al., 2020d). MiR-21-containing exosomes secreted by tumor-associated macrophages (TAMs) induce DDP resistance in GC (Zheng et al., 2017). MGC803/DDP-derived exosomes deliver miR-500a-3p targeting *FBXW7* *in vitro*, enhancing DDP resistance and stem cell properties of MGC803 recipient cells (Lin et al., 2020). MiR-155-5p is enriched in MGC-803R exons and can be delivered to MGC-803S cells (Wang et al., 2019). Exosomes containing miR-106a-5p and miR-421 are highly expressed and modulate *TFAP2E* methylation-induced chemotherapy (Jingyue et al., 2019). Exosomes containing miR-501 exhibit

resistance to doxorubicin by targeting *BLID* (Liu et al., 2019b). Exosomes secrete nanoparticles with anti-miR-214 activity to reverse GC cell chemoresistance (Wang et al., 2018c). Exosomes containing circPRRX1 enhance doxorubicin resistance by regulating miR-3064-5p/PTPN14 signaling (Table 2) (Wang et al., 2020b).

### 3.3.6 Glycolytic Pathway

Cancer cells undergo glycolysis in the presence of oxygen, which is called the Warburg effect. Accumulated evidence has displayed that the aberrant activation of glycolysis plays an important role in many kinds of diseases via various mechanisms, including the induction of cancer chemotherapy resistance, including GC (Broxterman et al., 2009; Ganapathy-Kanniappan and Geschwind, 2013). A study has determined that microRNA-4290 suppresses PDK1-mediated glycolysis to enhance the sensitivity of gastric cancer cell to cisplatin (Qian et al., 2020). Another research has studied that down-regulation of miR-7 in gastric cancer is able to inhibit the proliferation, colony formation, and glycolysis of GC cells owing to its regulation of LDH-A, it also associated with chemoresistance to cisplatin (Jin et al., 2020a).

## 3.4 The Resistance Mechanism of ncRNAs in HER2-Positive GC

*HER2* overexpression drives tumorigenesis by creating spontaneous receptor homoplasms or heterogenes with other *ERBB* family members, thereby generating the expression of active pathogenic downstream signals, such as PI3K/Akt/mTOR and MAPK, which promote cell proliferation, survival, and angiogenesis (Meric-Bernstam et al., 2019). Trastuzumab, gefitinib, and lapatinib were all shown to have significant curative effects in patients with HER2-positive GC, and these agents have become the key chemotherapy drugs in HER2-positive GC. Silencing of both *HER2* and *EGFR* has been shown to increase tumor chemosensitivity to gefitinib (Wang et al., 2018d).

In HER2-positive GC, miR-494 inhibits cancer-initiating cell phenotypes and reverses resistance to lapatinib by reducing the expression of fibroblast growth factor receptor 2 (FGFR2) (Yu et al., 2018). MiR-143 inhibits the growth of HER2-positive GC cells by inhibiting the KRAS network, including the RNA helicase DDX6 (Tokumaru et al., 2019). The miR-21/PTEN pathway affects the sensitivity of GC cells to trastuzumab by regulating the apoptosis of HER2-positive GC cells (Eto et al., 2014). Overexpression of miR-223 reduced the expression of *FBXW7* and the sensitivity of GC cells to terrazumab, whereas inhibition of miR-223 restored the expression of *FBXW7* and the sensitivity of GC cells to terrazumab (Table 3) (Eto et al., 2015).

## 3.5 Hypoxia

In addition, ncRNAs affect the sensitivity of GC to chemotherapy by regulating the hypoxia signal pathway. Hypoxia-inducible factor-1 (HIF-1) is the main transcription factor significantly activated by hypoxia (Liu et al., 2008). HIF-1 can inhibit drug-induced apoptosis and reduce the accumulation of drugs in cells, resulting in hypoxia-induced drug resistance. The abnormal

**TABLE 2 |** Exsomes ncRNAs AND drug resistance.

NcRNA	Host cells	Mechanism of function	References
miR-522	Cancer-associated fibroblasts (CAFs)	ferroptosis↓;lipid-ROS accumulation↓	Zhang et al. (2020d)
miR-21	tumor-associated macrophages (TAMs)	exosomal transfer;PTEN↓	Zheng et al. (2017)
miR-500a-3p	cisplatin-resistant GC cells	stemness properties↑	Lin et al. (2020)
miR-155-5p	paclitaxel-resistant GC cells	EMT↑;chemoresistant phenotypes↑	Wang et al. (2019)
miR-106a-5p and miR-421	5-fluorouracil-resistant GC cells	TFAP2E methylation↑	Jingyue et al. (2019)
miR-501	doxorubicin-resistant GC cells	BH3-like motif-containing protein(BLID)↑	Liu et al. (2019b)
miR-214	HEK293T	potential targets↑;apoptosis↑	Wang et al. (2018c)
circPRRX1	doxorubicin-resistant GC cells	miR-3064-5p↓;PTPN14↑	Wang et al. (2020b)

**TABLE 3 |** NcRNAs in HER2-positive GC.

ncRNA	Main contents	Mechanism of function	References
miR-494	lapatinib	fibroblast growth factor receptor 2(FGFR2)↓;cancer initiating cells (CICs)↓	Yu et al. (2018)
miR-143	growth	KRAS network↓;DDX6 RNA helicase↓	Tokumaru et al. (2019)
miR-21	trastuzumab	PTEN↓;AKT phosphorylation↑	Eto et al. (2014)
miR-223	terrazumab	F-box and WD repeat domain-containing 7 (FBXW7)↓	Eto et al. (2015)

expression of miR-20b, miR-27a, and miR-181a is related to the modulation of the chemotherapy response in GC by HIF-1 $\alpha$  (Danza et al., 2016). HIF-1 $\alpha$  induces the expression of miR-27a, which is closely associated with MDR in GC (Zhao et al., 2015). By binding to miR-376a, the lncRNA Nutm2a-AS1 positively modulates GC formation and drug resistance through the regulation of HIF-1 $\alpha$  (Wang et al., 2020c).

## 4 THE EFFECT OF SOME IMPORTANT NON-CODING RNAs ON GASTRIC CANCER

Comprehensive and in-depth analyses of several ncRNAs have revealed their roles in GC cell behavior and drug sensitivity (Figure 3).

### 4.1 MiR-21

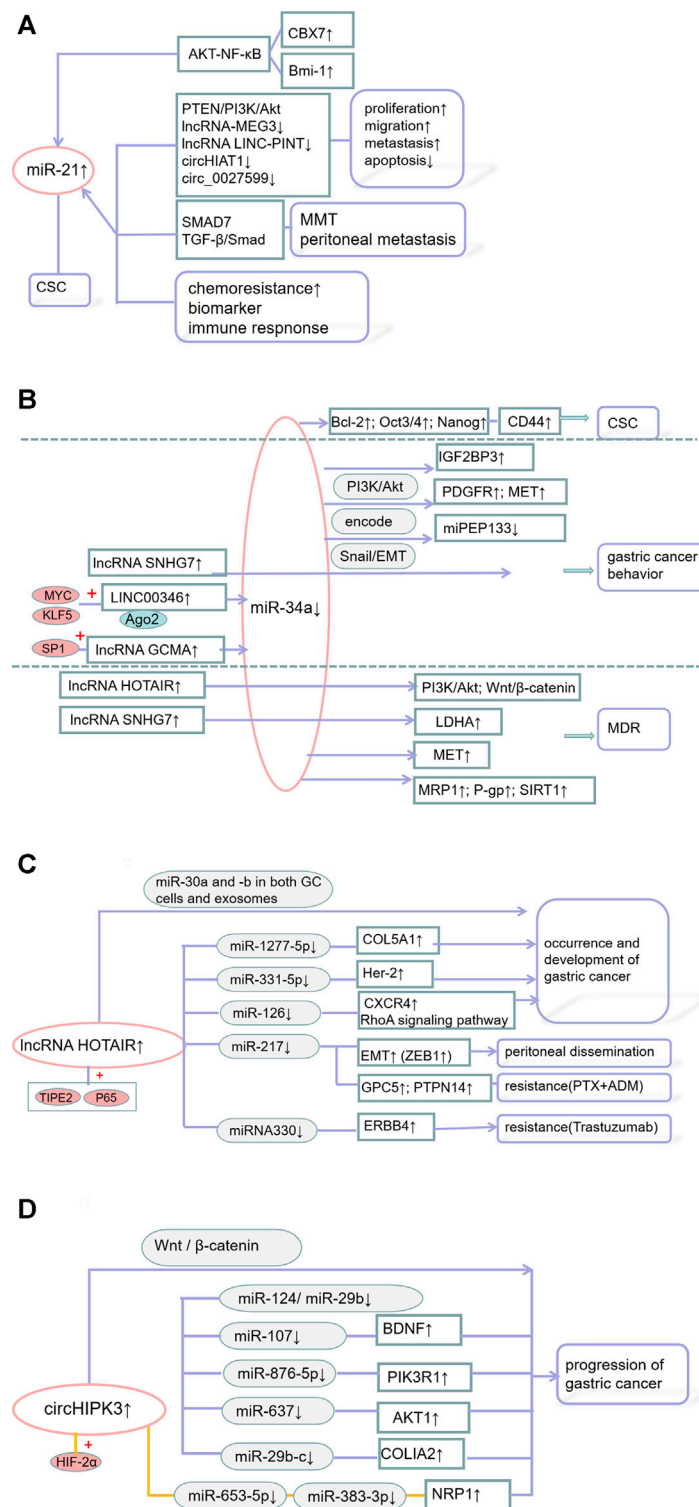
For example, a large-scale miRnome analysis of 540 samples, including solid tumors, such as GCs found that miR-21 is closely associated with the pathogenesis of solid tumors and poor patient prognosis (Volinia et al., 2006). MiR-21 affects the proliferation, metastasis, apoptosis, stem cell properties, and drug sensitivity of GCs. For example, miR-21-5p provides a favorable *in vitro* environment for the mesothelial-to-mesenchymal transformation (MMT) of peritoneal metastatic cancer cells by activating the TGF- $\beta$ /Smad pathway (Li et al., 2018b). Chromosomal protein homology (CBX) maintains the stem cell-like properties of GC cells by regulating the p16 and AKT-NF- $\kappa$ B-miR-21 pathways (Ni et al., 2018). B cell-specific moloney murine leukemia virus integration site 1 (Bmi-1) actively regulates the stem cell-like properties of GC cells by increasing miR-21, which targets the PTEN/Akt signaling pathway and promotes human GC cell proliferation, migration, and apoptosis (Zhang et al., 2012; Wang et al., 2016; Wang et al., 2018e). The lncRNAs-MEG3 (Dan et al., 2018) and LINC-PINT

(Feng et al., 2019), as well as circHIAT1 (Quan et al., 2020) and circ\_0027599 (Han et al., 2021), are able to bind miR-21 and play a negative role in GC progression. Exosomes carrying miR-21 can be transferred from macrophages to GC cells, inhibiting cell apoptosis, enhancing the activation of the PI3K/AKT signaling pathway, and inducing cisplatin resistance by down-regulating *PTEN*. MiR-21 enhances cell survival by targeting *PTEN*, inducing resistance to doxorubicin and cisplatin (Yang et al., 2013; Chen et al., 2018b).

Serum miR-21 is also a biomarker and a key regulator of the immune response in GC. Serum miR-21 concentration can indicate tumor recurrence in young GC patients (Park et al., 2016). MiR-21 is not only highly expressed in GCs, but is also highly expressed in the gastric juice of GC patients, affecting their prognosis (Motoyama et al., 2010; Zheng et al., 2011; Cui et al., 2013; Kim et al., 2013; Wu et al., 2015; Sierzega et al., 2017; Pereira et al., 2019; Zhang et al., 2020e). MiR-21 may also be a potential indicator of chemoresistance patients with metastatic GC (MGC) (Zheng et al., 2019). The overexpression of miR-21 increases the percentage of Th17 cells and reduces the percentage of Treg cells, resulting in an imbalance in the PD-1/PD-L1 pathway and regulating immune responses in GC (Liu et al., 2018). Synthetic circRNAs that target miR-21 can induce therapeutic dysfunction, inhibiting the proliferation of cancer cells and the activity of miR-21 against downstream protein targets, including the tumor protein DAXX (Qi et al., 2017b).

### 4.2 MiR-34a

MiR-34a was found to be a classic tumor suppressor miRNA in several malignancies (Hermeking, 2007). MiR-34a has also been shown to inhibit the growth, invasion, and metastasis of GC by targeting the expression of PDGFR and MET (Peng et al., 2014). Silencing of miR-34a is also partly responsible for activation of the cancer-promoting molecule IGF2BP3 (Zhou et al., 2017b). The novel microprotein miPEP133 encoded by the miR-34a precursor enhances *p53* transcription and miR-34a expression,



**FIGURE 3 | (A)** The mechanism of miR-21 in GC; **(B)** The mechanism of miR-34a in GC; **(C)** The mechanism of lncRNA HOTAIR in GC; **(D)** The mechanism of circular RNA circHIPK3 in GC.



thereby exerting a tumor suppressor effect (Kang et al., 2020). SNHG7 increases the invasion and migration of GC cells through the miR-34a-Snail-EMT axis (Zhang et al., 2020f). LINC00346 binds to and neutralizes miR-34a-5p, with KLF5 and MYC/LINC00346/miR-34a-5p being key effectors of GC tumorigenesis and progression (Xu et al., 2019a). SP1-activated lncRNA GCMA is a ceRNA that adsorbs miR-124 enhancing tumor metastasis and reducing GC progression (Tian et al., 2020b). Bmi-1 can actively regulate the stem cell-like properties of GC cells by increasing miR-34a expression (Wang et al., 2016). MiR-34a can regulate the sensitivity of human GC cells to DDP by targeting MET (Zhang et al., 2016b). MiR-34a-5p regulates the expression of Sirtuin 1 (SIRT1), P-glycoprotein (P-gp), and multidrug resistance-related protein 1 (MRP1) by directly binding to the 3'untranslated region (UTR) of *SIRT1*, thereby reversing the MDR of GC cells (Deng et al., 2021).

The lncRNAs HOTAIR and SNHG7 adsorb miR-34a. Anti-miR-34a antibody was found to reverse the effect of si-HOTAIR on DDP resistance, on apoptosis-related genes, and on the PI3K/Akt, and Wnt/ $\beta$ -catenin signaling pathways in anti-DDPGC cells, suggesting that the effect of HOTAIR depends on miR-34a (Cheng et al., 2018a). The level of the lncRNA SNHG7 negatively correlates with that of miR-34a, desensitizing GC cells to cisplatin (Pei et al., 2021). In addition, miR-34a was found to affect the expression of cancer stem cells (CSCs) overexpressing CD44, leading to tumorigenesis and recurrence, while inhibiting the proliferation, metastasis, and survival of CD44-positive CSCs (Jang et al., 2016).

### 4.3 LncRNA HOTAIR

Overexpression of the lncRNA HOTAIR is a biomarker for poor prognosis in patients with GC, as it may enhance malignant phenotype (Feng and Huang, 2017). High expression of HOTAIR is associated with tumor differentiation, lymph node and distant metastases, and higher clinical stage (Xu et al., 2019b). HOTAIR promotes GC by altering miRNA levels in cells and exosomes (Zhang et al., 2020g). HOTAIR effectively binds to and neutralizes miR-331-3p, thereby regulating the attenuation of HER2 levels and promoting GC progression (Liu et al., 2014). HOTAIR enhances GC growth by binding to a neutralizing miR-1277-5p and by up-regulating COL5A1 (Wei et al., 2020b). High HOTAIR expression promotes the proliferation and metastasis of GC through the miR-126/CXCR4 axis and SDF-1/CXCR4 signaling (Xiao et al., 2019). In mice, HOTAIR directly targets miR-217 and combines with the zinc finger electronic box binding home box 1 protein (ZEB1) to inhibit peritoneal diffusion of GC, significantly prolonging survival time (Takei et al., 2020). HOTAIR, which is stimulated by the NF- $\kappa$ B pathway, was found to promote GC progression by enhancing non-resolving inflammation (Zhang et al., 2019d). HOTAIR also affects GC chemoresistance. Overexpression of HOTAIR was found to enhance the resistance of GC cells to paclitaxel (PTX) and doxorubicin (ADR) (Wang et al., 2018f). In addition, the HOTAIR-miR-330-ERBB4 regulatory network with miRNA330 as its core was found to enhance the sensitivity of tumor cells to trastuzumab (Bie et al., 2020).

### 4.4 Circular RNA circHIPK3

CircHIPK3 is generated from exon 2 of the gene encoding homeodomain-interacting protein kinase 3 (HIPK3) (Xie et al., 2020b; Wen et al., 2020). The expression of circHIPK3 was found to be significantly higher in GC tissues than in adjacent normal tissues, suggesting that increased CircHIPK3 expression was associated with poor prognosis (Liu and Xu, 2019). CircHIPK3 forms an axis with miR-124 and miR-29b to that target COL1A1, COL4A1, and CDK6, which function in different histological growth patterns (Cheng et al., 2018b). CircHIPK3 adsorbs miR-107 (Wei et al., 2020c), miR-876-5p (Li et al., 2020c), miR-637 (Yang et al., 2021), playing a pivotal role in GC tumorigenesis and development. Hif-2 $\alpha$  is upregulated in hypoxic drug-resistant GC (HRGC) cells under a long-term hypoxic microenvironment and promotes GC metastasis through the miR-653-5p/miR-338-3P-NRP1 axis (Jin et al., 2020b). These circRNAs, which form complex RBP-circRNA-miRNA-mRNA interaction networks, such as the circHIPK3/miR-29b-c/COL1A2 network, are involved in GC development, progression, and reduced sensitivity to chemotherapy (Pereira et al., 2020).

## 5 CLINICAL TRIALS OF NCRNAS

Clinical studies are underway to evaluate applications of ncRNAs. For example, a study of tumor and adjacent normal tissues collected from 60 prospectively selected patients with GC found that Hsa\_circ\_0000745 played a crucial role in tumor development. A diagnostic index, involving plasma concentrations of ncRNAs and CEA, has been shown promising in the identifying patients with malignant tumors (Huang et al., 2017b). Four common miRNA polymorphisms, miR-146aC > G, miR-149T > C, miR-196a2T > C, and miR-499A > G, were found to be associated with susceptibility to and prognosis of GC in the Korean population (Ahn et al., 2013). A prospective clinical trial of GC patients treated with oxaliplatin/capecitabine (XELOX) chemotherapy found that plasma concentrations of miR-17-92 were closely associated with the progression of advanced GC and the effectiveness of XELOX chemotherapy (Fan et al., 2018). A study in 2010–2017 of Pralatrex and oxaliplatin in the treatment of unresectable or metastatic esophageal, gastric, or gastroesophageal junction carcinoma compared the mean expression of miR-215-5p in tumor tissues of responders and nonresponders using microprocessing devices of a gene chip and obtained reliable results. Another study in Singapore aims to test the predictive ability of multiple blood biomarkers, such as miRNAs, to detect early signs of disease at a stage at which tumors can be prevented or cured (Song and Meltzer, 2012).

Ongoing studies are evaluating the mechanisms of action of lncRNAs in gastrointestinal diseases and their possible relationships with *Helicobacter pylori* infection. In addition, levels of expression of miRNAs in GC tissue and blood and their association with responses to chemotherapy are being determined by next-generation sequencing, with validation by qRT-PCR, in multiple independent patient cohorts. The relationships between ncRNAs and cancer immune checkpoints are being explored to identify new uses for

ncRNAs in cancer immunotherapy, and differences in exosomal protein/ncRNA components are being evaluated in patients before and after combination chemotherapy with Apatinib and anti-PD-1 antibody.

## 6 DISCUSSION

Findings to date have shown that ncRNAs are extensively involved in gene regulatory networks. Many ncRNAs do not act as a single link, but as branching points with broad outputs that affect regulatory networks containing target genes or related signaling pathways. In GCs, ncRNAs have been identified as carcinogenic drivers and tumor suppressors.

In conclusion, this review provides strong evidence that ncRNAs can act as biomarkers for the diagnosis, prognosis, and chemotherapy resistance of GCs. Many studies to date have shown that ncRNAs play complex and important roles in GC proliferation, invasion, apoptosis, and angiogenesis. These ncRNAs or their resulting ceRNAs can alter the expression of target genes and/or affect classic signaling pathways. ncRNAs also participate in the regulation of resistance to radiotherapy and chemotherapy by affecting the expression of signaling pathways related to apoptosis and autophagy, and by regulating MDR-related genes and pathways. ncRNAs can also form networks in GC. Taken together, these findings indicate that targeting ncRNAs may be a promising method of enhancing sensitivity to chemotherapy, thereby improving the efficacy of treatment, in patients with GC. Studies evaluating the effects of ncRNAs on immunotherapy in GC are currently ongoing. The stability of ncRNAs in the circulation makes them suitable diagnostic and

prognostic markers for most cancers and determination of their concentrations in serum may be useful in the personalized management of patients. Better understanding of these ncRNAs and their target genes may provide new perspectives for the development of more complex and effective therapeutic agents for the treatment of GC. Techniques and tools to develop ncRNA-targeted and ncRNA-based drugs have been widely used in cancer treatment. Strategies including antisense oligonucleotide (ASO), RNA interference (RNAi) and CRISPR/Cas9 have been proposed to up-regulate the tumor inhibition of ncRNA using gene silencing techniques (Chen et al., 2021b). However, barriers to the translation of nucleic acid-based therapeutics into the clinic are related to their stability, specificity, delivery, and toxicity issues, such as “on-target” and “off-target” side effects (Hueso et al., 2021). It is very important to conduct further *in vitro* and *in vivo* studies and clinical studies on the efficacy and safety of ncRNA to achieve precision medicine.

## AUTHOR CONTRIBUTIONS

YY and XL wrote the review article. XQ participated in modification. LY and RW reviewed the manuscript. All authors read and approved the final manuscript.

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# New Insights into LINC00346 and its Role in Disease

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Accumulating evidence has shown that long intergenic non-protein-coding RNA 346 (LINC00346) functions as an oncogene in the tumorigenesis of several cancers. The expression level of LINC00346 has been shown to be obviously correlated with prognosis, lymphoma metastasis, histological grade, TNM stage, tumor size and pathologic stage. LINC00346 has been found to regulate specific cellular functions by interacting with several molecules and signaling pathways. In this review, we summarize recent evidence concerning the role of LINC00346 in the occurrence and development of diseases. We also discuss the potential clinical utility of LINC00346, thereby providing new insight into the diagnosis and treatment of diseases. In addition, we further discuss the potential clinical utility of LINC00346 in the diagnosis, prognostication, and treatment of diseases.

**Keywords:** lncRNA, LINC00346, biological function, mechanism, clinical utility

## INTRODUCTION

Human genome sequencing data have revealed that less than 2% of the human genome contains protein-coding genes, and the vast majority of genes give rise to noncoding RNAs (ncRNAs) (Li et al., 2018; He et al., 2019; Wei et al., 2019). ncRNAs, originally considered transcriptional noise (Yang X. et al., 2020; Wu et al., 2020), are considered essential regulators of gene expression; for example, they regulate transcription, mRNA stability, and mRNA translation (Fabian et al., 2010; Chen and Huang, 2018; Panni et al., 2020). There are multiple types of ncRNAs, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (Luo et al., 2017; Liu X. et al., 2019).

lncRNAs, typically longer than 200 nucleotides in length (Xu H. et al., 2020), lack an open reading frame of significant length (Liu X. et al., 2018; Li Y. et al., 2019; Parolia et al., 2019). Emerging evidence suggests that lncRNAs are key regulators of gene expression levels, posttranscriptional modifications, and binding to transcription factors or miRNAs (Zhao et al., 2019; Guo et al., 2021). lncRNAs are abnormally expressed in a plethora of diseases (Liu S. et al., 2019; Huang L. et al., 2019; Chen et al., 2019; Shen et al., 2019; Xing et al., 2019; Radhakrishnan and Kowluru, 2021). lncRNAs are correlated with a variety of clinical characteristics and are considered indispensable regulators of many cell activities, including cell proliferation, migration, invasion, and apoptosis (Yang et al., 2019; Li B. et al., 2021; Lei et al., 2021). lncRNAs play important roles in the occurrence and development of human diseases. These ideas provide new perspectives on the diagnosis and treatment of human diseases (Li L. et al., 2021; Ma et al., 2021; Morgan et al., 2021). Long intergenic non-protein-coding RNA 346 (LINC00346), a novel lncRNA, is encoded on chromosome 13q34. Several studies have revealed that LINC00346 is abnormally expressed in a variety of diseases and that aberrant LINC00346 expression is associated with many clinical features. LINC00346 has been found to

**TABLE 1 |** The expression and clinical features of LINC00346 in disease.

Type	Expression	Feature	Refs
Epilepsy	—	Vitamin D level	Mazdeh et al. (2019)
Schizophrenia	Upregulated	Sex difference	Ghafouri-Fard et al. (2021)
Gastric cancer	Upregulated	Tumor size, pathologic stage, and disease-free survival	Xu et al. (2019)
Colorectal cancer	—	Lymphoma metastasis, histological grade, and TNM stage	Li et al. (2020b)
Hepatocellular carcinoma	Upregulated	—	Jin et al. (2020)
Hepatocellular carcinoma	Upregulated	—	Zhang and Chen, (2020)
Hepatocellular carcinoma	Upregulated	—	Yin et al. (2020)
Pancreatic cancer	Upregulated	Disease-free survival and overall survival	Peng et al. (2019)
Pancreatic cancer	Upregulated	—	Shi et al. (2019)
Pancreatic cancer	Upregulated	Overall survival	Zhang et al. (2018)
Glioma	—	Overall survival	Geng et al. (2020)
Glioma	Upregulated	Disease-free survival and overall survival	Chen et al. (2020b)
Glioma	Upregulated	—	Yang et al. (2020a)
Lung cancer	Upregulated	Sex difference	Gheliji et al. (2020)
Lung cancer	Upregulated	—	Wang et al. (2017)
Lung cancer	—	Overall survival	Wang et al. (2021c)
Breast cancer	Upregulated	—	Li et al. (2020d)
Breast cancer	—	Overall survival	Liu et al. (2016)
Nasopharyngeal carcinoma	Upregulated	Overall survival and recurrence-free survival	Cui et al. (2020)
Cutaneous squamous cell carcinoma	Upregulated	—	Piipponen et al. (2020)

regulate specific cellular functions by interacting with several molecular and signaling pathways. LINC00346 has also been identified as a potential biomarker in the diagnosis, prognostication, and treatment of diseases. In this review, we summarize current evidence concerning the expression, clinical characteristics, functions, and related mechanisms of LINC00346 in the occurrence and development of diseases. We also discuss the potential clinical utility of LINC00346, thereby providing new insight into the diagnosis and treatment of diseases.

## ROLE OF LINC00346 IN DISEASE

### Expression of LINC00346 in Disease

Increasing evidence has revealed that the expression level of LINC00346 is significantly upregulated in schizophrenia (Ghafouri-Fard et al., 2021), nasopharyngeal carcinoma (Cui et al., 2020), lung cancer (Wang et al., 2017), hepatocellular carcinoma (HCC) (Jin et al., 2020; Yin et al., 2020; Zhang and Chen, 2020), glioma (Yang C. et al., 2020; Chen X. et al., 2020), colorectal cancer (CRC) (Tong et al., 2020), cutaneous squamous cell carcinoma (Piipponen et al., 2020), breast cancer (Li et al., 2020d), gastric cancer, and pancreatic cancer (Table 1). LINC00346 may have a pathogenic role in disease progression. Interestingly, Gheliji et al. (2020) found that LINC00346 expression was decreased in lung cancer tissues compared with adjacent normal tissues. The expression level of LINC00346 needs to be further explored in lung cancer.

### LINC00346 and Clinical Characteristics

Some research groups have reported a potential relationship between LINC00346 expression and clinicopathological features (Table 1). The expression of LINC00346 has the potential to indicate the prognosis of numerous diseases, such as nasopharyngeal carcinoma (Cui et al., 2020), lung cancer (Wang et al., 2017), glioma (Yang C.

et al., 2020; Chen X. et al., 2020), breast cancer (Li et al., 2020d), and pancreatic cancer (Zhang et al., 2018; Peng et al., 2019; Shi et al., 2019). In addition, LINC00346 expression was found to be strongly correlated with metastasis, histological grade, and TNM stage in (Li T. et al., 2020) CRC. An increased LINC00346 level predicted larger tumor size and poorer pathologic stage in gastric cancer (Xu et al., 2019). The level of LINC00346 was relatively correlated with sex in lung cancer and schizophrenia patients (Gheliji et al., 2020; Ghafouri-Fard et al., 2021). The LINC00346 expression level was negatively associated with the vitamin D level in epileptic patients (Mazdeh et al., 2019).

### Functional Roles of LINC00346 in Disease

A growing amount of evidence has shown that lncRNAs play an important role in human disease (Zheng et al., 2019; Yu et al., 2020; Luo et al., 2021). LINC00346 exerts a vital role in the development of the disease by regulating various cellular functions. The regulatory functions of LINC00346 are related to cell proliferation, migration, invasion, and apoptosis. In this section, we summarize the current findings on the functions of LINC00346 (Table 2).

#### The Role of LINC00346 in Cellular Growth

Malignant diseases are often caused by unregulated cell growth (Huang et al., 2016; Emmanuel et al., 2018; Tan et al., 2019). Controlling cell growth is critical for the treatment of some diseases. The upregulation of LINC00346 has been found to promote cell proliferation and inhibit cell apoptosis in many diseases. Silencing LINC00346 has been found to obviously inhibit cell proliferation and promote cell apoptosis in bladder cancer (Ye et al., 2017), lung cancer (Wang et al., 2017; Xu et al., 2021), (Jin et al., 2020; Yin et al., 2020) HCC, glioma (Chen X. et al., 2020), (Li T. et al., 2020) CRC, and breast cancer (Li et al., 2020d). Additionally, several studies have revealed that elevated LINC00346 expression enhances cell proliferation in gastric cancer (Xu et al.,

**TABLE 2 |** The functions and mechanisms of LINC00346 in disease.

Type	Role	Function	Related genes	Refs
Schizophrenia	Oncogene	—	—	Ghafouri-Fard et al. (2021)
Atherosclerosis	—	Inflammatory factors and functional injury	miR-148a-3p, HUVECs, and KLF5	Wang et al. (2021a)
Gastric cancer	Oncogene	Cell proliferation, cell migration, cell invasion, and cell cycle	KLF5, MYC and miR-34a-5p	Xu et al. (2019)
Colorectal cancer	—	Cell proliferation, apoptosis, cell migration, and cell invasion	miR-148b	Li et al. (2020b)
Colorectal cancer	—	Cancer stemness properties	miR-509-5p and WBSR22	Zhao et al. (2020)
Hepatocellular carcinoma	Oncogene	Apoptosis, cell migration, and cell cycle	miR-199a-3p, CDK1, CCNB1, and p53	Jin et al. (2020)
Hepatocellular carcinoma	Oncogene	Cell proliferation, cell migration, cell invasion	miR-542-3p, WDR18, Wnt/ $\beta$ -catenin pathway, and MYC	Zhang and Chen, (2020)
Hepatocellular carcinoma	Oncogene	Cell proliferation, apoptosis, cell migration, and cell invasion	JAK and STAT3	Yin et al. (2020)
Pancreatic cancer	Oncogene	Cell proliferation, cell migration, cell invasion	C-Myc and CTCF	Peng et al. (2019)
Pancreatic cancer	Oncogene	Cell proliferation, cell cycle, chemoresistance	miR-188-3p and BRD4	Shi et al. (2019)
Pancreatic cancer	Oncogene	Cell proliferation	—	Zhang et al. (2018)
Glioma	—	—	miR-128-3p and SZRD1	Geng et al. (2020)
Glioma	oncogene	Cell proliferation, apoptosis, cell migration, and cell invasion	miR-340-5p and ROCK1	Chen et al. (2020b)
Glioma	Oncogene	Angiogenesis	ANKHD1, LINC00346, and ZNF655	Yang et al. (2020a)
Lung cancer	Tumor suppressor	—	—	Ghelji et al. (2020)
Lung cancer	Oncogene	Cell proliferation, apoptosis, and cell cycle	JAK and STAT3	Wang et al. (2017)
Lung cancer	—	Cell proliferation, apoptosis, cell migration, cell invasion, and cell cycle	miR-30c-2-3 and MYBL2	Xu et al. (2021)
Lung cancer	—	Chemoresistance	—	Wang et al. (2021c)
Breast cancer	Oncogene	Cell proliferation, apoptosis, and glycolysis	miR-148a/b and GLUT1	Li et al. (2020d)
Breast cancer	—	—	—	Liu et al. (2016)
Bladder cancer	—	Cell proliferation, apoptosis, cell migration, and cell cycle	—	Ye et al. (2017)
Nasopharyngeal carcinoma	Oncogene	Chemoresistance	miR-342-5p	Cui et al. (2020)
Cutaneous squamous cell carcinoma	Oncogene	Cell invasion	STAT3 and MMP	Piipponen et al. (2020)

2019) and pancreatic cancer (Zhang et al., 2018; Peng et al., 2019; Shi et al., 2019). Cell cycle arrest induced by LINC00346 has been observed in bladder cancer (Ye et al., 2017), lung cancer (Wang et al., 2017; Xu et al., 2021), (Jin et al., 2020) HCC, gastric cancer (Xu et al., 2019), and pancreatic cancer (Shi et al., 2019). Knockdown of LINC00346 facilitates G1/G0 cell cycle arrest in bladder cancer (Ye et al., 2017), non-small-cell lung cancer (Wang et al., 2017; Xu et al., 2021), and (Jin et al., 2020)HCC. In gastric cancer, increased LINC00346 levels suppress cell cycle arrest at the G1–S phase (Xu et al., 2019) transition. The cell cycle is blocked in the G2/M phase with upregulated LINC00346 expression in pancreatic cancer (Shi et al., 2019) and lung adenocarcinoma (Xu et al., 2021).

### The Role of LINC00346 in Cell Motility

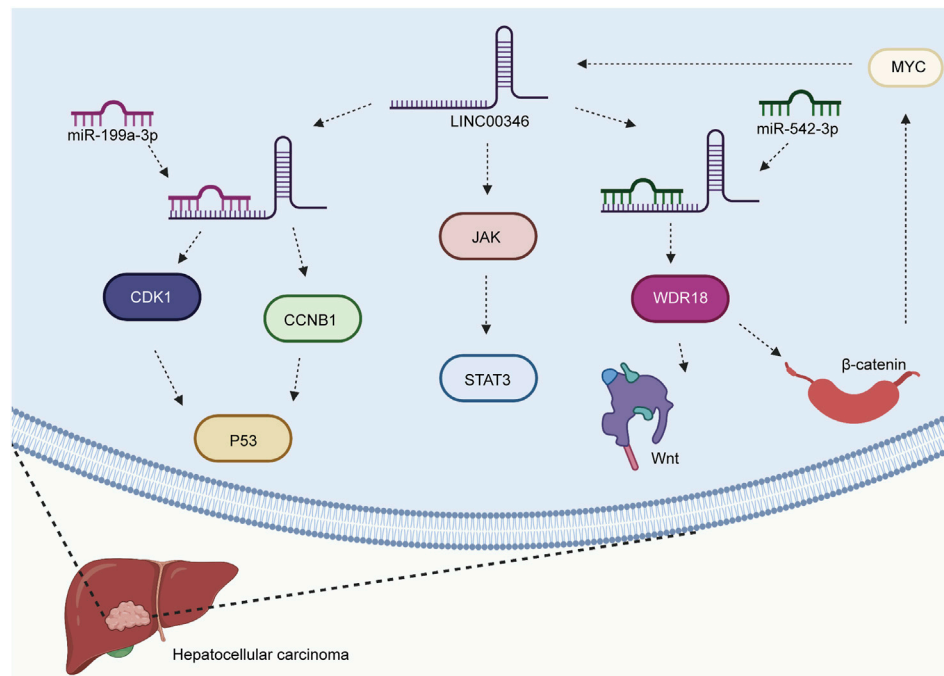
Metastasis to adjacent and distant sites, indicating poor prognosis, is a sign of malignant disease (Chakraborty et al., 2008; Okugawa et al., 2014; Lin et al., 2020). Cell motility is a highly regulated mechanical process important in wound healing, metastasis, and embryogenesis (Selmeczi et al., 2005; Yip et al., 2007; Mejean et al., 2009). Increased expression of LINC00346 has been found to promote tumor cell metastasis in bladder cancer (Ye et al., 2017), lung cancer (Xu et al., 2021), (Jin et al., 2020; Yin et al., 2020; Zhang and Chen, 2020) HCC, glioma (Chen X. et al., 2020), (Li T. et al., 2020; Zhao et al., 2020) CRC, cutaneous squamous cell carcinoma (Piipponen et al., 2020),

gastric cancer (Xu et al., 2019), and pancreatic cancer (Peng et al., 2019). LINC00346 significantly enhances cell migration and invasion in lung adenocarcinoma (Xu et al., 2021), (Jin et al., 2020; Yin et al., 2020; Zhang and Chen, 2020) HCC, glioma (Chen X. et al., 2020), (Li T. et al., 2020; Zhao et al., 2020)CRC, gastric cancer (Xu et al., 2019), and pancreatic cancer (Peng et al., 2019). LINC00346 also promotes cell migration in bladder cancer (Ye et al., 2017) and cell invasion in cutaneous squamous cell carcinoma (Piipponen et al., 2020).

### The Role of LINC00346 in Drug Resistance

The increasing frequency of DR has prompted substantial research interest (Shehata, 2005; Shi and Gao, 2016; Carné Trécesson et al., 2017). DR remains a significant obstacle in the treatment of cancer. It is important to investigate the underlying mechanisms of chemoresistance for cancer treatment. The expression of LINC00346 was found to be markedly correlated with DR in nasopharyngeal carcinoma (Cui et al., 2020) and pancreatic cancer (Shi et al., 2019). Knockdown of LINC00346 enhanced the sensitivity to cisplatin in nasopharyngeal carcinoma cell lines (Cui et al., 2020). Silencing LINC00346 attenuated the gemcitabine tolerance in pancreatic cancer cell lines (Shi et al., 2019). Monitoring and modulating LINC00346 expression may be potential therapeutic strategies for guiding clinical treatment.





**FIGURE 1 |** Mechanisms of LINC00346 in HCC (HCC). LINC00346 upregulates the expression levels of CDK1 and CCNB1 by sponging miR-199a-3p in HCC. It attenuates cell invasion and apoptosis and to regulates the cell cycle by regulating p53 and the miR-199a-3p/CDK1/CCNB1 axis. LINC00346 facilitates WDR18 expression and activates the Wnt/β-catenin pathway by acting as a sponge of miR-542-3p in HCC. β-catenin and LINC00346 form a positive feedback loop by interacting with MYC. Finally, LINC00346 affects cell proliferation and survival by activating the JAK-STAT3 signaling pathway.

## The Role of LINC00346 in Other Functions

Inflammation is considered a key factor in the pathophysiology of atherosclerosis (Sivapalaratnam et al., 2011; Paramel et al., 2020; Good et al., 2021). The upregulation of LINC00346 promoted inflammatory factor expression and functional injury in human umbilical vein endothelial cells (HUVECs) stimulated by OX-LDL (Wang F. et al., 2021). LINC00346 facilitated angiogenesis of glioma-associated endothelial cells (GECs), and *in vitro* LINC00346 knockdown experiments further verified this result (Yang C. et al., 2020). Glycolysis is a vital feature of tumor cells (Wang et al., 2013; Deng et al., 2020; Zhang et al., 2021). Inhibition of glycolysis is a promising therapeutic strategy for inhibiting tumors (Lee et al., 2017; Du et al., 2020). Increased LINC00346 levels were found to significantly promote glycolysis ability in breast cancer cell lines (Li et al., 2020d). In CRC, LINC00346 was found to regulate cancer stemness properties *in vitro* (Zhao et al., 2020).

## LINC00346 Regulatory Mechanisms in Disease

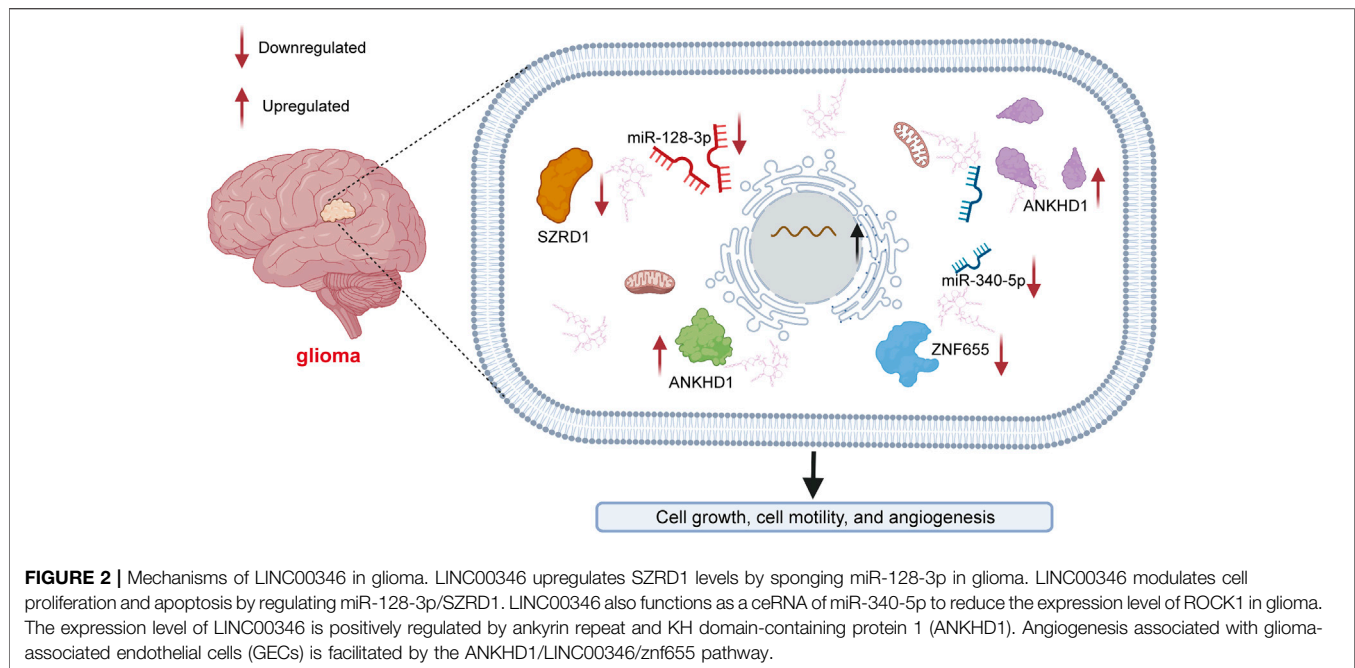
### Mechanisms of LINC00346 in Tumors

#### Mechanisms of LINC00346 in Digestive System Tumors

HCC is the most common type of liver cancer and has increasing mortality worldwide (Gomha et al., 2015; Shen et al., 2018; Yapasert et al., 2020). The underlying signaling mechanisms of HCC progression are poorly defined. A growing amount of evidence has shown that LINC00346 is significantly associated

with the progression of HCC. Upregulation of LINC00346 was found to promote cancer progression by regulating various biological functions in HCC. LINC00346 promoted the expression levels of CDK1 and CCNB1 by acting as a sponge of miR-199a-3p in HCC (Jin et al., 2020) (**Figure 1**). In addition, LINC00346 inhibited cell invasion and apoptosis and controlled the cell cycle by regulating p53 and the miR-199a-3p/CDK1/CCNB1 axis. LINC00346 facilitated WDR18 expression and the Wnt/β-catenin pathway by sponging miR-542-3p in HCC (Zhang and Chen, 2020). Researchers also observed that β-catenin and LINC00346 form a positive feedback loop by interacting with MYC. LINC00346 was also found to affect cell proliferation and survival by regulating the JAK-STAT3 signaling pathway (Yin et al., 2020).

Pancreatic carcinoma is one of the most malignant tumors and has an extremely poor prognosis (Xu D. et al., 2020; Wang et al., 2020; Chen et al., 2021). Chemotherapy is an important method of adjuvant therapy in the comprehensive treatment of pancreatic cancer (Rahman et al., 2017; Zhu et al., 2019). Therefore, the chemoresistance and pathogenesis of pancreatic cancer urgently need to be explored. LINC00346 was found to facilitate the transcription and expression of c-Myc by interacting with CTCF in pancreatic cancer (Peng et al., 2019). LINC00346, acts as a sponge of miR-188-3p and downregulated the level of BRD4 to increase gemcitabine resistance in pancreatic cancer (Shi et al., 2019). In CRC, LINC00346 promotes cell migration and invasion by reducing miR-148b levels (Li T. et al., 2020). LINC00346 also regulates the biological functions of CRC stem



cells by activating the Linc00346/miR-509-5p/wbscr22 pathway (Zhao et al., 2020). LINC00346 suppresses miR-34a-5p expression to affect CD44, Notch1, and AXL expression in gastric cancer (Xu et al., 2019). In addition, the expression of LINC00346 was found to be markedly upregulated by KLF5 and MYC in gastric cancer.

### Mechanisms of LINC00346 in Central Nervous System Tumors

Gliomas are the most common type of tumor of the central nervous system (Ceresa et al., 2019; Liu Y. et al., 2019; Stępnik et al., 2021).

Gliomas are the most aggressive type of brain tumor and have an extremely poor prognosis (Seliger and Hau, 2018; Chen W. et al., 2020). Glioma can be divided into astrocytoma, glioblastoma multiforme (GBM), oligodendroglioma and mixed tumors (Peng et al., 2018). Geng et al. (2020) found that LINC00346 inhibited the expression of miR-128-3p to upregulate SZRD1 levels in glioma (Figure 2). LINC00346 was found to affect cell proliferation and apoptosis through the regulation of miR-128-3p/SZRD1. LINC00346 was also found to act as a ceRNA (competing endogenous RNA) of miR-340-5p to suppress the expression of ROCK1 in glioma progression (Chen X. et al., 2020). Exploring the potential angiogenesis mechanisms is essential for the development of novel strategies for glioma treatment. LncRNAs play an essential role in tumor angiogenesis (Ruan et al., 2019; Cheng et al., 2020; Wang X. et al., 2021). In glioma, the expression level of LINC00346 was found to be positively regulated by ankyrin repeat and KH domain-containing protein 1 (Yang C. et al., 2020) (ANKHD1). The activation of ANKHD1/LINC00346/znf655 was found to facilitate angiogenesis in association with glioma-associated endothelial cells (GECs).

### Mechanisms of LINC00346 in Tumors of Other Systems

Lung cancer is the leading cause of cancer-related deaths worldwide (Liu J. et al., 2018; Harikrishnan et al., 2020; To et al., 2021). It can be divided into small-cell lung cancer and non-small-cell lung cancer (NSCLC) (Du et al., 2019; Li H. et al., 2020). LINC00346 was found to facilitate NSCLC progression via regulation of the JAK-STAT3 signaling pathway (Wang et al., 2017). LINC00346 promotes the expression of MYBL2 to regulate the cell cycle by acting as a sponge of miR-30c-2-3p in lung adenocarcinoma (Xu et al., 2021). Nasopharyngeal carcinoma (NPC) is a head and neck malignancy with a high incidence (Sun and Xu, 2015; Yao et al., 2020). Chemoresistance remains an obstacle in the treatment of NPC. LINC00346 attenuates cisplatin sensitivity by sponging miR-342-5p in NPC (Cui et al., 2020). In breast cancer, the expression of LINC00346 upregulates glucose transporter 1 levels by targeting miR-148a/b (Li et al., 2020d). Cutaneous squamous cell carcinoma is the second most frequent malignant skin cancer, and the incidence is increasing. LINC00346 increases the expression of matrix metalloproteinase by activating STAT3 signaling in cutaneous squamous cell carcinoma (Piipponen et al., 2020).

### Mechanisms of LINC00346 in Nontumor Disease

Atherosclerosis is a major cause of multiple diseases, such as coronary artery disease (CHD) (Martinus and Goldsbury, 2018; Xu et al., 2018), peripheral artery disease (PAD) (Biscetti et al., 2019), and atherosclerotic cerebrovascular disease (Sascău et al., 2021). However, the pathogenesis of atherosclerosis remains unclear. There is an urgent need to explore the mechanism of atherosclerosis. The level of LINC00346 was found to be negatively correlated with Krüppel-like factor 5 (KLF5) expression in atherosclerosis (Wang F. et al., 2021). Knockdown of LINC00346 inhibited inflammatory reactions and functional injury in the progression of atherosclerosis.

LINC00346 was found to affect the initiation and development of atherosclerosis by regulating the KLF5/LINC00346/miR-148a-3p pathway (Wang F. et al., 2021).

## Clinical Utility of LINC00346 in Disease

Despite improved technology and advances in modern medicine, malignant disease, especially cancer, remains one of the leading causes of death. Abnormal cell growth, metastasis, and drug assistance result in poor disease prognoses. Early diagnosis and targeted treatment are important for improving the prognosis of disease. LncRNAs may be potential diagnostic biomarkers and therapeutic targets. In this section, we will further discuss the potential clinical utility of LINC00346 in the diagnosis, prognostication, and treatment of diseases.

### LINC00346 as a Diagnostic Biomarker

The detection and diagnosis of disease are essential for disease management (Li et al., 2020c; Álvarez et al., 2020; Adhikari et al., 2021). Increasing evidence has revealed that lncRNAs are potential diagnostic biomarkers for several diseases (Wang et al., 2016; Jin et al., 2017; Huang G.-z. et al., 2019; Cheng et al., 2019). LINC00346 has found to be obviously upregulated in many tumors and nontumor diseases. Gheliji et al. (2020) observed that LINC00346 expression was downregulated in lung cancer tissues. The level of LINC00346 in lung cancer needs to be further explored. The sensitivity was 83.3%, and the specificity was 52.4% in an analysis of the ability to distinguish between lung cancer tissues and adjacent tissues (Gheliji et al., 2020). Importantly, the expression of LINC00346 in venous blood was found to be markedly upregulated in patients with schizophrenia or pancreatic cancer (Zhang et al., 2018; Ghafouri-Fard et al., 2021). LINC00346 has great value in the diagnosis of schizophrenia (Ghafouri-Fard et al., 2021) and pancreatic cancer (Zhang et al., 2018). Substances that are stably expressed in body fluids are more likely to be used as biomarkers in disease diagnosis.

### LINC00346 as a Prognostic Biomarker

Individualized therapy requires the identification of biomarkers to predict patient prognosis (Li Z. et al., 2019; Melling et al., 2019). The expression of LINC00346 was found to be significantly correlated with the poor prognosis of nasopharyngeal carcinoma (Cui et al., 2020), lung cancer (Wang et al., 2017), glioma (Yang C. et al., 2020; Chen X. et al., 2020), breast cancer (Li et al., 2020d), and pancreatic cancer (Zhang et al., 2018; Peng et al., 2019; Shi et al., 2019). The level of LINC00346 was found to be negatively associated with overall survival in nasopharyngeal carcinoma (Cui et al., 2020), lung adenocarcinoma (Wang Z. et al., 2021), glioma (Chen X. et al., 2020; Geng et al., 2020), breast cancer (Liu et al., 2016), and pancreatic cancer (Zhang et al., 2018; Peng et al., 2019). LINC00346 was also found to affect disease-free survival in patients with gastric cancer (Xu et al., 2019) and pancreatic cancer (Peng et al., 2019). The upregulation of LINC00346 was found to be correlated with shorter recurrence-free survival in nasopharyngeal carcinoma (Cui et al., 2020).

### LINC00346 as a Biomarker of Targeted Therapy

Molecular targeted therapy shows advantages for many diseases, especially malignancies (Baba et al., 2019; Becker et al., 2020; Xu P.

et al., 2020). LncRNAs contribute to disease progression through the regulation of cellular pathways (Li Z.-W. et al., 2020; Sun and Wu, 2020). They serve as an important therapeutic targets in the treatment of diseases. In **Section 2.4**, we introduced the mechanisms of LINC00346 in tumor and nontumor diseases. LINC00346 is a meaningful therapeutic biomarker in disease treatment. It is also considered an oncogene that contributes to tumorigenesis. Knockdown or silencing of LINC00346 can inhibit cell biological functions to suppress cancer progression in several cancers, such as bladder cancer (Ye et al., 2017), lung cancer (Wang et al., 2017), and glioma (Chen X. et al., 2020; Geng et al., 2020). The association of LINC00346 and chemoresistance has implications for treatment.

## CONCLUSIONS AND FUTURE PERSPECTIVES

LINC00346, a novel lncRNA, is encoded on chromosome 13q34. It is significantly upregulated in many diseases. However, some researchers have found that the level of LINC00346 is reduced in lung cancer. Therefore, the expression of LINC00346 in lung cancer needs to be further explored. Importantly, the expression of LINC00346 in venous blood was found to be elevated in patients with schizophrenia or pancreatic cancer. This finding is crucial for the successful clinical application of LINC00346. Substances stably expressed in bodily fluids have strong potential in the diagnosis of disease. The expression level of LINC00346 was found to be obviously correlated with prognosis, lymphoma metastasis, histological grade, TNM stage, tumor size and pathologic stage. LINC00346 expression has important guiding significance in the management of patients. Different strategies can be used for different patients if the prognosis can be accurately predicted. LINC00346 exerts a vital role by regulating cellular growth, cell motility, chemoresistance, and other functions in diseases. LINC00346 affects these biological functions by interacting with several pathways. Knockdown or silencing of LINC00346 inhibits the progression of several cancers. In conclusion, LINC00346 is a potential biomarker in the diagnosis, prognostication, and treatment of diseases. In terms of clinical applications, further basic experiments and multicenter research data are needed.

## AUTHOR CONTRIBUTIONS

LL and JL designed and guided the study. JL and ZX wrote and edited the manuscript. MX helped with reference collection. All authors read and approved the final manuscript.

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# Non-Coding RNAs Regulate the Resistance to Anti-EGFR Therapy in Colorectal Cancer

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Colorectal cancer (CRC) is the third prevalent cancer worldwide, the morbidity and mortality of which have been increasing in recent years. As molecular targeting agents, anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (McAbs) have significantly increased the progression-free survival (PFS) and overall survival (OS) of metastatic CRC (mCRC) patients. Nevertheless, most patients are eventually resistant to anti-EGFR McAbs. With the intensive study of the mechanism of anti-EGFR drug resistance, a variety of biomarkers and pathways have been found to participate in CRC resistance to anti-EGFR therapy. More and more studies have implicated non-coding RNAs (ncRNAs) primarily including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), are widely involved in tumorigenesis and tumor progression. They function as essential regulators controlling the expression and function of oncogenes. Increasing data have shown ncRNAs affect the resistance of molecular targeted drugs in CRC including anti-EGFR McAbs. In this paper, we have reviewed the advance in mechanisms of ncRNAs in regulating anti-EGFR McAbs therapy resistance in CRC. It provides insight into exploring ncRNAs as new molecular targets and prognostic markers for CRC.

**Keywords:** miRNA, lncRNA, circRNA, CRC, EGFR, drug resistance

## INTRODUCTION

CRC is the third most frequent cancer worldwide. Global cancer statistics in 2020 has shown there are about 1.932 million new cases and 935,000 deaths of CRC worldwide, accounting for 10.0% of the total new cases of cancer and 9.4% of the total cancer-related deaths, respectively (1). Following lung cancer, CRC causes the second highest mortality in cancer patients worldwide (1). The therapeutic strategies for CRC mainly include surgery, chemotherapy, radiotherapy and targeted therapy. Currently, surgery and chemotherapy are still the preferred treatment options for CRC. Nevertheless, patients with metastatic CRC (mCRC) have a poor prognosis (2). The combined chemotherapy and molecular targeted drugs can noticeably increase the progression-free survival (PFS) and overall survival (OS) of mCRC patients (3). As molecular targeted drugs, cetuximab and panitumumab can directly target epidermal growth factor receptor (EGFR). Combined with chemotherapeutic drugs, they are applied to effectively treat

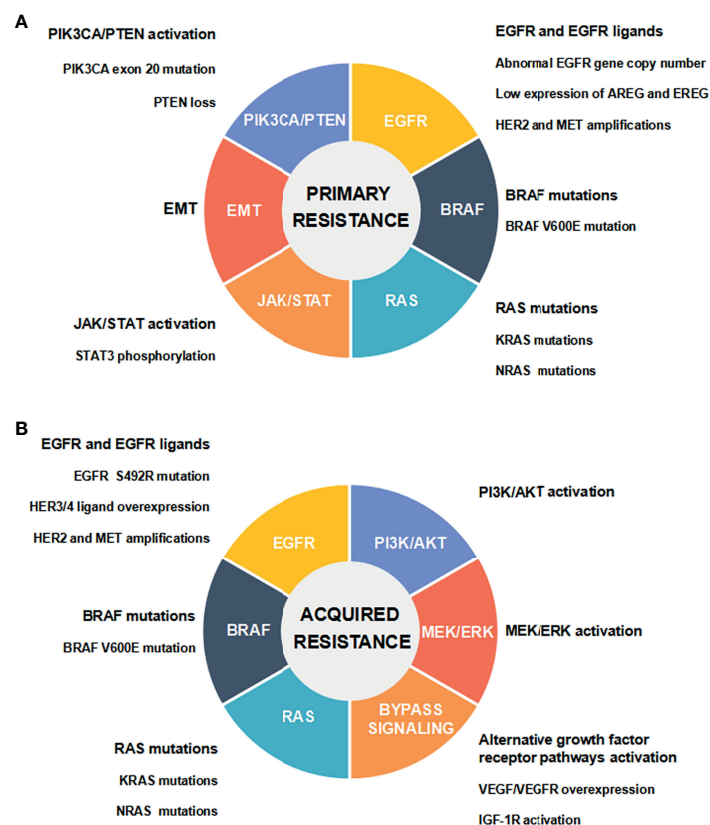


mCRC patients carrying wild-type *RAS* and *BRAF* (4). Unfortunately, few patients with mCRC are sensitive to anti-EGFR treatment, and most responding patients usually develop resistance to the therapy (5). In recent years, a variety of biomarkers and pathways have been found to participate in regulating the resistance to anti-EGFR therapy, and thus affecting the therapeutic effect and reducing the survival rate of CRC patients (6). Some studies have suggested the potential resistance mechanisms in order to explore strategies for overcoming anti-EGFR resistance (5, 7, 8) (**Figure 1**).

EGFR is a kind of HER tyrosine kinase receptor, which is composed of extracellular ligand binding domain, transmembrane hydrophobic domain, and intracellular tyrosine kinase domain. EGFR is selectively activated by binding to epidermal growth factor (EGF) as one of the major ligands. EGFR transmits signals from cytoplasm to nucleus through RAS/RAF/MEK/ERK/MAPK, PI3K/PTEN/AKT/mTOR, and some other intracellular signaling pathways which participate in regulating cancer cell proliferation, invasion, and angiogenesis (9). Abnormal expression and activation of any signal molecules mentioned above may lead to primary (*de novo*) or acquired (secondary) resistance to anti-EGFR therapy in mCRC (5). Abnormal EGFR gene copy number, protein expression of EGFR ligands, HER2 and MET gene amplifications, and activation of EGFR downstream cascade signaling pathways [including the mutations of RAS/BRAF/PIK3CA, the loss of

PTEN, STAT3 phosphorylation, and epithelial-mesenchymal transition (EMT)], have been demonstrated to be associated with the primary resistance to anti-EGFR therapy in CRC (5, 7, 8). It has been well documented that the acquired resistance is attributed to EGFR ectodomain mutation (S492R), genetic alterations in RAS/RAF and other downstream signaling molecules, and the activation of intracellular signaling pathways that are bypassing EGFR and mediated by IGF1R, HER2, MET, and VEGFR (5, 7, 8). Multiple genetic and nongenetic mechanisms drive resistance to anti-EGFR therapy in CRC, with a significant overlap in primary and acquired resistance (8) (**Figure 1**).

NcRNAs are a type of RNAs which have no protein-coding function. According to the length, they are divided into two classes: small non-coding RNAs (sncRNAs) with a length of 18–200 nt, and long noncoding RNAs (lncRNAs) with a length over 200 nt. NcRNAs are widely involved in cell proliferation, apoptosis, autophagy, EMT, and cell cycle progression (10–14). Accumulated studies have suggested ncRNAs play important roles in tumorigenesis, progression, and anti-EGFR monoclonal antibodies (McAbs) treatment resistance in CRC (15–21). In this review, we have focused on current progress in the underlying molecular mechanisms of ncRNAs in regulating the resistance to anti-EGFR therapy in CRC. We aim to fully explore the potentials of ncRNAs as novel molecular targets and prognostic markers for CRC.



**FIGURE 1** | Mechanisms of anti-EGFR drug resistance in CRC. **(A)** Primary resistance mechanisms. **(B)** Acquired resistance mechanisms.

## MIRNAS

### Biological Functions of MiRNAs

MiRNAs are single-stranded small ncRNAs with a length of 21–25 nt. The synthesis of miRNAs involves multiple biological steps. Firstly, primary miRNAs (pri-miRNAs) are encoded by DNA in the nucleus and transcribed by ribonucleic acid polymerase II. Secondly, long pri-miRNAs are processed by ribonuclease III Drosha, which produces precursor miRNAs (pre-miRNAs) with a length of 60–70 nt. Lastly, pre-miRNAs are cleaved into mature double-stranded miRNAs by ribonuclease III Dicer in the cytoplasm. Then, mature miRNAs participate in forming RNA-induced silencing complex (RISC) (22). MiRNAs induce messenger RNA (mRNA) degradation and translation repression by directly binding to the 3'-untranslated region (3'-UTR) of targeted mRNAs, and act as regulators at the post-transcriptional level during gene expression process (23). They are widely involved in cell proliferation, apoptosis, autophagy, and immune response (10, 11, 18, 21). Accumulated studies have suggested miRNAs participate in the pathogenesis of various diseases including cancers (24–28). MiRNAs act either as oncogenic miRNAs (onco-miRs) or tumor suppressive miRNAs (TS-miRs) with significant tissue- and organ-specificity (29, 30). Many studies have also found that miRNAs participate in regulating the drug resistance in CRC (31, 32). It has been demonstrated miR-31 negatively regulates breast cancer invasion and metastasis (33). However, it negatively regulates the expression of tumor suppressors and thus exerts oncogenic effects in lung cancer (34). In CRC, miR-31 has been documented to promote cancer progression by activating RAS signaling pathway and hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), respectively (35, 36). Taken together, miR-31 is involved in tumor progression and metastasis by serving as a TS-miR or an onco-miR in different malignancies. The diverse roles of miR-31 in cancer may be attributed to different types of cancer cells,

specific targets, and other complicated factors. Further research is required to reveal its specific functions in CRC.

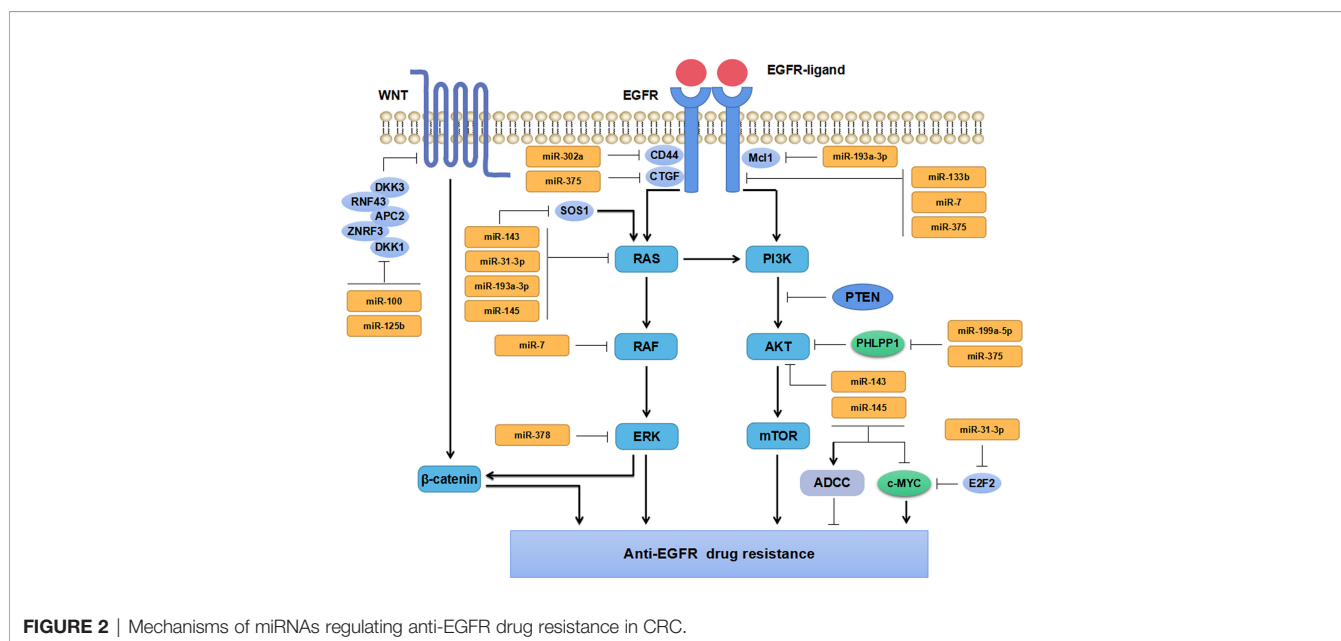
MiRNAs, aberrantly expressed in tumor tissues and tumor cells, exert their tumor suppressive- and oncogenic-functions by regulating different targeted genes (**Figure 2**). When the expression levels of TS-miRs decrease, negative regulation on targeted genes weakens. Besides, increasing expression levels of onco-miRs promotes tumor development, metastasis, and drug resistance through down-regulating tumor suppressive genes.

### MiRNAs Regulate Drug Resistance of Anti-EGFR Therapy in CRC

MiRNAs regulates anti-EGFR drug resistance by directly targeting tumor-related genes involved in EGFR-related signaling pathways in CRC. Abnormal expression of miRNAs is commonly observed in anti-EGFR treatment-resistant CRC cells. Recent studies have shown that miRNAs may predict the prognosis and drug therapeutic efficacy of CRC patients (37–39). The latest studies regarding miRNAs and drug resistance to anti-EGFR therapy in CRC have been described in the following subsections and briefly summarized in **Table 1**.

### Impact of MiRNAs on EGFR Signaling Pathway

EGFR signaling pathway has been confirmed to be aberrantly activated in multiple malignant tumors, which is associated with tumor progression and prognosis. Increasing evidence has implicated miRNAs participate in regulating EGFR signaling pathway and play vital roles in anti-EGFR drug resistance in CRC (**Figure 2**). Zhou et al. have found miR-133b regulated cell proliferation and invasion in CRC by targeting EGFR (40). Moreover, the combination of miR-133b mimics and cetuximab can effectively suppress the proliferation and invasion of cetuximab-resistant CRC cells (40). Suto et al. have found miR-7 is involved in regulating the EGFR signaling



**FIGURE 2** | Mechanisms of miRNAs regulating anti-EGFR drug resistance in CRC.

**TABLE 1 |** MiRNAs involved in anti-EGFR drugs resistance in CRC.

MiRNAs	Expression	Targets/Pathways	Drugs	References
MiR-133b	Down-regulated	EGFR pathway	Cetuximab	(40)
MiR-7	Down-regulated	EGFR/RAF pathway	Cetuximab	(41)
MiR-302a	Down-regulated	CD44/EGFR/RAS/MAPK pathway, CD44/EGFR/PI3K/AKT pathway	Cetuximab	(42)
MiR-143	Down-regulated	SOS1/RAS/ERK/MAPK pathway, AKT pathway	Cetuximab	(43)
		ADCC		(44)
		RAS-MAPK axis, c-MYC pathway		(45)
MiR-145	Down-regulated	ADCC	Cetuximab	(44)
		RAS-MAPK axis, c-MYC pathway		(45)
MiR-193a-3p	Down-regulated	KRAS/RAF/MEK/ERK pathway	Cetuximab	(46)
		Mcl1/EGFR/BRAF/MEK/MAPK pathway	Dabrafenib, Trametinib, Cetuximab	(47)
MiR-378	Down-regulated	ERK/MAPK pathway	Cetuximab	(48, 49)
MiR-31-3p	Up-regulated	RAS-MAPK axis, E2F2/c-MYC pathway	Cetuximab	(45)
MiR-100	Up-regulated	DKK1, ZNRF3/Wnt/ $\beta$ -catenin pathway	Cetuximab	(50)
MiR-125b	Up-regulated	ZNRF3, RNF43, DKK3, APC2/Wnt/ $\beta$ -catenin pathway	Cetuximab	(50)
MiR-199a-5p	Up-regulated	PHLPP1/AKT pathway	Cetuximab	(51)
MiR-375	Up-regulated	PHLPP1/AKT pathway	Cetuximab	(51)
	Down-regulated	CTGF/EGFR/PIK3CA/AKT pathway, EGFR/KRAS/BRAF/ERK1/2 pathway		(52)

pathway by down-regulating the expression of EGFR and RAF-1, which could inhibit CRC cells proliferation and reverse cetuximab resistance in CRC patients with mutant *KRAS* (41). Sun and the colleagues have found that miR-302a suppressed CRC metastasis by targeting nuclear factor I B (NFIB) and CD44 and decreasing the activation of NFIB/ITGA6 signaling pathway (42). MiR-302a has also been found to restore the response to cetuximab by inhibiting CD44-induced cancer stem cell (CSC)-like characteristics through EGFR-mediated MAPK and protein kinase B (AKT) signaling pathways (42). These studies have revealed that miRNAs can directly target EGFR (or RAF) in CRC cells, inhibit the activation of its downstream signaling pathways, and thus repress CRC cells growth and invasion. Besides, miR-100 and miR-125b have been found to cooperatively regulate the resistance to cetuximab in CRC through Wnt signaling pathway that has a cross-talk with EGFR pathway (50). MiRNAs are extensively involved in regulating the resistance to cetuximab. Accordingly, miRNAs might serve as markers for predicting anti-EGFR therapy in mCRC patients due to their regulatory effects on EGFR signaling pathway.

## Impact of MiRNAs on RAS Signaling Pathway

*KRAS*, a member of RAS family, has almost 40% mutation rate in CRC patients. *KRAS* mutations are predictive biomarkers for the treatment efficacy of anti-EGFR treatment and the outcome of patients with CRC (53). MiRNAs have been widely reported to regulate the therapeutic response and drug sensitivity of CRC patients through *KRAS* signaling pathway (43–45) (**Figure 2**).

Synthetic miR-143 (miR-143#12) inhibits *KRAS* signaling pathway activation and restores the sensitivity of cetuximab-resistant CRC cells by targeting the *KRAS* activating protein SOS1 (43). Overexpression of miR-143 or miR-145 can increase the sensitivity to cetuximab by enhancing cetuximab-mediated antibody-dependent cellular cytotoxicity (ADCC) in CRC cells (44). Strippoli et al. have demonstrated miR-31-3p, miR-143 and miR-145 are closely correlated with anti-EGFR treatment resistance in mCRC *via* regulating RAS-MAPK axis and c-MYC

pathway (45). Moreover, miR-143 and miR-145 have been well established to exert tumor-suppressive effects and are beneficial for the efficacy of anti-EGFR treatment in CRC, whereas miR-31-3p comes to the opposite. It has been shown that the overexpression of miR-193a-3p can promote *BRAF*-mutant CRC cells apoptosis by inhibiting the expression of *KRAS* and myeloid cell leukemia-1 (Mcl1) through MAPK signal (47). As a tumor suppressor, miR-193a-3p promotes the efficacy of *BRAF* inhibitor dabrafenib (DAB) and MEK inhibitor trametinib (TRA), and enhances the anti-proliferative effect of combined therapy of DAB, TRA with cetuximab in CRC (47). A recent study has shown that 4-acetyl-antroquinonol B (4-AAQB) inhibits CRC cell proliferation and induces cell apoptosis by up-regulating miR-193a-3p, down-regulating *KRAS* and inhibiting the activation of *KRAS* signaling pathway. The combined treatment of 4-AAQB with cetuximab can make *KRAS*-mutant CRC cells resensitized to cetuximab (46). In addition to *KRAS*, miR-193a-3p acts on multiple signaling pathways and plays a tumor-suppressive role by regulating the expression of interleukin 17 receptor D (IL17RD) and erb-b2 receptor tyrosine kinase 4 (ERBB4) in CRC (54, 55). And lower expression of miR-193a-3p in CRC tissues predicts poorer PFS independently of the status of *BRAF* mutation (56). Accordingly, miR-193a-3p may serve as a prognostic biomarker. Its combination with molecular targeted drugs may be a novel therapeutic strategy for *BRAF*-mutant CRC. Weng et al. have reported that lauric acid can induce miR-378 expression and increase the sensitivity of *BRAF*- and *KRAS*-mutant CRC cells to cetuximab by inhibiting *KRAS*, *BRAF*, MEK, ERK1/2 protein expressions through the MAPK signaling pathway (48). In addition, they have found that eicosapentaenoic acid ethyl ester (EPA) can also increase the expression of miR-378 in *BRAF*- and *KRAS*-mutant CRC cells and resensitize *KRAS*-mutant CRC cells to cetuximab (49). Taken together, miRNAs play vital roles in regulating the therapeutic response and drug sensitivity of *KRAS*- or *BRAF*-mutant CRC through RAS signaling pathway. Potential miRNAs and key molecules in the RAS signaling pathway may serve as promising biomarkers for predicting the efficacy and drug resistance during the targeted therapy in CRC.

## Impact of MiRNAs on PI3K/AKT Signaling Pathway

The PI3K/AKT signaling pathway is widely involved in carcinogenesis and cancer progression. Aberrant activation of PI3K-AKT can promote CRC invasion and metastasis (57). It has been reported that miRNAs can directly target the PI3K/AKT signaling molecules or signaling pathway regulators, including numerous regulatory proteins (51, 52, 57–60) (**Figure 2**). MiR-375 and miR-199a-5p promote cetuximab resistance in CRC patients by repressing the expression of PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) and positively regulating AKT signaling pathway (51). Nevertheless, some other studies have found miR-375 and miR-199a-5p inhibit CRC cells proliferation and invasion, suggesting their complicated functions in CRC (52, 58–60). It has been well documented that miR-375 suppressed CRC cell proliferation by targeting PIK3CA *via* the PI3K/AKT pathway (61), while miR-199a-5p inhibited CRC cell survival, proliferation, migration, and invasion by downregulating GCNT2 expression and inhibiting the AKT and ERK signal activation (62). Different roles of miR-375 and miR-199a-5p exerting in CRC, might be attributed to significant tumor heterogeneity among patients. Taken together, miRNAs regulate the progression and drug resistance of CRC by regulating tumor suppressors or oncogenes involved in various signaling pathways including PI3K/AKT signal. However, the precise mechanisms of miR-375 and miR-199a-5p underlying the resistance to anti-EGFR drugs in CRC warrant to be fully elucidated in more future research.

## Impact of MiRNAs on Tumor Immune Microenvironment

Tumor immune microenvironment is composed of a variety of cells, extracellular matrix and various signaling molecules (63). Imbalance of tumor immune microenvironment is essential for tumor growth, metastasis and prognosis (64). MiRNA-mediated regulation of tumor microenvironment (TME) has been demonstrated to affect cancer growth, angiogenesis, metastasis, and drug resistance exerting either antitumor or tumorigenic effects (65). For instance, a recent study has shown miR-34a promoted the expression of B7-H3 and TNF- $\alpha$  in tumor microenvironment and negatively regulated T cell-mediated immune response, which thus induced immunosuppression and immune escape in CRC (66). MiR-148a-3p and miR-448 respectively down-regulate the expression of calnexin (CANX) and indoleamine 2,3-dioxygenase 1 (IDO1), which enhances CD8<sup>+</sup> T cell-mediated immune response in CRC (67, 68).

Cetuximab can induce ADCC by binding to EGFR on cancer cells and CD16 receptor on natural killer (NK) cells and dendritic cells (DCs) (69–71). It stimulates the production of proinflammatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , and activates cytotoxic T cells in the TME, thereby exerting tumor immunosuppressive effects (69–71). It has been suggested that anti-EGFR therapy and immunotherapy have synergetic and complementary mechanisms. The combination of immune checkpoint inhibitors, chemotherapy with anti-EGFR McAbs in mCRC has shown an encouraging clinical outcome (72). Nevertheless, littler is known about the role of

miRNAs in regulating tumor immune microenvironment and thus affecting anti-EGFR drugs resistance in CRC.

## LNCRNAS

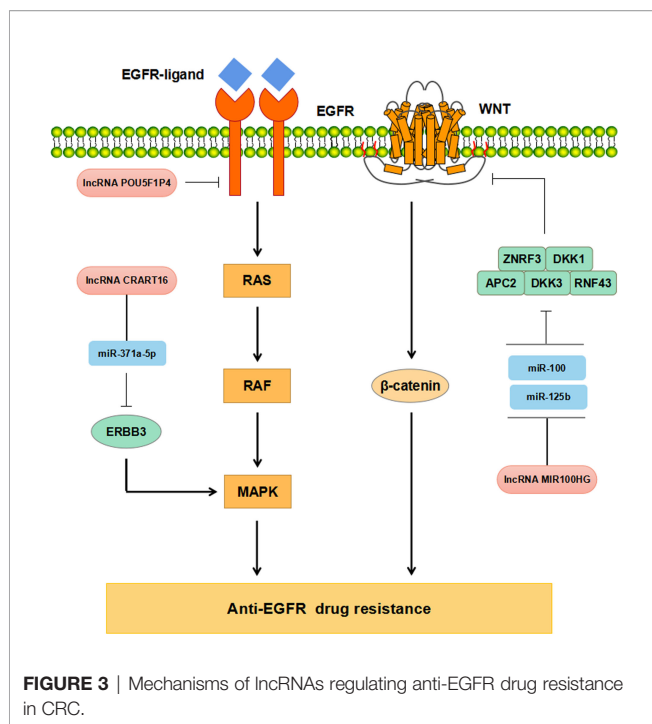
### Biological Functions of LncRNAs

LncRNAs are a type of ncRNAs over 200 nt in length. They are mainly formed by RNA polymerase II-catalyzed transcription typically containing a cleavable 3' poly-A tail (73). According to genomic localization, lncRNAs are grouped into five classes: sense lncRNA, antisense lncRNA, intronic lncRNA, bidirectional lncRNA, and intergenic lncRNA (74). LncRNAs have low sequence conservation and high tissue and organ specificity. As competitive endogenous RNAs (ceRNAs), lncRNAs can directly sponge miRNAs and inhibit their expression. LncRNAs interact with DNA, RNA and protein, acting as regulators of gene expression at multiple levels and play roles in various cell processes, such as genomic imprinting, epigenetic regulation, transcriptional regulation, chromosome conformation, and cell cycle regulation (75). A great deal of data has suggested lncRNAs participate in the pathogenesis of various diseases, including cancer (75–78). Linc00152, SNHG1, SCARNA2, DLEU1 and XIST contribute to colorectal carcinogenesis, metastasis and prognosis of CRC (54, 79–83). In addition, a number of studies have implicated lncRNAs lead to associated with primary or acquired drug resistance in CRC, thereby reducing drug efficacy (84, 85). Nonetheless, the regulatory mechanisms of lncRNAs underlying anti-EGFR therapy resistance in CRC are not clear yet.

### LncRNAs Regulate Drug Resistance of Anti-EGFR Therapy in CRC

Increasing evidence has supported that lncRNAs participate in regulating CRC resistance to anti-EGFR McAbs through multiple signaling pathways (**Figure 3**). The study by Peng et al. has found that down-regulation of POU5F1P4 in cetuximab-sensitive CRC cells can reduce their sensitivity in mCRC (86). LNC00973 and several other lncRNAs may be involved in cetuximab resistance by regulating glucose metabolism (87). Down-regulation of LNC00973 can improve cetuximab resistance in drug-resistant CRC cells (87). Lu et al. have elaborated that the overexpression of lncRNA MIR100HG-derived miR-100 and miR-125b promotes cetuximab resistance through Wnt/ $\beta$ -catenin pathway in CRC (50) (**Tables 1, 2**). Recent studies have reported that lncRNA CRART16 is up-regulated in CRC cells with secondary cetuximab-resistance. CRART16 contributes to cetuximab resistance in CRC by up-regulating ERBB3 through miR-371a-5p/MAPK signaling pathway (88). LncRNA HCG18 promotes cell proliferation, migration, and cetuximab resistance in CRC by up-regulating PD-L1 and down-regulating CD8<sup>+</sup> T lymphocytes *via* sponging miR-20b-5p (89). Besides, the study by Yang et al. has shown the evidence that up-regulation of UCA1 in cetuximab-resistant CRC cells and the produced exosomes (90). Moreover, exosomal UCA1 is observed to cause drug resistance in cetuximab-sensitive CRC cells (90). Due to its non-invasive and relatively stable content in





serum, exosomal UCA1 is hopefully used as a new biomarker for CRC in the future (Table 2).

Accumulated studies have suggested lncRNAs have been elucidated to serve as ceRNAs by sponging miRNAs, which subsequently regulates miRNAs-mediated anti-EGFR therapy resistance in CRC. In addition, lncRNAs play vital roles in CRC progression, metastasis, and drug resistance. These findings provide therapeutic targets and potential prognostic markers for CRC with regard to lncRNAs. Future studies are warranted to reveal the specific mechanism of lncRNAs involved in CRC progression, metastasis, and drug resistance.

## CIRCARNAS

### Biological Functions of CircRNAs

CircRNAs are novel covalently closed circular single-stranded ncRNAs discovered in recent years, mainly formed by exon reverse splicing of pre-mRNA. According to the sequence origin,

circRNAs are grouped into exonic circRNAs, circular intronic RNAs, and exon-intron circRNAs (91). They exist stably in plasma, serum, saliva, and other body fluids, and are widely expressed in various types of cells with cell- and tissue-specificity (92, 93). Acting as ceRNAs, circRNAs can competitively bind with miRNAs and regulate gene expression *via* interacting with miRNAs or RNA-binding proteins (RBPs). They exert essential effects on the progression of multiple diseases including cancer (17, 94–99).

### CircRNAs Regulate Drug Resistance in CRC

Increasing evidence has supported that circRNAs participate in regulating tumorigenesis and drug resistance of CRC (100, 101). Chen et al. have found that circ-PRKDC acted as a miR-375 sponge and targeted FOXM1, and enhanced CRC cells resistance to 5-fluorouracil (5-FU) through the Wnt/β-catenin signaling pathway (102) (Table 3, Figure 4). CircRNAs of circ\_0007031, circ\_0007006, and circ\_0000504 have been found to modulate 5-FU resistance of CRC cells by regulating AKT3 *via* the AKT signaling pathway, while circ\_0048234 can sponge miR-671-5p in 5-FU-resistant CRC cells *via* the EGFR signaling pathway (103) (Table 3, Figure 4). ATP-binding cassette (ABC) transporters, such as ABCB1, ABCC1, and ABCG2, have been reported to play crucial roles in CRC drug resistance by increasing drug efflux out of cancer cells (105). Inhibition expression of ABC transporters is an effective approach to reverse drug resistance in cancer cells (105, 106). A number of ncRNAs have been demonstrated to be involved in regulating ABC transporters in drug-resistant cancer cells by regulating EGFR and its downstream signaling pathways (107, 108). Circ\_0007031 has been documented to induce 5-FU resistance by modulating the expression of ABC transporter ABCC5 through miR-133b/ABCC5 axis in CRC (100). MiR-7 functions as a regulator of anti-EGFR therapy resistance in CRC. It has been shown that ciRS-7 regulated CRC cell growth and invasion by sponging miR-7 and upregulating EGFR and IGF-1R expression (109). Similarly, CiRS-7 can function as ceRNA for miR-7 to activate EGFR/RAF1/MAPK pathway in CRC (110). The study by Zeng et al. has reported circHIPK3 sponged miR-7 to upregulate the expression of several oncogenes, such as FAK, IGF1R, EGFR, and YY1, through the PI3K/AKT and MEK/ERK signaling pathways that contributing to drug resistance in CRC (104). Additionally, inhibition of circHIPK3 can reverse the

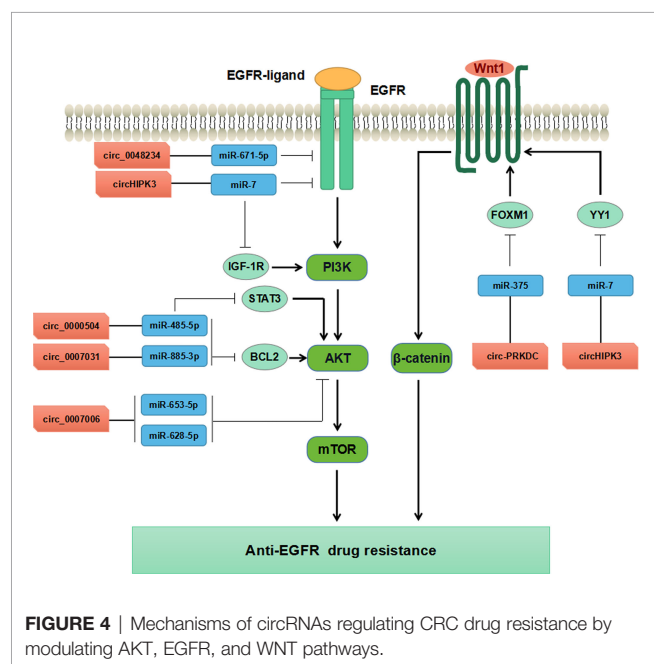
**TABLE 2 |** lncRNAs involved in anti-EGFR drugs resistance in CRC.

lncRNAs	Expression	Targets/Pathways	Drugs	References
POU5F1P4	Down-regulated	EGFR pathway	Cetuximab	(86)
LNC00973	Up-regulated	/	Cetuximab	(87)
MIR100HG	Up-regulated	MIR100/DKK1, ZNRF3/Wnt/β-catenin pathway, MIR-125b/ZNRF3, RNF43, DKK3, APC2/Wnt/β-catenin pathway	Cetuximab	(50)
CRART16	Up-regulated	MIR-371a-5p/ERBB3/MAPK pathway	Cetuximab	(88)
HCG18	Up-regulated	MIR-20b-5p/PD-L1	Cetuximab	(89)
UCA1	Up-regulated	/	Cetuximab	(90)

/, unmentioned in the reference.

**TABLE 3 |** CircRNAs involved in drugs resistance in CRC.

CircRNAs	Expression	Targets/Pathways	Drugs	References
Circ-PRKDC	Up-regulated	MIR-375/FOXM1/Wnt/ $\beta$ -catenin pathway	5-FU	(102)
Circ_0007031	Up-regulated	MIR-885-3p/BCL2/AKT pathway	5-FU	(103)
		MIR-133b/ABCC5 axis		(100)
Circ_0007006	Up-regulated	MIR-653-5p, miR-628-5p/AKT pathway	5-FU	(103)
Circ_0000504	Up-regulated	MIR-485-5P/STAT3, BCL2/AKT pathway	5-FU	(103)
Circ_0048234	Down-regulated	MIR-671-5p/EGFR pathway	5-FU	(103)
CircHIPK3	Up-regulated	MIR-7/IGF-1R/PI3K/AKT pathway, MIR-7/EGFR/MEK/ERK pathway, MIR-7/YY1/Wnt pathway	Cetuximab	(104)



resistance to cetuximab by targeting miR-7 in CRC cells (104) (Figure 4). All these findings have provided novel insights into the understanding of drug resistance mechanisms regarding circRNAs. Nevertheless, more studies are warranted to estimating the involvement and mechanism of circRNAs in regulating the resistance to anti-EGFR therapy in CRC.

## PERSPECTIVES

Drug resistance remains a major challenge for CRC treatment. The mechanisms underlying CRC resistance to anti-EGFR

therapy are complicated. Increasing studies have shown that ncRNAs play crucial roles in regulating the resistance to anti-EGFR therapy in CRC, primarily including miRNAs, lncRNAs and circRNAs, which have been identified as either oncogenes or tumor suppressors (111). Currently available studies have supported ncRNAs participate in modulating anti-EGFR drug resistance based on miRNAs-mRNAs, lncRNAs-miRNAs-mRNAs, or circRNAs-miRNAs-mRNAs regulatory networks through the EGFR signaling pathway, RAS signaling pathway, and PI3K/AKT signaling pathway. Accordingly, ncRNAs may function as novel biomarkers in predicting the efficacy and resistance of anti-EGFR therapy in CRC. Nevertheless, the molecular mechanisms of ncRNAs involved in anti-EGFR therapy resistance still warrant to be further elucidated in CRC. Further studies need to focus on investigating new therapeutic strategies based on ncRNAs regulatory networks combining with anti-EGFR targeted therapy in CRC.

## AUTHOR CONTRIBUTIONS

SY, DX, and JC wrote the draft and revised it. JC, XF, ZS, LG, WD, HL, XY, JD, LLZ, and LZ collected the data and designed the tables and figures. All authors read and approved the submitted version.

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# The Emerging Roles of Circular RNAs in the Chemoresistance of Gastrointestinal Cancer

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Gastrointestinal (GI) cancer represents a major global health problem due to its aggressive characteristics and poor prognosis. Despite the progress achieved in the development of treatment regimens, the clinical outcomes and therapeutic responses of patients with GI cancer remain unsatisfactory. Chemoresistance arising throughout the clinical intervention is undoubtedly a critical barrier for the successful treatment of GI cancer. However, the precise mechanisms associated with chemoresistance in GI cancer remain unclear. In the past decade, accumulating evidence has indicated that circular RNAs (circRNAs) play a key role in regulating cancer progression and chemoresistance. Notably, circRNAs function as molecular sponges that sequester microRNAs (miRNAs) and/or proteins, and thus indirectly control the expression of specific genes, which eventually promote or suppress drug resistance in GI cancer. Therefore, circRNAs may represent potential therapeutic targets for overcoming drug resistance in patients with GI cancer. This review comprehensively summarizes the regulatory roles of circRNAs in the development of chemoresistance in different GI cancers, including colorectal cancer, gastric cancer and esophageal cancer, as well as deciphers the underlying mechanisms and key molecules involved. Increasing knowledge of the important functions of circRNAs underlying drug resistance will provide new opportunities for developing efficacious therapeutic strategies against GI cancer.

**Keywords:** gastrointestinal cancer, chemoresistance, circular RNAs, molecular sponges, microRNAs, therapeutic targets

## 1 INTRODUCTION

Gastrointestinal (GI) cancers are among the most important causes of cancer-related death worldwide and mainly include colorectal cancer (CRC), gastric cancer (GC) and esophageal cancer (EC) (Wang D.-K. et al., 2021). These GI cancers pose a huge threat to human health worldwide (Fang et al., 2021). Conventional treatments, including chemotherapy, radiotherapy and surgery, have been the mainstays of cancer therapy (Buckley et al., 2020). However, these therapeutic approaches have limitations and lead to unsatisfactory clinical outcomes, as evidenced by the high mortality rate of patients with GI cancers (D'Eliseo and Velotti, 2016; Hsu et al., 2020). Chemoresistance is a significant factor accounting for treatment failure in patients with GI cancer (Wang Y. et al., 2020). Therefore, a comprehensive investigation of the detailed mechanisms underlying cancer chemoresistance is essential to improve the efficacy of chemotherapy against GI cancer.

To date, a number of studies have suggested a linkage between noncoding RNAs (ncRNAs) and chemoresistance in GI cancer. Circular RNAs (circRNAs) are a class of endogenous ncRNA molecules with a covalently closed loop configuration (Patop et al., 2019). They are produced from exons and/or introns via the back-splicing pattern. CircRNAs were initially regarded as mis-splicing products without any genuine function and have not been adequately explored until recently (Sanger et al., 1976). The development of high-throughput sequencing technology and associated bioinformatics enables a thorough investigation of circRNAs. CircRNAs have been shown to play a crucial role in the chemoresistance of GI cancer. For instance, the circRNA circDDX17 sensitized CRC cells to 5-fluorouracil (5-FU) and promoted apoptosis via the miR-31-5p/kidney ankyrin repeat-containing protein 1 (KANK1) axis (Ren et al., 2020). CircHIPK3 acted as a molecular sponge for miR-637 to stimulate the downstream signal transducer and activator of transcription 3 (STAT3)/B-cell lymphoma-2 (Bcl-2)/Beclin 1 signaling cascade in CRC cells (Zhang et al., 2019). This event resulted in the suppression of autophagic cell death and enhanced oxaliplatin (OXA) resistance in CRC cells. However, current research aiming to understand the molecular mechanisms by which circRNAs regulate GI cancer chemoresistance is still in its initial stage. Further studies are warranted to enrich our knowledge of ncRNA-mediated mechanisms related to drug resistance in different GI cancers. In this review, we summarize the most recent evidence for the roles of circRNAs in regulating the drug resistance of GI cancer and discuss possible mechanisms of action. Future directions are suggested to better apprehend their exact roles and their usefulness as therapeutic targets.

## 2 CIRCULAR RNAs

Circular RNAs (circRNAs) are a class of single-stranded closed-loop RNA molecules that lack free 5' and 3' ends. Although circRNAs were first identified in viruses in 1976, they were originally considered splicing intermediates or byproducts of aberrant mRNA splicing events without specific functions and thus did not attract considerable scientific attention for many years (Sanger et al., 1976). With the advent of high-throughput sequencing techniques, a multitude of endogenous circRNAs have been discovered in various species. CircRNAs exhibit cell type-, tissue- or developmental stage-specific expression patterns, suggesting their possible biological significance. Accumulating evidence has verified that circRNAs are vital players in the initiation and progression of multiple human diseases, especially cancer (Verduci et al., 2021).

### 2.1 Biogenesis of Circular RNAs

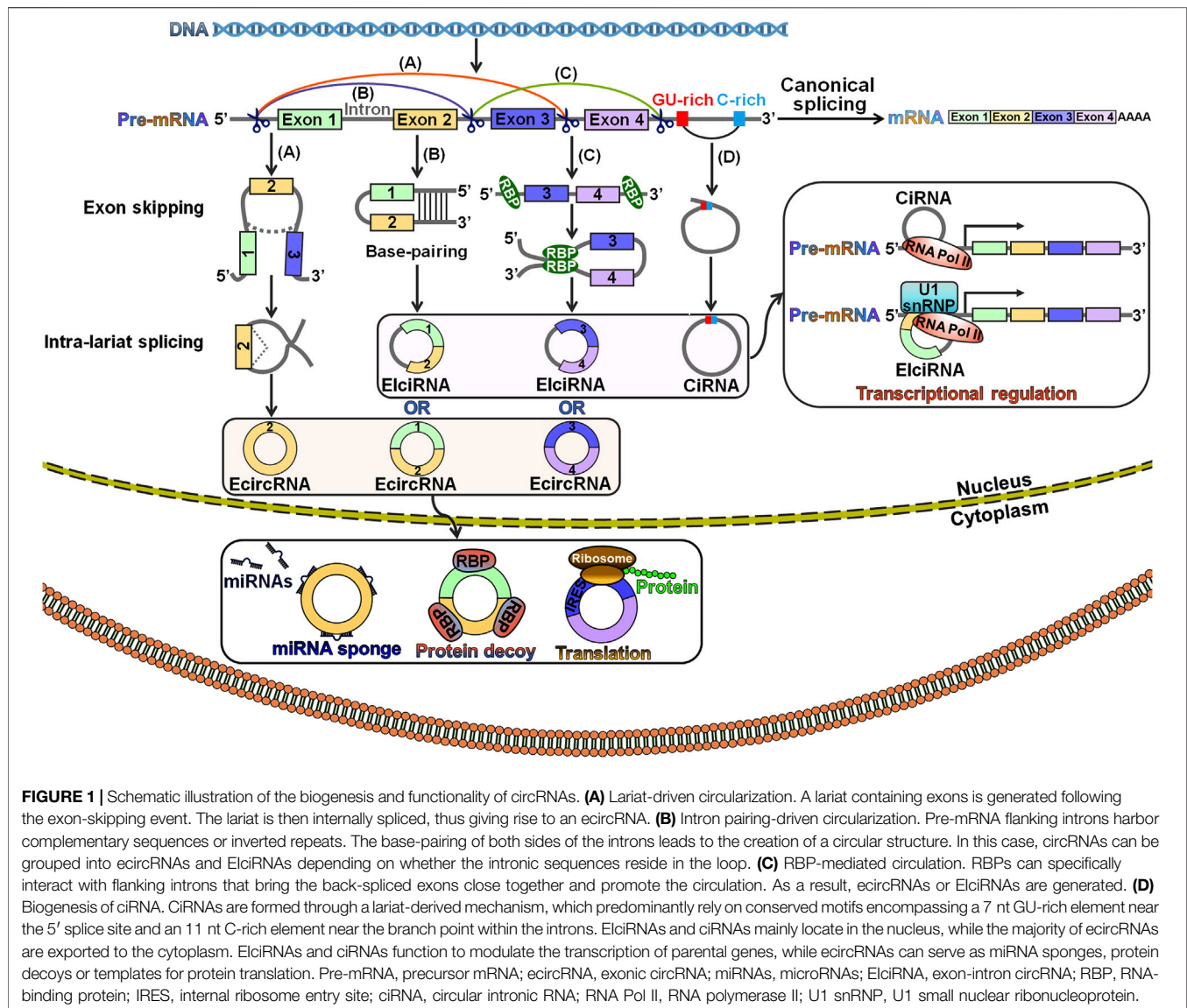
The exact mechanisms of circRNA biogenesis remain largely unknown. CircRNAs are generated from precursor mRNAs (pre-mRNAs) via a back-splicing process, which attaches a downstream splice donor (5' splice site) to an upstream splice acceptor (3' splice site) (Wilusz, 2018). The pre-mRNA is spliced into linear RNA by deleting introns. In contrast, back-splicing occurs in an inverse orientation to produce an RNA

molecule with a single or multiple exons (Zhang et al., 2016). CircRNAs are derived from exons, introns, untranslated regions (UTRs), antisense transcripts and intergenic regions (Memczak et al., 2013; Lu et al., 2015). According to previous studies, canonical splicing signals and spliceosome machinery were required for circRNA biogenesis (Ashwal-Fluss et al., 2014; Liang et al., 2017). However, canonical splicing is a primary choice for gene splicing under most circumstances. Depletion of core spliceosome components resulted in the shift from linear mRNA production toward preferred output of circRNAs (Liang et al., 2017). Based on their distribution and biogenesis, circRNAs are categorized into three main subclasses: exonic circRNAs (ecircRNAs), exon-intron circRNAs (EicRNAs) and circular intronic RNAs (ciRNAs) (Ashwal-Fluss et al., 2014; Chen et al., 2015; Kelly et al., 2015). EicRNAs and ciRNAs mainly reside in the nucleus, while the majority of ecircRNAs are exported to the cytoplasm (Zhang et al., 2013; Li et al., 2015; Huang et al., 2018). Three classical models for circRNA biogenesis have been proposed, including lariat-driven circularization, intron pairing-driven circularization and RNA-binding protein (RBP)-mediated circularization (Liang and Wilusz, 2014; Barrett et al., 2015; Conn et al., 2015) (**Figure 1**).

Lariat-driven circularization is also known as the exon-skipping mechanism (Wu et al., 2019). In this model, exon skipping occurs during canonical splicing (**Figure 1A**). Specifically, the folding of the pre-mRNA during transcription brings the 5' donor site of the upstream intron and the 3' receptor site of the downstream intron into closer proximity, leading to the formation of a linear RNA with skipped exons and an exon-containing lariat precursor. This lariat precursor then undergoes internal back-splicing to produce a circRNA.

Intron pairing-driven circularization, also known as the direct back-splicing mechanism, is mediated by cis-acting regulatory elements that contain reverse complementary motifs specifically located in the flanking introns, leading to direct base pairings of flanking introns (Liang and Wilusz, 2014) (**Figure 1B**). Flanking complementary sequences (e.g., Alu sequences) play a critical role in exon circularization (Dubin et al., 1995). In the RBP-mediated circularization process, RBPs recognize and dock on particular sequence motifs of bordering introns (**Figure 1C**). This docking brings the flanking introns of the back-spliced exons close together, hence facilitating exon circularization. Muscleblind (MBL) and quaking (QKI) proteins promoted the formation of circMbl and circQKI by interacting with specific intronic motifs, respectively (Ashwal-Fluss et al., 2014; Conn et al., 2015). In all the models, the intronic segments are completely removed or retained to form ecircRNAs or EicRNAs, respectively (Bahn et al., 2015).

During canonical splicing, introns are normally excised in lariat forms, which are quickly debranched and degraded by exonucleolytic enzymes (Cao et al., 2021). However, the excised intronic lariats occasionally escape debranching and degeneration processes, and thus form stable ciRNAs with a 2',5'-phosphodiester bond between the splicing donor and the branchpoint (**Figure 1D**). A consensus motif containing both a 7 nt GU-rich element near the 5' splice



site and an 11 nt C-rich element adjacent to the branchpoint site is essential for ciRNA biogenesis (Zhang et al., 2013).

The biogenesis of circRNAs is a highly orchestrated process in which many cis/trans-acting elements are involved. The explicit mechanisms by which these regulators control circRNA abundance are worthy of further study. More cis/trans-regulators implicated in circRNA biogenesis remain to be characterized. Genetic or epigenetic factors have been proposed to regulate circRNA formation. Genomic abnormalities, such as short nucleotide variants and chromosome translocation, may cause sequence alterations in DNA segments flanking the encircled region, hence influencing the generation of circRNAs (Sudmant et al., 2015). Epigenetic modifications within genes and histones affect alternative splicing and may indirectly influence circRNA formation (Bentley, 2014). The epigenetic mechanisms contributing to circRNA formation warrant more detailed exploration.

## 2.2 Biological Functions of Circular RNAs

CircRNAs have emerged as crucial participants in various physiological and pathological processes (Ely et al., 2021). Currently, circRNAs have been the spotlight of cancer research, as numerous studies have revealed pivotal biological roles for various circRNAs in cancer. CircRNAs affect various hallmarks of cancer by functioning as miRNA sponges, interacting with RBPs, governing transcription and splicing and encoding functional peptides or proteins (Li et al., 2020) (Figure 1). Among these functions, miRNA sponging is the best-characterized regulatory mechanism for circRNAs. For instance, the circRNA ciRS-7 served as a molecular sponge for tumor-suppressive miR-7 and regulated the progression of various cancers, including esophageal squamous cell carcinoma (ESCC), GC and non-small-cell lung cancer (NSCLC) (Li R.-c. et al., 2018; Pan et al., 2018; Su et al., 2018). CircRNAs are capable of interacting with RBPs. The circRNA *cIARS* abrogated



alkylation repair homologue 5 (ALKBH5)-mediated autophagy inhibition in hepatocellular carcinoma (HCC) by physically interacting with ALKBH5 (Liu Z. et al., 2020). CircZKSCAN1 inhibited the malignant characteristics of HCC cells by binding the RBP fragile X mental retardation protein (FMRP) to block the Wnt/ $\beta$ -catenin signaling pathway (Zhu et al., 2019). As cellular proteins affect multiple hallmarks of cancer, circRNAs indirectly regulate cancer development by altering protein structure and function through their functions as protein decoys, recruiters or scaffolds. Intriguingly, the roles of circRNAs in cancer may be partially attributed to their regulation of transcription and splicing. For example, oncogenic circERBB2 modulated ribosomal DNA transcription, a key step in ribosome biogenesis and cell proliferation, by altering the nucleolar localization of proliferation-associated protein 2G4 (PA2G4) (Huang et al., 2019a). As a result, circERBB2 exerted a positive effect on gallbladder cancer cell proliferation. CircNOL10 retarded the proliferation and promoted the apoptosis of lung cancer cells by enhancing the transcriptional regulatory effect of sex comb on midleg-like 1 (SCML1) on the humanin (HN) polypeptide family (Nan et al., 2019). CircURI1 was shown to inhibit GC metastasis (Wang et al., 2021d). In terms of mechanism, circURI1 dominated the alternative splicing of cell migration-related genes by directly binding heterogeneous nuclear ribonucleoprotein M (hnRNPM). Additionally, some circRNAs contain open reading frames (ORFs) that can be translated into peptides or proteins with key biological importance. CircRNA-encoded proteins serve as oncoproteins or tumor suppressors in cancer. CircPPP1R12A encoded the functional protein circPPP1R12A-73aa, which facilitated the growth and metastasis of colon cancer via the Hippo/yes-associated protein (YAP) signal transduction cascade (Zheng et al., 2019). Experimental evidence revealed that circFNDC3B-218aa encoded by circFNDC3B prevented colon cancer progression by limiting Snail expression (Pan et al., 2020). The biological functions of circRNA-encoded proteins have not yet been completely defined. Studies aiming to clarify whether circRNA-produced proteins and their full-length protein counterparts encoded by the linear transcript have the same capabilities are essential. The modulatory mechanisms underlying circRNA translation deserve continued study.

Altogether, circRNAs play multifaceted roles in cancer development and progression through diverse mechanisms. Presumably, particular circRNAs may simultaneously perform multiple functions and modulate the same cancer-related pathway through different modes of action. The identification of the potential concerted mechanisms of circRNAs might help provide a comprehensive landscape of oncogenic signaling cascades. On the other hand, one circRNA may be implicated in various cancer-associated processes by employing the same mechanism of action. Thus, significant work is needed to reveal the complex relationship between circRNAs and cancer pathogenesis. In addition, many important questions in relation to circRNA function remain unanswered. CircRNAs have been reported to interact with miRNAs and RBPs. Researchers have not yet determined whether circRNAs bind other types of ncRNAs or

molecules. The effect of circRNAs on the translocation of cellular components (e.g., RNAs and proteins) is also unclear. Increased efforts are needed to systematically characterize the versatile functionalities of circRNAs. In-depth investigations of circRNAs would foster the development of effective approaches for cancer diagnosis and treatment.

## 2.3 Circular RNA Expression Profiles in Gastrointestinal Cancer

With the help of circRNA microarray analysis and high-throughput sequencing technology, various deregulated circRNAs have been discovered in GI cancer, suggesting their important functions in the occurrence and development of GI cancer. For instance, 21,458 circRNAs were discovered in four paired CRC tissues and adjacent normal mucosa tissues using high-throughput RNA sequencing (RNA-seq) (Li X.-N. et al., 2018). Further analysis indicated that 448 circRNAs were differentially expressed in CRC tissues compared with normal mucosa tissues, including 394 upregulated and 54 downregulated circRNAs. These differentially expressed circRNAs were involved in the regulation of cell communication, autophagosome and GTPase binding. They might also be correlated with CRC-relevant signaling pathways, such as deleted in colorectal carcinoma (DCC)-mediated attractive signaling and the Netrin-1 signaling pathway. Accordingly, circRNAs played an important role in CRC carcinogenesis. The circRNA-seq data from 40 CRC samples revealed 113 (92 upregulated and 21 downregulated) dysregulated circRNAs in CRC patients with liver metastasis compared with CRC patients (Xu et al., 2019). In particular, two upregulated circRNAs, circRNA\_0001178 and circRNA\_0000826, presented potential diagnostic value in CRC patients with liver metastasis. By performing RNA-seq in combination with bioinformatics analysis, Sun Y. et al. (2020) identified 1,055 circRNAs that were abnormally expressed in three pairs of ESCC and adjacent normal tissues, 418 of which were upregulated and 637 were downregulated. Circ\_0000075, circ\_0000513, circ\_0000530, circ\_0001005, circ\_0001121, circ\_0001904 and circ\_0002255 were predicted to be key circRNAs involved in ESCC pathogenesis. These circRNAs might be involved in ESCC progression through regulation of adherens junction and angiogenesis. A systematic meta-analysis based on circRNA microarrays revealed 64 differentially expressed circRNAs between GC tissues and adjacent normal tissues (Ding et al., 2020). Among these, hsa\_circ\_0005927 and hsa\_circ\_0067934 were identified as potential biomarkers for GC screening. The expression profile of circRNAs in liver cancer was previously assessed (Wang M. et al., 2020). Based on circRNA-seq data and bioinformatics analysis, 13,124 unique circRNAs were identified in three paired liver cancer tissues and adjacent normal tissues, 2,996 of which showed different expression patterns. These abnormally expressed circRNAs might exert regulatory effects on tumor growth and immunity in liver cancer. In another study, by performing circRNA-seq of 30 primary HCC tissues, Hu et al. (2020) detected 72,277 circRNAs that were expressed in at least one sample. The authors further screened 144 upregulated

and 76 downregulated circRNAs in metastatic HCC compared with non-metastatic HCC, indicating their potential association with HCC metastasis. The dysregulated circRNAs in pancreatic cancer were previously investigated using a circRNA array analysis (Guo et al., 2018). The results indicated that 128 circRNAs were upregulated and 161 circRNAs were downregulated in pancreatic cancer tissues compared to adjacent normal tissues. Some differentially expressed circRNAs (e.g., circRNA\_000780, circRNA\_100435, circRNA\_101252 and circRNA\_103076) might be involved in pancreatic cancer progression by acting as miRNA sponges.

Given their close association with cancer biology, exploring deregulated circRNAs and their roles in cancer has been a research hotspot. In recent years, an expanding number of dysregulated circRNAs have been gradually identified in GI cancer, but their detailed functions and mechanisms in the development and progression of GI cancer are still equivocal. Further studies are needed to fully elucidate the regulatory mechanisms and clinical significance of circRNAs in GI cancer. In the case of circRNAs that are upregulated in GI cancer, depletion of oncogenic circRNAs might be achieved by RNA interference. For circRNAs that are downregulated in GI cancer, ectopic expression of tumor-suppressive circRNAs may represent a potential treatment option. Illumination of the therapeutic potential of circRNA-based approaches against cancer has become an important area of biomedical research. One of the central issues with circRNA-based therapies is discovering the optimal method to efficiently transport small interfering RNAs (siRNAs) or artificial circRNAs *in vivo*. Lipid nanoparticles represent an appropriate delivery system that protects siRNAs from degradation and facilitates their uptake by host cells (Tam et al., 2013). However, the efficiency of nanoparticle-mediated delivery of siRNAs or circRNA expression vectors is relatively low. Nanoparticle delivery systems must be optimized to overcome this limitation. Moreover, exosomes have exhibited great promise as transport vehicles for circRNA-targeting siRNAs or circRNA expression vectors (Ha et al., 2016). Exosomes appear to be superior drug carriers compared to synthetic nanoparticles. Nevertheless, exosomes have the challenges of the manufacturing scale and homogeneity. Studies aiming to perform an in-depth characterization of the factors and molecular pathways responsible for circRNA biogenesis will be helpful for the development of advanced delivery systems. Limited preclinical reports concerning the utility of circRNAs for treating cancer are available. Much more work is needed to validate the safety and effectiveness of circRNA-based therapeutics before their clinical use.

### 3 CIRCULAR RNAs AND GASTROINTESTINAL CANCER CHEMORESISTANCE

Drug resistance is still a major clinical challenge in effective cancer therapy. The development of drug resistance in cancer involves diverse mechanisms, including DNA damage repair, modulation of

cell viability and proliferation, manipulation of cell death-relevant pathways, regulation of glucose metabolism, induction of the cancer stem cell (CSC) phenotype, and modification of drug efflux and metabolism (Micallef and Baron, 2021). CircRNAs play an important role in regulating the chemoresistance of GI cancer by interfering with these pathways (Figure 2).

#### 3.1 DNA Damage Repair Pathway

CRC is ranked as the fourth leading cause of cancer-related death worldwide (Ren et al., 2021). CRC is commonly diagnosed at an advanced stage, and chemotherapy represents an important treatment option for patients with advanced CRC. CircRNAs have been shown to affect the sensitivity of CRC cells to different therapeutic drugs, such as 5-FU, OXA and irinotecan. In a recent report, Yao F. et al. (2021) detected circRNA expression profiles and identified differentially expressed circRNAs in 5-FU- and cisplatin (DDP)-resistant CRC cells compared with parental CRC cells by performing RNA-seq and bioinformatics analyses. The results showed that 7,393 and 7,385 circRNAs were expressed in 5-FU- and DDP-resistant CRC cells, respectively. In addition, 48 and 90 differentially expressed circRNAs were identified among the discovered circRNAs in 5-FU- and DDP-resistant CRC cells compared with sensitive CRC cells. Importantly, differentially expressed circRNAs might be associated with drug resistance-relevant pathways, including the DNA repair pathway and the Hippo signal transduction cascade. *In vitro* experimental results verified that hsa\_circ\_002482 upregulation markedly increased the chemosensitivity of CRC cells (Table 1). Paradoxically, hsa\_circ\_002482 reduced the expression of drug-sensitizing miR-503-5p (Yang et al., 2017). It was unclear whether miR-503-5p participated in hsa\_circ\_002482-regulated drug susceptibility. Considerable research efforts should be devoted to fully elucidating the definite mechanisms by which hsa\_circ\_002482 altered CRC chemoresistance. Existing evidence suggested that the impact of circRNAs on CRC drug resistance was partially attributable to the regulation of the DNA repair pathway. Additional investigation is still needed to define the interaction between chemoresistance-associated circRNAs and the DNA repair pathway in CRC.

GC is the second most common cause of cancer-associated mortality worldwide (Bray et al., 2018). The main GC treatment remains cytotoxic chemotherapy. DDP is a commonly used chemotherapeutic agent in clinical GC treatment. Nevertheless, DDP resistance has become a serious obstacle undermining its therapeutic efficacy. CircRNAs are critical regulators of GC chemoresistance, suggesting their clinical implications as novel therapeutic targets for GC (Wei et al., 2020). The linkage between circRNAs and DDP resistance has been gradually disclosed, providing novel insights into the mechanisms associated with GC chemoresistance. Circ\_0026359 was shown to be overexpressed in DDP-resistant GC compared with sensitive tissues (Zhang Z. et al., 2020). Circ\_0026359 absence increased miR-1200 activity and thus lowered the expression of DNA polymerase  $\delta$  subunit 4 (POLD4). POLD4 downregulation caused DNA fragmentation and genomic instability in DDP-resistant GC cells, thus reducing cell viability and promoting apoptosis and DDP sensitivity in GC cells. The miR-1200/POLD4 pathway mediated

**TABLE 1 |** Chemoresistance-relevant circRNAs in gastrointestinal cancer.

Cancer	CircRNA	Drugs	Expression	Targets	Function	References
Colorectal cancer	Hsa_circ_002482	5-Fluorouracil, cisplatin	Downregulation	miR-503-5p	Sensitivity	Yao et al. (2021a)
Gastric cancer	Circ_0026359	Cisplatin	Upregulation	miR-1200/POLD4	Resistance	Zhang et al. (2020b)
Gastric cancer	CircAKT3	Cisplatin	Upregulation	miR-198/PIK3R1	Resistance	Huang et al. (2019b)
Gastric cancer	Hsa_circ_0001546	Oxaliplatin	Downregulation	miR-421/ATM/Chk2/p53	Sensitivity	Wu et al. (2020)
Colorectal cancer	CircCCDC66	Oxaliplatin	Upregulation	Cell proliferation/survival-associated genes	Resistance	Lin et al. (2020)
Gastric cancer	Circ-PVT1	Cisplatin	Upregulation	miR-152-3p/HDGF, Bax, cleaved caspase-3, Bcl-2	Resistance	Wang et al. (2021e)
Esophageal squamous cell carcinoma	CircPSMC3	Gefitinib	Downregulation	miR-10a-5p/PTEN	Sensitivity	Zhu et al. (2021)
Colorectal cancer	Circ_0000338	5-Fluorouracil	Upregulation	miR-217, miR-485-3p	Resistance	Zhao et al. (2021)
Colorectal cancer	Circ_0071589	Cisplatin	Upregulation	miR-526b-3p/KLF12, Cyclin D1, Bcl-2, cleaved caspase-3	Resistance	Zhang et al. (2021b)
Gastric cancer	CircVAPA	Cisplatin	Upregulation	miR-125b-5p/STAT3, Bcl-xL, Mcl-1, Survivin	Resistance	Deng et al. (2021)
Gastric cancer	CircDONSON	Cisplatin	Upregulation	miR-802/BMI1, cleaved caspase-3/-9, p27, Cyclin D1	Resistance	Liu et al. (2020b)
Gastric cancer	Hsa_circ_0000520	Herceptin	Downregulation	PI3K/Akt, Bax, Bcl-2	Sensitivity	Lv et al. (2020)
Gastric cancer	CircCCDC66	Cisplatin	Upregulation	miR-618/Bcl-2	Resistance	Zhang et al. (2020a)
Gastric cancer	CircHECTD1	Diosbulbin-B	Upregulation	miR-137/PBX3, Bax, Bcl-2	Resistance	Lu et al. (2021)
Gastric cancer	CircMCTP2	Cisplatin	Downregulation	miR-99a-5p/MTMR3, P62, LC3-II	Sensitivity	Sun et al. (2020a)
Gastric cancer	CircCUL2	Cisplatin	Downregulation	miR-142-3p/ROCK2, P62, Beclin 1, LC3	Sensitivity	Peng et al. (2020)
Esophageal squamous cell carcinoma	cDOPEY2	Cisplatin	Downregulation	CPEB4, Mcl-1	Sensitivity	Liu et al. (2021)
Gastric cancer	CircNRIP1	5-Fluorouracil	Upregulation	miR-138-5p/HIF-1 $\alpha$	Resistance	Xu et al. (2020)
Colorectal cancer	Hsa_circ_001680	Irinotecan	Upregulation	miR-340/BMI1	Resistance	Jian et al. (2020)
Gastric cancer	CircFAM73A	Cisplatin	Upregulation	miR-490-3p/HMGA2, Nanog, OCT4, SOX2, $\beta$ -catenin	Resistance	Xia et al. (2021)
Colorectal cancer	Circ-PRKDC	5-Fluorouracil	Upregulation	miR-375/FOXO1	Resistance	Chen et al. (2020)
Gastric cancer	Circ-PVT1	Paclitaxel	Upregulation	miR-124-3p/ZEB1	Resistance	Liu et al. (2019)
Gastric cancer	Circ_ASAP2	Cisplatin	Upregulation	miR-330-3p/NT5E	Resistance	Sun et al. (2021)
Gastric cancer	Circ_0000260	Cisplatin	Upregulation	miR-129-5p/MMP11	Resistance	Liu et al. (2020a)
Esophageal squamous cell carcinoma	Circ_0006168	Paclitaxel	Upregulation	miR-194-5p/JMJD1C	Resistance	Qu et al. (2021)
Colorectal cancer	Circ_0007031	5-Fluorouracil	Upregulation	miR-133b/ABCC5	Resistance	He et al. (2020)
Colorectal cancer	Circ_0000338	5-Fluorouracil	Upregulation	Cell viability	Resistance	Hon et al. (2019)
Colorectal cancer	CIRS-122	Oxaliplatin	Upregulation	miR-122/PKM2	Resistance	Wang et al. (2020c)
Colorectal cancer	Circ-FBXW7	Oxaliplatin	Downregulation	miR-18b-5p, MRP1, Mcl-1, cleaved caspase-3	Sensitivity	Xu et al. (2021)
Gastric cancer	Circ_0032821	Oxaliplatin	Upregulation	miR-515-5p/SOX9	Resistance	Zhong et al. (2021)
Gastric cancer	Circ-PVT1	Cisplatin	Upregulation	miR-30a-5p/YAP1, LC3-II/I, P-gp, P62	Resistance	Yao et al. (2021b)
Esophageal squamous cell carcinoma	Circ_0000337	Cisplatin	Upregulation	miR-337-3p/JAK2	Resistance	Zang et al. (2021)

the function of circ\_0026359 in the DDP resistance of GC. The expression of circAKT3 was overtly upregulated in DDP-resistant GC tissues and cells compared to sensitive samples (Huang et al., 2019b). Mechanistically, circAKT3 functioned as a miR-198 sponge to upregulate phosphatidylinositol 3-kinase regulatory subunit 1 (PIK3R1), which activated the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling cascade. Activation of the PI3K/Akt pathway caused the upregulation of breast cancer type 1 susceptibility protein (BRCA1), thereby inducing DNA damage repair and contributing to increased DDP resistance in GC cells. These findings highlighted a novel therapeutic option for DDP-

resistant GC. The exact mechanism by which BRCA1 modulates DNA damage repair in GC remains to be elucidated. In contrast, hsa\_circ\_0001546 was apparently downregulated in GC tissues compared with adjacent normal tissues (Wu et al., 2020). Hsa\_circ\_0001546 increased the expression of the DNA damage-inducible kinase ataxia telangiectasia mutated (ATM) by absorbing miR-421. ATM/checkpoint kinase 2 (Chk2)/p53 signaling played a pivotal role in the DNA damage response, cell apoptosis and drug resistance in cancer (Shi et al., 2012; Yao et al., 2017). As a result, hsa\_circ\_0001546 suppressed cell proliferation and sensitized OXA-resistant GC cells to OXA by activating the ATM/Chk2/p53

signaling pathway. Based on these data, hsa\_circ\_0001546 might represent a therapeutic target for the treatment of OXA-resistant GC. Collectively, circRNAs could stimulate or suppress the DNA damage repair pathway, leading to altered sensitivity of GC cells to chemotherapy.

Chemotherapeutic drugs may induce cancer cell death by triggering DNA damage. Cancer cells activate DNA damage repair pathways to resist chemotherapeutic agents. Numerous genes and signaling pathways are involved in DNA damage repair. Alterations in their expression levels or activities significantly affect the responsiveness of cancer cells to chemotherapy and cancer progression. Multiple lines of evidence have highlighted the emerging roles of circRNAs in GI cancer chemoresistance through the regulation of DNA damage-responsive genes or signaling cascades. Targeting circRNAs that modify DNA damage repair pathways may be a potential therapeutic option to address drug resistance in GI cancer. DNA damage repair is mainly triggered by PI3K-related kinase (PIKK) family proteins including ATM, ATM- and Rad3-related (ATR) kinase and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), followed by the activation of downstream reactions (Lukas et al., 2011). The regulatory function of circRNAs in the ATR and DNA-PKcs pathways is yet to be determined. The interplay between circRNAs and DNA damage repair pathways is worthy of further study. Current studies predominantly focus on the roles of circRNAs in nuclear DNA damage repair pathways in GI cancer. The involvement of circRNAs in the mitochondrial DNA (mtDNA) repair pathway remains unknown. Inhibition of the mtDNA repair pathway may diminish unfavorable effects on normal cells and shows promise as a more effective anticancer therapy. Future studies are warranted to characterize circRNA-associated regulatory networks implicated in the mtDNA repair pathway.

### 3.2 Cell Viability and Proliferation

DEXH-box helicase 9 (DHX9) functions as a critical regulator of circRNA biogenesis and modulates the base-pairing interaction of intronic sequences bracketing the circularized region (Aktas et al., 2017). OXA exposure enhanced circCCDC66 expression through PI3KK-mediated DHX9 phosphorylation in CRC cells (Lin et al., 2020). CircCCDC66 knockdown evidently reduced the survival of OXA-resistant CRC cells, thereby blocking the development of chemoresistance. A genome-wide analysis of circCCDC66 knockdown-resistant cells and control resistant cells using RNA-seq revealed that circCCDC66 modulated the expression of various genes associated with cell proliferation and survival by competitively binding multiple miRNAs. The upregulation of circCCDC66 might confer a survival advantage to OXA-resistant CRC cells in response to OXA treatment. Nevertheless, the signaling pathways implicated in circCCDC66-induced chemoresistance are worthy of continued exploration. It seems that circRNAs play crucial roles in fine-tuning the expression of genes associated with cell proliferation by sponging diverse miRNAs. The role of circRNA/miRNA/

mRNA interaction networks in GI cancer cell proliferation and chemoresistance needs to be systematically studied.

Circ-PVT1 was apparently upregulated in DDP-resistant GC tissues and cells compared to chemosensitive samples (Wang et al., 2021e). Downregulation of circ-PVT1 reduced the resistance of GC cells to DDP, thereby attenuating the malignant features of DDP-resistant GC cells. Mechanistically, circ-PVT1 was able to directly target miR-152-3p, which specifically decreased the expression of hepatoma-derived growth factor (HDGF). HDGF functions as an oncogene with abnormally elevated activity in various cancers (Chu et al., 2019). Circ-PVT1 interference promoted the apoptosis of DDP-resistant GC cells by increasing the levels of Bcl-2-associated X protein (Bax) and cleaved caspase-3 and decreasing the level of Bcl-2. Upregulation of miR-152-3p or knockdown of HDGF suppressed the chemoresistance and malignancy of DDP-resistant GC cells, whereas these effects were reversed by circ-PVT1 overexpression. Thus, circ-PVT1 controlled GC chemoresistance via the miR-152-3p/HDGF pathway. Targeting circ-PVT1 in GC may be an attractive treatment strategy.

EC has the sixth highest mortality rate among all malignancies worldwide and can be divided into two major histological types, ESCC and esophageal adenocarcinoma (EAC) (Uhlenhopp et al., 2020). The expression level of circPSMC3 was declined in gefitinib-resistant ESCC cells compared to sensitive cells (Zhu et al., 2021). Upregulation of circPSMC3 improved the sensitivity of ESCC cells to gefitinib. The functional downstream pathway of circPSMC3 was also identified. CircPSMC3 elevated the expression of phosphatase and tensin homolog (PTEN) by lowering miR-10a-5p levels. Restoration of miR-10a-5p expression or knockdown of PTEN significantly counteracted the effect of circPSMC3 overexpression on increasing gefitinib sensitivity in ESCC. Thus, the circPSMC3/miR-10a-5p/PTEN axis might constitute the molecular mechanisms involved in ESCC chemoresistance. PTEN is a well-characterized tumor suppressor with a crucial role in modulating the anti-apoptotic and survival pathways (McLoughlin et al., 2018). Inhibition of PTEN-targeting oncogenic miRNAs is of prime interest as an anticancer treatment. CircPSMC3 post-transcriptionally coordinated PTEN expression via acting as a miRNA sponge. Reactivation of circPSMC3 may potentially be beneficial for the treatment of chemoresistant ESCC.

Uncontrolled cell proliferation plays a crucial role in cancer progression. Many anticancer agents, including alkylating drugs (e.g., DDP and OXA), antimetabolic drugs (e.g., 5-FU) and DNA crosslinking drugs (e.g., DDP and mitomycin C), suppress cancer cell proliferation. Chemoresistant cancer cells can tolerate anticancer drugs to a certain extent. CircRNAs are capable of regulating drug resistance in GI cancer by targeting important genes involved in cell proliferation pathways. Various miRNAs and proteins have been identified as key participants in cancer cell proliferation (Zhang N. et al., 2021). CircRNAs may alter cell proliferation and drug sensitivity in GI cancer by interacting with these miRNAs and proteins. The intertwined competitive endogenous RNA (ceRNA) regulatory axes underlying GI cancer chemoresistance require further study.



### 3.3 Cell Death Pathways

In contrast to 5-FU-sensitive tissues, circ\_0000338 was upregulated in 5-FU-resistant CRC tissues (Zhao et al., 2021). Depletion of circ\_0000338 sensitized 5-FU-resistant CRC cells to 5-FU by promoting the apoptosis and inhibiting the proliferation of CRC cells. Remarkably, circ\_0000338 acted as a ceRNA to soak up miR-217 and miR-485-3p. Silencing of miR-217 or miR-485-3p attenuated circ\_0000338 knockdown-mediated increase in the chemosensitivity of 5-FU-resistant CRC cells. The miRNA sponging activity was crucial for the effect of circ\_0000338 on 5-FU resistance in CRC. However, downstream targets of these two miRNAs were not identified in this study. Thus, further functional and mechanistic studies are required to define the function of circ\_0000338-miR-217/miR-485-3p feedback loops in CRC chemoresistance. Similarly, circ\_0071589 expression was significantly elevated in DDP-resistant CRC tissues compared with sensitive tissues (Zhang W. et al., 2021). Circ\_0071589 increased the expression of the oncogene kruppel-like factor 12 (KLF12) by interacting with miR-526b-3p. Moreover, downregulation of circ\_0071589 inhibited the proliferation and promoted the apoptosis of DDP-resistant CRC cells by downregulating Cyclin D1 and Bcl-2 and upregulating cleaved caspase-3. These effects contributed to the suppression of chemoresistance in DDP-resistant CRC cells. Silencing of miR-526b-3p abolished the effect of circ\_0071589 knockdown on DDP resistance and cancer malignancy in DDP-resistant CRC cells. In addition, circ\_0071589 interference aggravated DDP-induced tumor inhibition in a murine xenograft model. Accordingly, circ\_0071589 was proposed to impair the intrinsic apoptotic pathway and might serve as a novel target for improving the efficacy of chemotherapy in CRC.

CircVAPA was expressed at high levels in GC tissues, and its downregulation enhanced the susceptibility of chemoresistant GC cells to DDP (Deng et al., 2021). CircVAPA upregulated STAT3 by interacting with miR-125b-5p. Consistently, STAT3 downstream proteins, including B-cell lymphoma-extra large (Bcl-xL), myeloid cell leukemia-1 (Mcl-1) and Survivin, were upregulated in DDP-resistant GC cells. As a result, circVAPA promoted the proliferation and inhibited the apoptosis of GC cells. The miR-125b-5p inhibitor or STAT3 upregulation reversed circVAPA knockdown-induced chemosensitivity in GC cells. It was thus proposed that the miR-125b-5p/STAT3 axis mediated the regulatory effects of circVAPA on GC chemoresistance. Another upregulated circRNA in DDP-resistant GC tissues and cells, circDONSON, acted as a molecular sponge by competing for miR-802 binding to affect the expression of its target, B lymphoma Mo-MLV insertion region 1 (BMI1) (Liu Y. et al., 2020). Functionally, circDONSON knockdown sensitized GC cells to DDP via the miR-802/BMI1 axis *in vitro* and *in vivo*. CircDONSON deficiency elevated the levels of cleaved caspase-3, cleaved caspase-9 and p27, while reducing the level of Cyclin D1 in DDP-resistant GC cells. Thus, circDONSON downregulation reduced cell viability and accelerated cell apoptosis in DDP-resistant cells. These results suggested the important role of circDONSON in GC, indicating that it represented a

promising therapeutic target for improving chemotherapy effectiveness in GC patients. In contrast, hsa\_circ\_0000520 was downregulated in Herceptin-resistant GC cells compared with GC cells (Lv et al., 2020). Hsa\_circ\_0000520 overexpression apparently decreased the viability and promoted the apoptosis of chemoresistant GC cells by upregulating Bax and downregulating Bcl-2. Hsa\_circ\_0000520-mediated inactivation of the PI3K/Akt signaling pathway contributed to its anticancer activity. Conversely, induction of the PI3K/Akt pathway eliminated the inhibitory effect of hsa\_circ\_0000520 on Herceptin resistance in GC. Altogether, hsa\_circ\_0000520 enhanced Herceptin sensitivity in GC cells by blocking the PI3K/Akt signaling cascade. CircCCDC66 was upregulated in DDP-resistant GC tissues and cells compared to sensitive samples (Zhang Q. et al., 2020). *In vitro* and *in vivo* experiments indicated that circCCDC66 suppressed GC cell apoptosis and induced DDP resistance in GC cells by targeting miR-618 to facilitate Bcl-2 release. CircHECTD1 was overexpressed in GC tissues in contrast with adjacent normal tissues (Lu et al., 2021). CircHECTD1 deletion expedited the apoptosis of GC cells by upregulating Bax and downregulating Bcl-2. CircHECTD1 increased the expression of pre-B-cell leukemia transcription factor 3 (PBX3) by sponging miR-137. PBX3 is a cancer-relevant protein that has been reported to be associated with cancer cell metastasis (Han et al., 2014). CircHECTD1 absence reinforced diosbulbin-B (DB) sensitivity in GC cells, and this effect was alleviated by a miR-137 inhibitor. CircHECTD1 altered cell viability, apoptosis and drug resistance in DB-induced GC cells, which provided theoretical support for its application as a promising therapeutic target for GC. The key mediators of the intrinsic apoptotic pathway participate in carcinogenesis and chemotherapy resistance and can be targeted for anticancer therapeutic approaches. The multi-targeted strategies may be more efficacious to combat drug resistance in cancer. CircRNAs have the ability to target diverse apoptosis-associated proteins. Substitution of pro-apoptotic circRNAs or inhibition of anti-apoptotic circRNAs could be used to design and develop multi-target-based anticancer strategies for the treatment of GI cancer.

CircMCTP2 was downregulated in DDP-resistant GC tissues and cells (Sun G. et al., 2020). CircMCTP2 inhibited autophagic cell death in DDP-resistant cells by modulating P62 and LC3-II levels. Mechanistically, circMCTP2 increased the level of the autophagy inhibitor myotubularin-related protein 3 (MTMR3) by interacting with miR-99a-5p. CircMCTP2 sensitized GC cells to DDP through suppressing autophagy by restoration of MTMR3 expression. Another downregulated circRNA, circCUL2, induced the upregulation of rho-associated coiled-coil-containing protein kinase-2 (ROCK2) by sponging miR-142-3p (Peng et al., 2020). CircCUL2 inhibited autophagy by regulating miR-142-3p, as reflected by the altered expression of autophagy-related markers, including P62, Beclin 1 and LC3. Consequently, circCUL2 enhanced DDP sensitivity in GC cells via miR-142-3p/ROCK2-mediated

autophagy. Particularly, circCUL2 restricted protective autophagy to increase the chemosensitivity of GC cells. In some cases, autophagy performs an anti-carcinogenic function. The roles of circRNAs in autophagy regulation deserve further attention. Specifically, it is important to discern whether circRNA-mediated autophagy is beneficial or harmful. Additional efforts are required to investigate how circRNAs regulate varied forms of autophagy. The mechanisms by which circRNA-regulated autophagy governs cancer development and chemoresistance have not yet been fully elucidated. Further work is needed to understand the crosstalk between circRNA-associated ceRNA networks and the autophagic flux in chemoresistant GI cancer.

Hsa\_circ\_0008078, also named cDOPEY2, was apparently downregulated in DDP-resistant ESCC cells compared with chemosensitive cells (Liu et al., 2021). cDOPEY2 upregulation markedly strengthened the cytotoxicity of DDP toward DDP-resistant ESCC cells by promoting cell apoptosis, as reflected by the downregulation of the anti-apoptotic protein Mcl-1. Furthermore, cDOPEY2 served as a scaffolding molecule to facilitate the interaction between cytoplasmic polyadenylation element-binding protein 4 (CPEB4) and the E3 ligase tripartite motif-containing protein 25 (TRIM25), leading to the ubiquitination and degradation of CPEB4. The binding of CPEB4 to the *Mcl-1* mRNA enhanced the expression of Mcl-1. cDOPEY2-mediated degradation of CPEB4 abrogated this effect. Gain-of-function experiments showed that the restoration of cDOPEY2 expression alleviated DDP resistance in ESCC by repressing CPEB4-mediated Mcl-1 translation. This study established a previously uncharacterized mechanism underpinning a critical role of circRNAs in altering cancer chemoresistance.

Apoptosis and autophagy are closely associated with carcinogenesis. The crosstalk between apoptosis and autophagy has been characterized (Xie et al., 2020). Apoptosis represents one of the predominant ways of cancer cell death. Autophagy exerts opposite effects on cancer pathogenesis. Autophagy can initiate cancer cell apoptosis in combination with anticancer drugs. In some circumstances, autophagy suppresses chemotherapeutic agent-induced apoptosis in cancer. Autophagy constitutes a major factor contributing to cancer drug resistance. Studies revealing the interplay between apoptosis and autophagy facilitate improvements in the therapeutic efficacy of anticancer agents against cancer. The cellular apoptosis and autophagy pathways may be modulated by shared regulatory factors and signal transduction cascades, including ncRNAs, p53 and the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway. CircRNAs have emerged as upstream effectors of cell death pathways in GI cancer. CircRNAs possess the ability to tip the balance between cell death signalings and hence may improve the efficacy of anticancer medications. It is unclear whether circRNAs simultaneously regulate the apoptotic and autophagic pathways in GI cancer by controlling the same factor or signaling pathway. Crosstalk between ncRNAs affecting apoptosis and autophagy may exist.

Thus, the impact of circRNAs on the interaction pattern between apoptosis and autophagy in GI cancer necessitates further exploration, which will provide a basis for the development of more effective therapeutic strategies to combat chemoresistant GI cancer.

### 3.4 Glycolysis

Hypoxia-induced chemoresistance has been considered a major hurdle to the development of successful therapy for GC (Karakashev and Reginato, 2015). Xu et al. (Xu et al., 2020) found that circNRIP1 was expressed at high levels in hypoxic GC cells and promoted hypoxia-induced 5-FU resistance in GC cells. Mechanistically, circNRIP1 acted as a miR-138-5p sponge and modulated its target, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). Glycolysis is the predominant energy-generating pathway in hypoxic cancer cells, leading to the development of hypoxia-induced chemoresistance and the survival of cancer cells under hypoxic conditions (Tavares-Valente et al., 2013; Bhattacharya et al., 2014). HIF-1 $\alpha$  plays a key role in modulating glycolysis and is involved in cancer drug resistance (Shukla et al., 2017). Thus, HIF-1 $\alpha$ -dependent glucose metabolism might contribute to hypoxia-induced chemoresistance in GC. Consistent with this hypothesis, pharmacological blockade of glycolysis reversed the effects of circNRIP1 on hypoxia-induced 5-FU resistance in GC cells, suggesting that circNRIP1 had emerged as an important regulator of hypoxia-induced chemoresistance via HIF-1 $\alpha$ -mediated glucose metabolism in GC by targeting miR-138-5p (Xu et al., 2020).

Hypoxia is a common microenvironmental feature in solid tumors (Masoud and Li, 2015). Cancer cells can activate the transcription factor HIF-1 $\alpha$  under hypoxic conditions. HIF-1 $\alpha$  mediates metabolic switching by upregulating glucose transporters and glycolytic enzymes, favoring the adaptation of cancer cells to hypoxia and eventually inducing drug resistance (Zheng et al., 2021). Glycolysis meets the energetic demands for cellular functions and establishes biological blocks for cancer cells, leading to the rapid growth of cancer cells (Beltran-Anaya et al., 2016). A number of enzymes are implicated in glucose metabolism, such as hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH). Targeting glycolytic enzymes with ncRNAs would add to the complexity of the glucose metabolism process in hypoxic cancer cells. Existing evidence has suggested the effect of the circRNA/miRNA/HIF-1 $\alpha$  regulatory axis on hypoxia-induced drug resistance in GC. The expression and activity of the downstream genes responsible for catalyzing glucose metabolism remain to be validated. In addition, the direct impact of circRNAs on glycolytic enzymes merits additional research.

### 3.5 Self-Renewal of Cancer Stem Cells

CSCs, also known as tumor-initiating cells (TICs), are a unique subpopulation of self-renewing cells with high carcinogenic potential and higher resistance to conventional therapies than other cells within a tumor (Najafi et al., 2019). Due to their self-renewing property and ability to differentiate into heterogeneous lineages of cancer cells, CSCs are responsible for tumor

progression and recurrence (Phi et al., 2018). CSC-mediated chemoresistance can be partially explained by their quiescence or dormancy, increased drug efflux and avoidance of harmful stresses (Li et al., 2021). Recent evidence suggests that circRNAs are critical regulators of CSC growth that modify drug resistance in GI cancer. A higher expression level of hsa\_circ\_001680 was observed in CRC tissues than in matched adjacent normal tissues (Jian et al., 2020). *In vitro* and *in vivo* evidence showed that hsa\_circ\_001680 overexpression promoted the proliferation and migration of CRC cells. BMI1 is a critical transcription factor required for the maintenance and self-renewal of CSCs (Wang et al., 2016). Hsa\_circ\_001680 enhanced the CSC population of CRC cells and thus contributed to irinotecan chemotherapy resistance in CRC cells by sponging miR-340 to increase BMI1 expression (Jian et al., 2020). Hsa\_circ\_001680 were able to control the growth of CSCs in CRC by inducing the key regulator of CSC self-renewal, which supported the maintenance of CSC stemness and chemoresistance.

CircFAM73A was apparently upregulated in GC, and its upregulation was strongly correlated with the poor prognosis of GC patients (Xia et al., 2021). CircFAM73A fostered GC cell proliferation, migration and DDP resistance. Moreover, circFAM73A boosted stem cell-like properties in GC cells by upregulating stemness-related transcription factors (e.g., Nanog, OCT4 and SOX2). A subsequent functional study indicated that circFAM73A coordinated the expression of high mobility group A2 (HMGA2) by sequestering miR-490-3p. *In vitro* and *in vivo* experimental results revealed that the positive effect of circFAM73A on GC cell self-renewal and malignancy was counteracted by HMGA2 depletion. Thus, circFAM73A regulated the CSC-like properties and malignant behaviors of GC by increasing HMGA2 expression. Additionally, circFAM73A bound to heterogeneous nuclear ribonucleoprotein K (HNRNPK) and facilitated  $\beta$ -catenin stabilization, hence aggrandizing the CSC-like properties of GC. CircFAM73A targeted stemness-related proteins through its miRNA sponging and protein binding activities, suggesting its broad implication in CSC self-renewal. Altogether, circFAM73A strengthened the self-renewal capability of GC cells, leading to DDP resistance and cancer progression.

CSCs are implicated in cancer initiation and development, as well as the acquisition of drug resistance. CSCs have been considered a promising therapeutic target for conquering cancer drug resistance. CircRNAs regulate the growth of CSCs in GI cancer by targeting stemness-related transcription factors and relevant miRNAs. Nevertheless, the multifaceted contributions of circRNAs to regulating CSC biology have not been fully delineated thus far. Additional studies should be conducted to elucidate the functional activity of circRNAs in modifying CSC features in GI cancer. Various cellular molecules and signal transduction cascades have been reported to be involved in the regulation of CSC functions, including Bcl-2, STAT3 and transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling (Kyriazi et al., 2020). The mechanisms by which CSCs facilitate cancer drug resistance include the induction of the epithelial-mesenchymal transition (EMT) program, upregulation of multidrug resistance (MDR) proteins, and

regulation of the tumor environment (e.g., hypoxia and inflammation) (Phi et al., 2018). Ongoing studies are critical to explore whether circRNAs interfere with a cascade of signaling events contributing to CSC-mediated drug resistance and subsequent cellular effects in GI cancer. CSC-targeted therapeutic approaches have tremendous potential to completely eliminate cancer cells. CircRNAs have been identified as novel targets for affecting CSC properties in GI cancer. Therapies based on modulation of stemness-associated circRNAs may be feasible and effective in specifically eradicating progenitor cells and CSCs. Nevertheless, it should be noted that the tumor heterogeneity and ever-changing tumor microenvironment pose huge challenges for therapies targeting CSCs. Therefore, a detailed understanding of CSC characteristics and tumor microenvironment is a significant prerequisite for developing CSC-targeted therapeutic approaches against GI cancer.

### 3.6 Epithelial-Mesenchymal Transition, Invasion and Metastasis

The expression of circ-PRKDC was significantly upregulated in 5-FU-resistant CRC tissues and cells compared with sensitive CRC samples (Chen et al., 2020). Silencing of circ-PRKDC sensitized 5-FU-resistant CRC cells to 5-FU and inhibited cell invasion. Circ-PRKDC deficiency repressed the Wnt/ $\beta$ -catenin pathway by regulating the miR-375/forkhead fox protein M1 (FOXO1) axis. Deletion of miR-375 abolished the inhibitory effects of circ-PRKDC knockdown on CRC chemoresistance and cell invasion in 5-FU-resistant cells. Consequently, circ-PRKDC exerted a positive role in 5-FU resistance in CRC by orchestrating the miR-375/FOXO1 axis and the Wnt/ $\beta$ -catenin pathway. Zinc finger E-box-binding homeobox 1 (ZEB1) is an important transcriptional repressor of E-cadherin that accelerates the EMT program, migration and invasion in GC (Jia et al., 2012). Circ-PVT1 was overexpressed in paclitaxel (PTX)-resistant GC cells and increased ZEB1 expression by tethering miR-124-3p (Liu et al., 2019). Silencing of circ-PVT1 reduced PTX resistance, enhanced PTX-induced apoptosis and blocked the invasion of GC cells, while these effects were counteracted by miR-124-3p downregulation. Inhibition of ZEB1 improved the sensitivity of PTX-resistant GC cells to PTX. Approaches targeting circ-PVT1 might represent a promising therapeutic strategy for GC. The Wnt/ $\beta$ -catenin signaling cascade is considered critical to the activation of EMT in cancer. CircRNAs can hijack the Wnt/ $\beta$ -catenin pathway to regulate cancer cell invasion, metastasis and drug resistance by targeting key proteins and transcription factors involved in this pathway.

Circ\_ASAP2 was expressed at high levels in DDP-resistant GC tissues and cells (Sun et al., 2021). Circ\_ASAP2 silencing enhanced DDP sensitivity and apoptosis, and retarded the proliferation, migration and invasion of DDP-resistant GC cells. In terms of mechanism, circ\_ASAP2 directly targeted miR-330-3p to upregulate the expression of ecto-5'-nucleotidase (NT5E), which is associated with tumor invasion and metastasis (Wang et al., 2008). Circ\_ASAP2 enhanced DDP

resistance and promoted the functional behaviors of resistant GC cells by targeting the miR-330-3p/NT5E axis. Circ\_0000260 also showed higher expression levels in DDP-resistant GC tissues than in sensitive tumor tissues (Liu S. et al., 2020). Matrix metalloproteinase 11 (MMP11) functions as a key driver of cancer development and metastasis (Yang et al., 2019). *In vitro* and *in vivo* experimental studies verified that circ\_0000260 knockdown reduced DDP resistance and impeded the malignancy of resistant GC cells by modulating the expression of miR-129-5p and its target MMP11. These results revealed a vital mechanism underlying the role of circ\_0000260 in DDP resistance of GC. Circ\_0006168 was upregulated in PTX-resistant ESCC tissues compared to oesophageal epithelial cells and sensitive ESCC cells (Qu et al., 2021). Circ\_0006168 depletion enhanced the cytotoxicity of PTX toward resistant ESCC cells. It sponged miR-194-5p to upregulate jumonji domain containing 1C (JMJD1C). Downregulation of JMJD1C improved PTX sensitivity and suppressed the malignant behaviors of PTX-resistant ESCC cells. Circ\_0006168 deficiency restrained tumor growth *in vivo* by increasing miR-194-5p expression and reducing JMJD1C expression. The specific physiological mechanisms underlying the roles of circ\_0006168 in PTX resistance of ESCC require additional exploration. The clinical significance of circ\_0006168 remains to be further validated. In some cases, metastatic cancer cells are more resistant to anticancer drugs relative to non-metastatic cells (Liang et al., 2002). Conversely, chemoresistant cancer cells are prone to be more invasive and metastatic than sensitive cancer cells. Increasing knowledge on the participation of circRNAs in controlling cancer invasion/metastasis could help to understand the mechanisms underlying circRNA-mediated drug resistance in GI cancer.

EMT is a complicated process in which epithelial cells acquire the characteristics of invasive mesenchymal cells (Du and Shim, 2016). EMT is indispensable for cancer cell invasion and metastasis. The causal linkage between EMT and cancer chemoresistance has been increasingly recognized. Cancer cells undergoing EMT display a chemoresistant phenotype similar to CSCs (Du and Shim, 2016). EMT-driven chemoresistance involves the acquisition of resistance to anticancer agent-induced apoptosis. In addition, the tumor microenvironment (e.g., fibroblasts and hypoxia) is a significant factor contributing to EMT-mediated chemoresistance. Multiple lines of evidence have shown that circRNAs are important regulators of the EMT program, invasion and metastasis of GI cancer cells. Not surprisingly, these circRNAs influence the sensitivity of GI cancer cells to chemotherapy. However, the specific mechanisms by which circRNA-regulated EMT alters drug resistance in GI cancer are largely equivocal, and thus follow-up investigations are required.

### 3.7 Drug Efflux

Adenosine triphosphate (ATP)-binding cassette (ABC) subfamily C member 5 (ABCC5), also known as multidrug resistance protein 5 (MRP5), is a member of the ABC transporter family that regulates the efflux of toxins and drugs (Jansen et al., 2015). The expression of circ\_0007031 was positively associated with 5-

FU resistance in CRC (He et al., 2020). Circ\_0007031 downregulation inhibited CRC cell proliferation and malignancy, and enhanced 5-FU sensitivity. Circ\_0007031 functioned as a ceRNA to increase ABCC5 expression by competitively binding to miR-133b in CRC. The effect of circ\_0007031 on enhancing drug resistance in CRC was likely attributable to ABCC5-mediated drug efflux. Nevertheless, intracellular 5-FU accumulation in circ\_0007031-knockdown CRC cells should be determined in future studies to provide direct evidence supporting the specific role of circ\_0007031 in drug efflux.

The induction of drug efflux is a well-characterized mechanism underlying cancer chemoresistance. ABC transporters are a large and common superfamily of proteins that exploit the energy produced by ATP hydrolysis to export various cytotoxic substances from cells (Zappe and Cichna-Markl, 2020). These efflux pumps diminish the intracellular accumulation of anticancer agents and protect cancer cells from chemotherapeutic medications. Reportedly, chemoresistant cancers exhibited alleviated epigenetic inhibition of *MDR1* through promoter hypomethylation and histone acetylation (Sharma et al., 2010; Toth et al., 2012). CircRNAs play a critical role in the epigenetic modulation of gene expression (Zeng et al., 2021). It is intriguing whether circRNAs drive the epigenetic alteration of efflux pump genes. Further efforts are urgently needed to elucidate the epigenetic mechanism by which circRNAs control the expression of drug efflux genes.

### 3.8 Exosomal Circular RNA-Mediated Chemoresistance

Exosomes are nano-sized extracellular biovesicles of endocytic origin that are shed by most types of cells and circulate in body fluids (Chivet et al., 2014). Exosomes carry a variety of molecular and genetic components of their cells of origin, including lipids, proteins and ncRNAs (Chen et al., 2021). These vesicles may transmit multiple signals that affect cancer development and chemoresistance (Wang et al., 2019). Importantly, exosomes transport chemoresistance-relevant ncRNAs between cancer cells. Exosomal circRNAs have been shown to play a key role in mediating drug resistance transfer in GI cancer (Wang H. et al., 2021). The combination of leucovorin (LV) and 5-FU with OXA (FOLFOX) is a first-line therapeutic regimen for CRC (Guan et al., 2020). The microarray profiles of exosomal circRNAs in FOLFOX-resistant and sensitive cells were previously explored (Hon et al., 2019). In total, 139 circRNAs were aberrantly expressed in FOLFOX-resistant CRC cell-derived exosomes, including 105 upregulated and 34 downregulated circRNAs. Among these, circ\_0000338 was markedly upregulated in FOLFOX-resistant CRC cell-derived exosomes compared with sensitive cell-originated exosomes. Chemoresistant CRC cell-derived exosomes transported circRNAs into sensitive CRC cells and increased the viability of recipient cells in the presence of 5-FU. Thus, FOLFOX-resistant CRC cell-secreted exosomes conferred drug resistance to sensitive CRC cells via the selective transfer of circRNAs. The loss-of-function study showed



that knockdown of circ\_0000338 decreased the viability of FOLFOX-resistant CRC cells in the presence of 5-FU. Thus, circ\_0000338 might be involved in CRC chemoresistance. The exact roles of circ\_0000338 in CRC chemoresistance deserve further study. Moreover, the impact of circ\_0000338 on cellular signaling cascades remains to be studied.

The production of ATP through aerobic glycolysis is required for the growth and acquisition of chemoresistance in CRC (Wang X. et al., 2020). The M2 isoform of pyruvate kinase (PKM2) plays an important role in aerobic glycolysis (Chaneton and Gottlieb, 2012). The circRNA ciRS-122 was reported to increase PKM2 expression by decoying miR-122 (Wang X. et al., 2020). Both *in vitro* and *in vivo* studies demonstrated that OXA-resistant CRC cells transferred ciRS-122 to sensitive cells via exosomes, and exosomal ciRS-122 fostered glycolysis and OXA resistance in sensitive cells, as reflected by increases in glucose uptake, lactate, ATP production and tumor growth. In summary, exosomal ciRS-122 weakened drug susceptibility in recipient CRC cells by targeting the miR-122/PKM2 pathway. PKM2 expedites cancer growth, metastasis and chemoresistance by altering cancer cell metabolism or cellular signaling pathways. Blockade of PKM2 activity was shown to repress glycolysis and override drug resistance in cancer (Li et al., 2016). Hence, ciRS-122 might serve as a promising target for sensitizing CRC cells to chemotherapy. The expression of circ-FBXW7 was declined in OXA-resistant CRC tissues and cells compared with sensitive samples (Xu et al., 2021). Exosome-mediated delivery of circ-FBXW7 from normal colon cells to CRC cells reduced drug efflux and conferred chemosensitivity to OXA-resistant CRC cells by reducing the expression of MRP1 and Mcl-1. Exosomal circ-FBXW7 promoted OXA-induced apoptosis by increasing the levels of cleaved caspase-3. It also impeded the migration and invasion of OXA-resistant CRC cells by blocking the EMT program. Overexpression of miR-18b-5p, the downstream target of circ-FBXW7, overturned circ-FBXW7-induced sensitivity to OXA and thus attenuated the anticancer effects of circ-FBXW7. *In vivo* experimental evidence also confirmed that exosomal circ-FBXW7 reversed OXA resistance and inhibited CRC growth partially by tethering miR-18b-5p. Circ-FBXW7 induced chemosensitization of CRC cells by governing drug transport, cell apoptosis and the EMT process. Circ-FBXW7 might have a potential application in CRC therapy. However, additional studies are needed to screen and validate the downstream effectors of the circ-FBXW7/miR-18b-5p regulatory axis.

Circ\_0032821, a circRNA that was expressed at high levels in OXA-resistant GC cells, was also associated with GC chemoresistance (Zhong et al., 2021). Circ\_0032821 was mainly secreted from GC cells via exosomes. The expression level of exosomal circ\_0032821 secreted by resistant cells was significantly higher than that secreted by sensitive cells. OXA-resistant GC cell-derived exosomal circ\_0032821 might be incorporated into sensitive GC cells. Exosomal circ\_0032821 aggravated OXA resistance, cell proliferation, migration and invasion in sensitive GC cells. Mechanistically, circ\_0032821 upregulated the tumor promoter SRY-box transcription factor 9 (SOX9) by sequestering miR-515-5p. Notably, miR-515-5p

was previously identified to function as a repressor of GC progression (Wang D. et al., 2020). It was proposed that circ\_0032821 modulated cancer progression and the development of OXA resistance in GC through the miR-515-5p/SOX9 axis. Circ-PVT1 was highly expressed in exosomes from serum samples of DDP-resistant GC patients and from DDP-resistant GC cells (Yao W. et al., 2021). YAP1 is a crucial effector of the Hippo signaling pathway and participates in carcinogenesis (Shibata et al., 2018). Circ-PVT1 regulated YAP1 expression by targeting miR-30a-5p. Circ-PVT1 interference reduced LC3-II/I and P-glycoprotein (P-gp) expression but increased P62 expression in DDP-resistant cells. These events led to decreased DDP resistance in DDP-resistant GC cells, which could be reversed by a miR-30a-5p inhibitor or YAP1 overexpression. Thus, circ-PVT1 deletion increased DDP sensitivity in DDP-resistant GC cells by facilitating apoptosis and inhibiting autophagy or invasion through the miR-30a-5p/YAP1 axis. Exosomal circRNAs enhanced chemoresistance of GC cells through promotion of malignancy and cancer progression, inhibition of apoptosis or enhancement of autophagy.

The expression level of circ\_0000337 was higher in exosomes from DDP-resistant ESCC cells than in those from sensitive ESCC cells (Zang et al., 2021). Exosomes derived from DDP-resistant ESCC cells induced sensitive cells to develop DDP resistance by delivering circ\_0000337. Mechanistic investigation indicated that circ\_0000337 interacted with miR-337-3p in ESCC cells, and miR-337-3p overexpression mitigated exosomal circ\_0000337-mediated DDP resistance by targeting the oncogenic Janus kinase 2 (JAK2). Coincidentally, xenograft results proved that exosomal circ\_0000337 facilitated tumor growth and DDP resistance in ESCC *in vivo*. These results pointed to a potential role of exosomal circ\_0000337 in the development of DDP resistance in ESCC.

Collectively, exosomes contribute to spreading chemoresistant phenotypes from chemoresistant cancer cells to chemosensitive cells by delivering circRNAs. Exosomal circRNAs are anticipated to be promising therapeutic targets for the treatment of drug-resistant GI cancer due to their high stability, tissue/cell specificity and functional diversity. Even with these encouraging results, many obstacles must be addressed. Standardized approaches for isolating, collecting and quantifying exosomes and their ncRNA cargos must be developed. Based on accumulating evidence, exosomal circRNAs perform biological functions by decoying miRNAs. It is elusive whether they can act as protein scaffolds or translation templates. The detailed mechanisms associated with the enrichment of circRNAs in exosomes remain largely unknown. Given the low abundance of exosomal circRNAs, much work is required to ascertain the genuine contribution of exosomal circRNAs to the transmission of drug-resistant phenotypes among cancer cells. Finally, clinical studies with large cohorts should be undertaken to clarify the therapeutic potential of exosomal circRNA-based anticancer therapies. Despite their great



prospects, there is still a long way to go before turning the promise of exosomal circRNA-based therapeutics into clinical reality.

## 4 CONCLUSION AND FUTURE PERSPECTIVES

Chemoresistance has become a major hurdle undermining the efficacy of cancer chemotherapy. It is essential to elucidate the mechanisms associated with cancer chemoresistance, which will accelerate the development of improved therapeutic approaches for cancer. According to the body of evidence described above, circRNAs play key roles in GI cancer chemoresistance. Specifically, circRNAs affect DNA damage repair pathways, cell viability and proliferation, cell death pathways, glucose metabolism, stem cell-like properties, EMT, cell invasion and metastasis, and drug efflux in several GI cancers (Figure 2). The mechanisms underpinning the roles of circRNAs in GI cancer chemoresistance are quite complicated, and concerted research efforts are warranted to obtain a thorough understanding of the relationships between circRNAs and chemoresistance in GI cancer.

The profound impacts of circRNAs on drug resistance in GI cancer make them promising therapeutic targets for GI cancer treatment. However, there are still several issues that need to be addressed before circRNA-based therapeutics can be clinically applied to treat GI cancer. First, comprehensive profiles of the expression patterns of circRNAs between chemoresistant and sensitive GI cancers are needed. Due to the close linkage between aberrantly expressed circRNAs and the drug resistance of GI cancers, screening and identification of key deregulated circRNAs will be conducive to seeking potential therapeutic targets for chemoresistant GI cancer. Moreover, further studies should focus on validating the expression and function of deregulated circRNAs in GI cancer. Second, the mechanisms of action of circRNAs in chemoresistant GI cancer remain largely obscure, and more studies are needed to explain the underlying mechanisms. Remarkably, current studies examining the molecular mechanisms of circRNA-associated chemoresistance have mainly focused on the miRNA sponging function of circRNAs. It is of great importance to substantiate whether circRNAs regulate GI cancer chemoresistance through other mechanisms, including transcriptional modulation or the generation of functional proteins. Extensive investigations should be conducted to completely unveil the mechanisms responsible for the implication of circRNAs in GI cancer chemoresistance. Third, the roles of specific circRNAs in MDR of GI cancer require in-depth research. MDR is defined as the resistance of cancer cells to various chemotherapeutic agents with distinct structures and modes of action (Baguley, 2010). MDR severely weakens the efficacy of clinical chemotherapy in cancer treatment (Bukowski et al., 2020). Nevertheless, relatively few studies have described the role of circRNAs in regulating MDR in GI cancer. It stresses a critical need of the characterization of key circRNAs in GI cancer exhibiting the MDR phenotype. Because circRNAs may target the same molecule or signaling pathway in different cancers, certain circRNAs will likely have an identical regulatory role in various GI cancers exposed to different chemotherapies. Therefore, future studies should be dedicated to discovering common circRNAs in different GI

cancers and to defining their contributions to cancer chemotherapy responsiveness, which will expand our comprehension of the explicit mechanisms behind drug resistance in GI cancer.

Finally, the circRNA-associated regulatory axes that are activated during the development of chemoresistance in GI cancer must be systematically delineated. It has been generally accepted that ncRNAs play a momentous role in regulating nearly all cancer hallmarks. Deciphering intricate ncRNA regulatory networks has become a significant critical research direction in the field of oncology. The upstream effectors of circRNAs involved in GI cancer chemoresistance have yet to be identified. The molecules that regulate circRNA biogenesis may be potentially related to the acquisition of drug resistance in GI cancer. Considering the involvement of RBPs in circRNA formation, continuous studies are required to determine whether RBPs can modify drug susceptibility in GI cancer by regulating the abundance of circRNAs. At present, many gaps exist in our knowledge of the mechanisms regulating circRNA biogenesis. Thus, considerable efforts are warranted to delve into the biogenetic process of circRNAs. Significant improvements in the knowledge of circRNA biology will broaden our understanding of the impact of the mechanisms regulating circRNA biogenesis on chemotherapy resistance in cancer. The ceRNA regulatory network plays an important role in cancer pathogenesis. Long noncoding RNAs (lncRNAs) and circRNAs control the expression of diverse mRNAs by acting as ceRNAs for miRNAs. They may compete with the same miRNA response element (MRE) to dominate the expression of miRNA downstream target genes. A large number of reports have indicated a causal relationship between lncRNA dysregulation and chemotherapy responsiveness in cancer. The crosstalk between the circRNA/miRNA/mRNA and lncRNA/miRNA/mRNA pathways is still fairly elusive. The intricate ceRNA regulatory circuits involved in cancer chemoresistance merit intensive investigation. A comprehensive exploration of the circRNA-associated ceRNA network will help to characterize the reciprocal interactions between ncRNAs and mRNAs and provide novel insights into the molecular mechanisms underlying chemoresistance in GI cancer. Even with these challenges, it is also believed that circRNA-based therapeutics could be an effective supplement to conventional treatment to overcome drug resistance in cancer patients.

## AUTHOR CONTRIBUTIONS

MW and KW conceived this study. MW drafted the manuscript and prepared the figures. FY, YZ, and LZ collected the related papers and helped to prepare the figures. KW and WC revised the manuscript. All authors reviewed and approved the final manuscript.

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# miR-30d-5p: A Non-Coding RNA With Potential Diagnostic, Prognostic and Therapeutic Applications

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Cancer is a great challenge facing global public health. Scholars have made plentiful efforts in the research of cancer therapy, but the results are still not satisfactory. In relevant literature, the role of miRNA in cancer has been widely concerned. MicroRNAs (miRNAs) are a non-coding, endogenous, single-stranded RNAs that regulate a variety of biological functions. The abnormal level of miR-30d-5p, a type of miRNAs, has been associated with various human tumor types, including lung cancer, colorectal cancer, esophageal cancer, prostate cancer, liver cancer, cervical cancer, breast cancer and other types of human tumors. This reflects the vital function of miR-30d-5p in tumor prognosis. miR-30d-5p can be identified either as an inhibitor hindering the development of, or a promoter accelerating the occurrence of tumors. In addition, the role of miR-30d-5p in cell proliferation, motility, apoptosis, autophagy, tumorigenesis, and chemoresistance are also noteworthy. The multiple roles of miR-30d-5p in human cancer suggest that it has broad feasibility as a biomarker and therapeutic target. This review describes the connection between miR-30d-5p and the clinical indications of tumors, and summarizes the mechanisms by which miR-30d-5p mediates cancer progression.

**Keywords:** MiR-30d-5p, human cancer, cancer therapy, tumor progression, prognosis

## INTRODUCTION

Cancer remains an intractable disease worldwide, owing to the fact that its causes are not fully understood, and tumors are often found at an advanced stage, which makes treatment arduous (Siegel et al., 2019; Wu et al., 2019). Several methods have been conceived to treat malignant tumors, such as surgery, radiation, chemotherapy, immunotherapy, or photodynamic therapy (Mun et al., 2018; Zehnder et al., 2018). In particular, cancer immunotherapy has expanded the scope of tumor-targeted therapies (Van Den Bulk et al., 2018). In recent years, cancer therapy has evolved from relatively nonspecific cytotoxic drugs to selective, mechanism-based approaches (Vanneman and Dranoff, 2012). However, the drug resistance that cancer cells acquire during treatment makes the treatment less effective (Cui et al., 2018). Epigenetic abnormalities have been shown to be associated with cancer progression and have been the focus of some researches (Qu et al., 2013; Kanwal et al., 2015). In addition, some of the above treatments are only suitable for patients diagnosed at an early stage (Anwanwan et al., 2020). Timely diagnosis is therefore crucial, nevertheless, it is not frequently achieved in many cancers, such as liver and gastric cancer (Song et al., 2017). There is growing evidence that cancer screening assists with the reduction of cancer-related morbidity and mortality (Loud and



Murphy, 2017). Thus, finding biomarkers of malignancy is pivotal for prompt diagnosis and subsequent treatment.

Non-coding RNAs are a class of RNAs that do not participate editing proteins and can be divided into three categories according to length. One is less than 50 nt, including microRNA, siRNA, piRNA, etc. The second is 50 nt to 500 NT, including rRNA, tRNA, snRNA, snoRNA, SLRNA, SRPRNA, and so on. The third is greater than 500 nt (Ma et al., 2013; Cech and Steitz, 2014; Hombach and Kretz, 2016; Paskeh et al., 2021). As miRNA sponges, circRNAs inhibit the activity of miRNAs and regulate gene expression (Panda, 2018; Yu and Kuo, 2019). And the regulation of lncRNAs and miRNAs have also been reported in a variety of cancers (Dong et al., 2019; Xu et al., 2019; Ghafouri-Fard et al., 2021). MiRNAs, a kind of endogenous non-coding RNAs consisting of 21-23 nucleotide sequences, participate in the regulation of gene expression (Zamani et al., 2020; Mirzaei et al., 2021a). They control post-transcriptional regulatory factors of gene expression and the translation process of target mRNAs via attachment of mRNA molecules at the 3'-UTR bases, thus reducing transcription of proteins (Lagos-Quintana et al., 2001; Bartel, 2004; Pu et al., 2019; Alizadeh-Fanalou et al., 2020). In addition, miRNAs bind to the 5'-UTR of mRNAs, which is a path to reduce protein transcription (O'Brien et al., 2018; Ashrafizadeh et al., 2021). MiRNAs were shown to be involved in cell proliferation (Cai et al., 2009; Wang et al., 2019b), differentiation (Shi et al., 2016; Salunkhe and Vaidya, 2020), apoptosis (Zhang et al., 2019) and other cellular activities in tumors (Mishra et al., 2016; Mirzaei et al., 2021b). In addition, There are mRNAs, miRNAs and ncRNAs in the lumen of exosomes, and exosomes fuse with cells to regulate recipient cells (Momen-Heravi et al., 2014; Zhang et al., 2015). The role of exosome miRNAs in tumors have been gradually revealed (Ashrafizadeh et al., 2021). For example, Li et al. confirmed the influence of dysregulation of exosome miRNAs on hepatocellular carcinoma and discovered the clinical significance of exosome miRNAs in the diagnosis of hepatocellular carcinoma (Li et al., 2018). Overall, the abnormalities of miRNAs have been associated with the occurrence and development of numerous diseases, especially human cancers (McGuire et al., 2015; Annamareddy and Eapen, 2017).

MiR-30d-5p is located on chromosome 8q24.22, and the genetic symbol is SLC7A5 (Li et al., 2017; Zhang et al., 2017), and is regulated by LINCRNA in a variety of cancers. The microRNA Cluster was miR-30 b/d, and the seed family of miR-30d-5p included miR-30abcdef/384-5p (Li et al., 2017). MiR-30d-5p is regulated by LncRNA PVT1 in gallbladder cancer (Yu et al., 2018), LncRNA SOX2-OT/miR-30d-5p is associated with the progression of non-small cell lung cancer (Chen et al., 2021), and LINC00284 to miR-30d-5p regulation is associated with the development of thyroid cancer (Hu C. et al., 2021). Growing evidences have highlighted the influence of miR-30d-5p on cell activities and its role in the occurrence and development of different cancer types, therefore, it could serve as a novel biomarker of diagnosis. For example, miR-30d-5p was targeted to inhibit cell activity in non-small cell lung cancer (Kranjc et al., 2020), and was also involved in the construction of

prostate cancer diagnosis and a disease stratification model (Song et al., 2018). MiR-30d-5p was also reported to participate in the progression in esophageal squamous cell carcinoma (Zhu et al., 2017). Several studies demonstrated the connection between the dysregulation of tumor progression and miR-30d-5p, which was therefore considered as a potentially pivotal tumor biomarker.

This review aims to provide additional clues for future research on miR-30d-5p, and consists of two parts: the first one describes the connection between miR-30d-5p and clinical processes in different types of cancer, and the other summarizes the regulatory mechanisms involving miR-30d-5p.

## EXPRESSION OF MIR-30D-5P AND CLINICAL CHARACTERISTICS OF MULTIPLE HUMAN CANCERS

Growing evidence have revealed miR-30d-5p is likely to be related to clinical features. The following is an overview of the association between different types of cancers and miR-30d-5p. A summary of miR-30d-5p expression and cancer-related clinical indicators is provided in **Table 1**.

### Lung Cancer

The global incidence of lung cancer is extremely high. Pathologically, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and the vast majority of lung cancer patients are diagnosed with the latter (Chen et al., 2014). Lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) are the common types of NSCLC, which is considered as clinically the most commonly encountered type of lung tumor (Kinoshita et al., 2015; Goodwin et al., 2017; Gelatti et al., 2019). Despite the premise of continuous exploration, the prognosis of this disease is poor, and its 5-year survival rate is unsatisfactory (Garon et al., 2019). According to relevant reports, the ectopic level of miR-30d-5p impeded tumor progression via inhibiting the invasion and motility of tumor (Chen et al., 2015). By targeting CCNE2, miR-30d-5p was shown to have an anticancer role (Li et al., 2017; Hosseini et al., 2018). More importantly, since the expression level of miR-30d-5p in tumors clearly differed from that in surrounding tissues, the expression of miR-30d-5p in tumor tissue decreased significantly (Zhao et al., 2021). Therefore, it was considered as a possibly important factor connected with the clinical diagnosis and treatment of NSCLC (Zhang et al., 2017; Gao et al., 2018). Interestingly, the content of miR-30d-5p varied between different grades of NSCLC, indicating that miR-30d-5p was associated with the occurrence and progression of NSCLC (Xue et al., 2021; Zhao et al., 2021). In addition, Qi et al. indicated that the expression of miR-30d-5p in LUSC cells was significantly downregulated, and the proliferation, migration and invasion of tumor cells were subsequently reduced, indicating that miR-30d-5p had a clear antitumor effect (Qi et al., 2021). Luciferase experiments also verified the targeted binding of miR-30d-

**TABLE 1 |** The relationship between miR-30d-5p expression and clinical characteristics in multiple cancers.

Cancer type	Property	Studied species and biomaterial	Expression	Prognosis	Number of cases	Refs
Lung cancer	Suppressor	Human tumorous tissue	Down	Favorable	24	Hosseini et al. (2018)
	Oncogene	NSCLC cell lines and body fluid	Down	Poor	—	Li et al. (2017)
	Suppressor	Human NSCLC tissue and cell lines	Down	Favorable	80	Zeng et al. (2020)
	—	HEK 293T cells	Down	—	—	Chen et al. (2015)
	—	Human NSCLC tissue and cell lines	Down	—	—	Gao et al. (2018)
	Suppressor	Human NSCLC tissue	Down	Favorable	81	Zhang et al. (2017)
	Suppressor	Human LUSC cell lines	Down	Favorable	—	Qi et al. (2021)
	Suppressor	Human LUAD tissue and cell lines	Down	Favorable	20	Zhao et al. (2021)
	Suppressor	Human NSCLC cell lines	Down	—	—	Chen et al. (2021)
Gallbladder carcinoma	Suppressor	Human GBC tissue and cell lines	Down	Favorable	53	He et al. (2018)
Cholangiocarcinoma	Oncogene	Human bile and serum	Up	Poor	106	Han et al. (2020a)
Rectal cancer	—	Human CRC tissue and cell lines	Down	—	—	Bjørnstrøm et al. (2019)
Colon cancer	—	Human tumorous tissue	—	—	396	Jacob et al. (2017)
	Suppressor	Human tumorous tissue and cell lines	Down	Favorable	60	Yu et al. (2018)
Prostate cancer	Suppressor	Human PCa tissue and PC cell lines	Down	Favorable	40	Song et al. (2018)
	—	Human PCa cell lines	Down	—	—	Kumar et al. (2016)
	—	Human seminal fluid and PCa cell lines	Down	Favorable	24	Barceló et al. (2020)
Esophageal squamous cell carcinoma	—	Human serum	Up	Poor	30	Zhu et al. (2017)
	Suppressor	Human ESCC tissue and cell lines	Down	Favorable	144	Guo et al. (2021)
	Suppressor	Human ESCC tissue and cell lines	Down	Favorable	64	Wang et al. (2020)
Ovarian cancer	Suppressor	The meta-analysis	Down	Favorable	—	Shi et al. (2018)
Cervical cancer	—	Human tumorous tissue	Down	Favorable	121	Zheng et al. (2019)
Breast cancer	—	Human tumorous tissue	Down	Favorable	96	Kunc et al. (2020)
Renal cell carcinoma	Suppressor	Human RCC tissue and cell lines	Down	Favorable	25	Liang et al. (2021)
Pancreatic cancer	Suppressor	Pancreatic cancer cell	Down	Favorable	—	Zheng et al. (2021)
Osteosarcoma	Oncogene	Human cell line HFOB1.19	Up	Poor	—	Hu Y. et al. (2021)
Thyroid cancer	Suppressor	Cell lines	Down	—	—	Hu C. et al. (2021)
Hepatocellular carcinoma	Suppressor	Human HCC tissue	Down	Favorable	36	Yu et al. (2019)
	Suppressor	Human HCC tissue and cell lines	Down	Favorable	25	Zhuang et al. (2019)

5p with DBF4's 3'-UTR region, which inhibited various physiological functions of cancer cells (Qi et al., 2021).

## Cholangiocarcinoma (CCA) and Gallbladder Carcinoma (GBC)

Cholangiocarcinoma (CCA) is a remarkable type of hepatic malignancy, whose incidence has been on the rise year by year in recent decades (Rizvi and Gores, 2013; Labib et al., 2019). Many patients still face a poor prognosis even after effective surgery (Esnaola et al., 2016; Oliverius et al., 2019). Studies have validated that miR-30d-5p shows an increasing trend in CCA; its expression level was markedly higher than in other benign diseases (Han et al., 2020a). Compared with other miRNAs, miR-30d-5p had higher specificity and sensitivity to distinguish benign and malignant diseases of the biliary tract. Therefore, miR-30d-5p is likely to be regarded as a potential biomarker for CCA (Han et al., 2020a). Gallbladder carcinoma is a common biliary tract cancer in China (Gomes et al., 2020; Murimwa et al., 2021). Lactate dehydrogenase A (LDHA) is found in many tissues and cells of the human body, and was found capable of facilitating the Warburg effect (Pathria et al., 2018) to produce lactic acid and ATP under aerobic conditions. He et al. stated that miR-30d-5p was often lowly expressed in GBC as opposed to the expression of LDHA. When miR-30d-5p had decreased expression, the survival rate of patients was also reduced. In addition, miR-30d-5p was overexpressed by targeting

LDHA to reduce glycolysis in malignant tumors, and thus inhibited cancer development (He et al., 2018). In both gallbladder cancer and bile duct cancer, expression levels of miR-30d-5p were different from those in healthy tissues, which was worthy of further study in subsequent experiments.

## Colorectal Cancer

While the overall incidence of colorectal cancer has declined in recent years (Akgül et al., 2014), the number of patients below 50 years of age has rather been on the rise, which should be highly concerning (Haraldsdóttir et al., 2014). One study linked miR-30d-5p with carcinogenesis in colon cancer through the long non-coding RNA, PVT1 (Patel and Ahnen, 2018). Specifically, PTV1 prevented miR-30d-5p from exhibiting its own function, resulting in the increased expression of the downstream factor RUNX2, a novel oncogene (Patel and Ahnen, 2018). The LASSO regression analysis revealed a 16-miRNA signature, including miR-30d-5p, which was then considered as an effective prognostic biomarker for stage 2 and 3 colorectal cancer, indicating the future direction of research on therapeutic targets for colorectal cancer (Jacob et al., 2017). Furthermore, miR-30d-5p was regarded as an indicator of cellular hypoxia in rectal cancer, which might promote cancer metastasis to specific organs (Bjørnstrøm et al., 2019). Overall, miR-30d-5p is important for the diagnosis and treatment of colorectal cancer, but more clinical data are needed to support it.

## Esophageal Cancer

Esophageal cancer is a common malignancy with high morbidity and mortality due to its rapid development and invasiveness (Huang and Yu, 2018), while its early diagnosis is challenging and has poor prognosis (Bollschweiler et al., 2017; Short et al., 2017). In one study, the serum miR-30d-5p levels were significantly increased in esophageal squamous cell carcinoma patients, but they evidently declined after surgery. Additionally, miR-30d-5p could be associated with the TNM staging of esophageal cancer, suggesting its capability to become a potential biomarker in scientific research and subsequent clinical work (Zhu et al., 2017; Wang et al., 2020). Wang et al. indicated that LOC440173 could weaken the proliferation and invasion of malignant tumors through sponging miR-30d-5p (Wang et al., 2020). In other words, miR-30d-5p interacted with other molecules to influence the clinical indications of esophageal cancer. Additionally, Guo et al. showed that miR-30d-5p was decreased in esophageal cancer tissues, while its upregulation inhibited cancer progression (Guo et al., 2021). Although the mechanism of miR-30d-5p in esophageal cancer had been proven, more data are needed to support whether miR-30d-5p can be applied to clinical work.

## Prostate Cancer (PCa)

Prostate cancer is a common malignancy in males, with its incidence increasing with age (Grozescu and Popa, 2017). The course of the disease is slow, however, the mortality risk is considerable (Chang et al., 2014; Srougi et al., 2017; Teo et al., 2019). One report indicated that the negative regulation of NT5E in the tumor via miR-30d-5p tended to inhibit prostate cancer progression (Song et al., 2018). Specifically, the reduction of miR-30d-5p had a fundamental impact on the proliferation and migration of PCa cells. In addition, NT5E gene methylation has been shown to be associated with cancer (Lo Nigro et al., 2012; Jeong et al., 2020). A recent study reported that, during the development of the abnormal expression of PCa, related miRNA was applied to the seminal plasma, and the level of miR-30d-5p indicated the prognosis of prostate cancer, that is, its downregulation predicted poor prognosis (Barceló et al., 2020). The [PSA + miR-30d-5p + miR-93-5p] and [PSA + miR-30d-5p] models were also used for the prediction and diagnosis of prostate cancer (Barceló et al., 2020). Notably, when miR-30d-5p was upregulated, the androgen receptor activity was lower, and these two factors were often reversed (Kumar et al., 2016). Moreover, the level of miR-30d-5p expression was abnormal in chronic prostatitis (Chen et al., 2018), suggesting that miR-30d-5p could be considered a biomarker in benign and malignant prostate diseases.

## Other Cancers

In liver cancer, miR-30d-5p expression was associated with the cancer cell migration rate and patient survival rate, and thus considered useful for guiding the treatment of advanced liver cancer (Yu et al., 2019). In hepatocellular carcinoma (HCC), the target of miR-30d-5p, glycine decarboxylase (GLDC) (Zhuang et al., 2019), was closely related to the prognosis of HCC patients, and was considered as a separate factor for analysis in all

probability. In female reproductive system tumors, namely ovarian cancer, a high expression of miR-30d-5p was able to attain better prognosis (Shi et al., 2018). In cervical cancer (Zheng et al., 2019), miR-30d-5p had the potential as an extraordinary diagnostic biomarker not only for invasive screening of tumors, but also their precursors. What is more, miR-30d-5p showed a diminished level in breast cancer (Kunc et al., 2020). miR-30d-5p also functioned to impede cell activity through the downstream factor ATG5 in renal cell carcinoma (Liang et al., 2021). One study suggested that miR-30d-5p contributed to the construction of pancreatic cancer regulatory network, thus providing a solution for the treatment of pancreatic cancer (Zheng et al., 2021). In addition, miR-30d-5p was validated to be associated with the occurrence and development of osteosarcoma (Hu Y. et al., 2021). miR-30d-5p was down-regulated in thyroid cancer and impeded tumor development through competitive binding with LINC00284 (Hu C. et al., 2021). Overall, in the cancers described above, miR-30d-5p had clinical significance and was nominated as a potential biomarker for research.

## THE FUNCTIONAL ROLES OF MIR-30D-5P IN CANCERS

miR-30d-5p generally exerts its effects through molecular mechanisms, such as those related to cancer cell proliferation, and some upstream and downstream targets. In addition to the above discussion on the connection between miR-30d-5p expression and clinical pathological features in various tumors, this paper reviews the molecular mechanisms that have been associated with miR-30d-5p below (Table 2).

### Cell Proliferation

In tumors, cell proliferation is obviously increased and is in close connection with cancer progression. The flow cytometry analysis revealed that miR-30d-5p overexpression gave rise to cell cycle arrest at the G0/G1 phase in prostate cancer (Song et al., 2018). miR-30d-5p directly targeted CCNE2 to impede tumor cell activity in NSCLC (Chen et al., 2015). Furthermore, in NSCLC cell lines, the overexpression of POU3F3 led to the negative regulation of miR-30d-5p to facilitate the proliferation of cancer cells. However, for cells with only POU3F3 overexpressed, the cell proliferation activity was much lower than that of cells with the deregulation of both. POU3F3 (Zeng et al., 2020) acted as an upstream regulator of miR-30d-5p to control CCNE2 downstream in NSCLC (Chen et al., 2015). Thus, the POU3F3/miR-30d-5p-CCNE2 signal was likely to be a new signaling pathway in NSCLC (Figure 1). In addition, one study reported that DBF4, a downstream gene of miR-30d-5p (Lankhaar et al., 2021), was overexpressed to activate the proliferation of cell lines in lung squamous cell carcinoma.

miR-30d-5p was also mentioned in connection with cell proliferation in other human cancers. In vitro studies, LOC440173 played a positive regulatory role in the development of esophageal cancer (Wang et al., 2020). The level of HDAC9, the target gene of miR-30d-5p, had the same trend as LOC440173 (Wang et al., 2020). The up-regulation of

**TABLE 2 |** The biological functions and molecular mechanisms of miR-30d-5p.

Cancer type	Property	Functions	Genes/proteins/pathways	Refs
Lung cancer	Suppressor	Cell proliferation and motility inhibitor	Targeting CCNE2	Chen et al. (2015)
	Suppressor	Cell proliferation, migration, and invasion inhibitor	Targeted by POU3F3	Zeng et al. (2020)
	Suppressor	Cell proliferation, migration, and invasion inhibitor	Targeting DBF4	Qi et al. (2021)
	Suppressor	Cell proliferation, invasion and stemness inhibitor	circCD151/miR-30d-5p/GLI2 axis	Zhao et al. (2021)
	Suppressor	Malignant progression inhibitor and immune escape	LncRNA SOX2OT/miR-30d-5p/PDK1 driving PD-L1 through the mTOR signaling pathway	Chen et al. (2021)
Gallbladder carcinoma	Suppressor	Cell apoptosis promoter, and migration inhibitor	Targeting LDHA	He et al. (2018)
Rectal cancer	Suppressor	Organ-specificity of metastasis inhibitor	—	Bjømestø et al. (2019)
Colon cancer	Suppressor	TNM	—	Jacob et al. (2017)
	Suppressor	Cell proliferation, metastasis and lymph node inhibitor, tumor stage	LncRNA PVT1/miR-30d-5p/RUNX2 axis	Yu et al. (2018)
Prostate cancer	Suppressor	Cell proliferation, and migration inhibitor	Targeting NT5E	Song et al. (2018)
	Suppressor	Cell proliferation inhibitor	AR (Androgen receptor) regulating	Kumar et al. (2016)
Esophageal squamous cell carcinoma	Suppressor	Cell proliferation, migration, invasion inhibitor, and EMT inhibitor	LOC440173/miR-30d-5p/HDAC9 axis	Wang et al. (2020)
	Suppressor	Cell proliferation and invasion inhibitor, EMT inhibitor	Sponged by DDX11-AS1, regulating SNAI1/ZEB2 and Wnt/ $\beta$ -catenin pathway	Guo et al. (2021)
Renal cell carcinoma	Suppressor	Cell proliferation inhibitor, and autophagy promoter	Targeting ATG5	Liang et al. (2021)
Pancreatic cancer	Suppressor	Metastasis	miR-30d-5p/GJA1 CTNNA1, CTNNB1, CTNND1	Zheng et al. (2021)
Osteosarcoma	Oncogene	Cell migration, invasion, proliferation inhibitor, apoptosis promoter, and EMT	Regulating SOCS3/JAK2/STAT3 pathway	Hu Y. et al. (2021)
Thyroid cancer	Suppressor	Tumorigenesis	LINC00284/miR-30d-5p/ADAM12 regulating Notch signaling pathway	Hu C. et al. (2021)
Hepatocellular carcinoma	Suppressor	Cell migration inhibitor	—	Yu et al. (2019)
	Suppressor	Autophagy	Targeting GLDC	Zhuang et al. (2019)

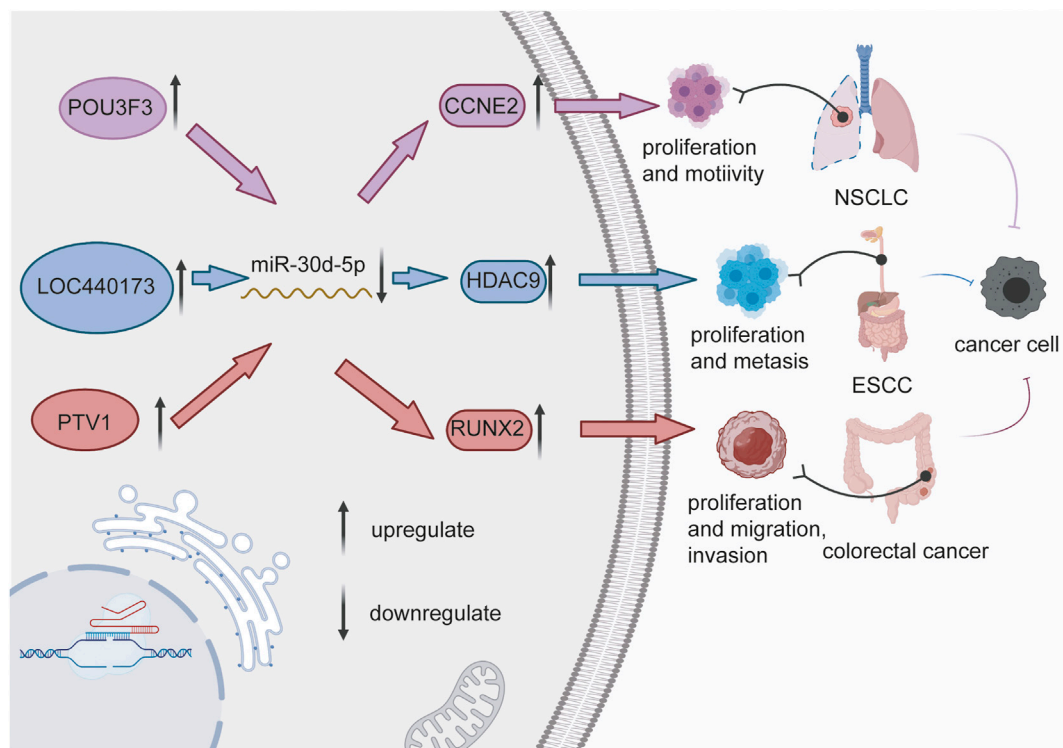
miR-30d-5p had a blocking effect on the proliferation of esophageal cancer cells, and this effect had also been verified in the invasion of cancer cells (Guo et al., 2021) (**Figure 2**). Compared with thymidine kinase 1 (TK1), a key enzyme in cell proliferation, miR-30d-5p had a higher sensitivity and specificity to the abnormal proliferation of esophageal squamous cell carcinoma cells (Zhu et al., 2017). Moreover, miR-30d-5p overexpression reduced the level of ATG5 (Liang et al., 2021), thus obviously inhibited the proliferation of tumor cells in renal cell carcinoma, and slowed the transition from the G1 to the S phase of the cell cycle. Furthermore, the high expression of miR-30d-5p weakened granuloma cell proliferation via targeting Smad2 (Yu and Liu, 2020). Wu et al., 2018 established that DGCR5 upregulated the level of Runx2 via miR-30d-5p and induced the osteoblast differentiation in human mesenchymal stem cells. In general, miR-30d-5p has been strongly connected with cell proliferation in various cancers.

## Cell Motility

In tumors, cellular activity signifies the invasion and metastasis of cancer cells. PVT1 directly regulates the progression of colorectal cancer via the miR-30d-5p/RUNX2 axis (**Figure 1**) (Yu et al., 2018). Experiments have shown that

the silencing of PTV1 induced miR-30d-5p regulation, which remained a significant suppression impact on the invasion of colorectal cancer. The high expression of miR-30d-5p significantly inhibited the migration of PCa cells, while the overexpression of NT5E (Rodemer-Lenz, 1989) reduced the impact of miR-30d-5p on the invasiveness of PCa cells. Similarly, the elevated expression of CCNE2 boosted the invasion, and migration of NSCLC cells (Chen et al., 2015). In addition, in the upstream mechanism, POU3F3 (Zeng et al., 2020) overexpression activated the invasion and migration of cancer cells via miR-30d-5p, which was obviously associated with the depth of tumor invasion and the degree of lymph node metastasis in esophageal carcinoma. Zhu et al., 2017; Zheng et al., 2021 illustrated a new lncRNA/Pseudogene-hsa-miR-30d-5p-GJA1 regulatory network associated with pancreatic cancer metastasis, and found that CTNNA1, CTNNB1 and CTNND1 were likely to participate in this network. In gallbladder cancer cells, LDHA activity inhibited cellular activities (He et al., 2018). After FC-11 treatment, LDHA activity was decreased and the invasion of tumor cells was also significantly reduced. MiR-30d-5p directly combined with the 3'-UTR site of LDHA, hence the imbalance of miR-30d-5p affected the movement and invasion





**FIGURE 1** | LncRNAs (POU3F3, LOC440173, and PTV1) sponge miR-30d-5p, thereby increasing the expression of downstream factors (CCNE2, HDAC9, and RUNX2), which in turn facilitate the activity of cancer cells, and promote the development of non-small cell lung cancer, esophageal squamous cell carcinoma and colorectal cancer.

of tumor cells. Yu and Liu, (2020) proposed that miR-30d-5p prevented the growth of ovarian granulosa cells and facilitated apoptosis, while the overexpression of Smad2 reversed this effect.

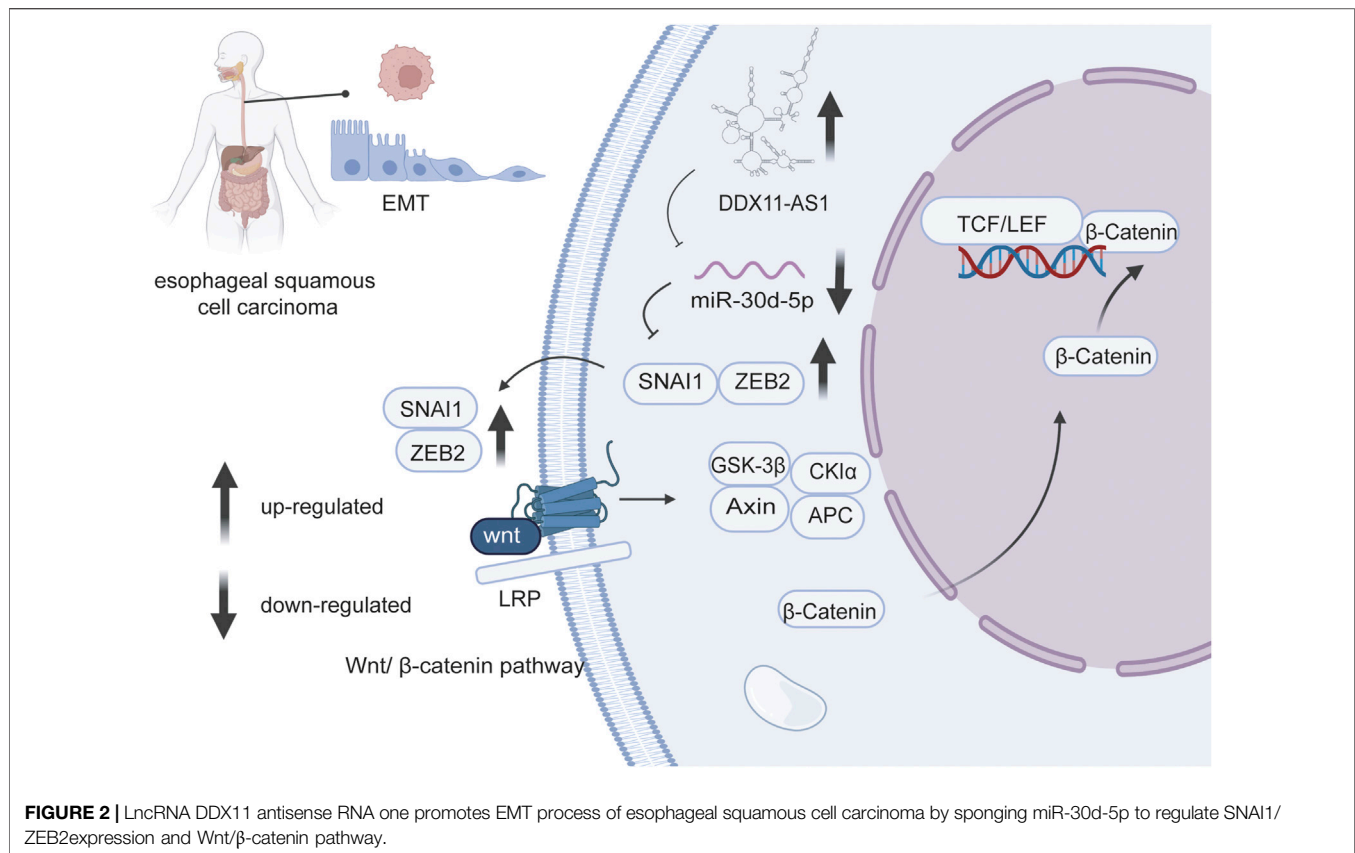
## Cell Autophagy and Apoptosis

As supported by experiments, autophagy is closely related to cancer cell metastasis (Qin et al., 2015). Autophagy has been linked to ROS and mercaptan REDOX status (Desideri et al., 2012; Peiris-Pagès et al., 2015). In hepatocellular carcinoma cells, GLDC regulated the autophagy and invasion of cells via silencing miR-30d-5p (Zhuang et al., 2019). Therefore, GLDC overexpression inhibited intrahepatic tumor metastasis. In cholangiocarcinoma, LDHA silencing promoted apoptosis through miR-30d-5p/LDHA axis (He et al., 2018). In addition to its prominent role in cancer, miR-30d-5p is involved in the autophagy process in brain injury. Jiang et al., 2018 found that exosomes loaded with miR-30d-5p reversed ischemia-induced autophagy-mediated brain damage via facilitating M2 microglia polarization, which is a feasible direction towards mitigating brain injury. Moreover, a recent study reported that various miRNAs, especially miR-30d-5p, were downregulated in brain hypoxia-ischemia (HI) (Wu et al., 2018). miR-30d-5p targeted the 3'-UTR site of Beclin1 mRNA and participated in autophagy in a newborn rat HI brain via regulating Beclin1. The C2dat2/miR-30d-5p/DDIT4/mTOR axis formed a new signaling pathway

facilitating autophagy induced by cerebral ischemia reperfusion injury (Xu et al., 2021). Specifically, C2dat2 blocked the targeting function of miR-30d-5p on DDIT4, and promoted the upregulation of DDIT4 and Beclin-1 levels.

## Tumorigenesis

Tumorigenesis and tumor development may occur due to various reasons (Karki and Kanneganti, 2019), with the abnormal expression of oncogenes widely believed to be the principal cause (Tan et al., 2020; Jiang et al., 2021; Saito et al., 2021). Many studies reported that miRNAs are involved in tumor development under the action of oncogenes. Relevant studies revealed that PVT1 is an oncogene (Yu et al., 2018) that facilitates the evolvement of colon cancer through the miR-30d-5p/RUNX2 axis. The level of RUNX2, the downstream target of miR-30d-5p, was consistent with the expression trend of PVT1 in tumor tissues. *In vitro*, LOC440173 competitively sponged miR-30d-5p (Wang et al., 2020). Furthermore, HDAC9, a target gene of miR-30d-5p (Cheetham et al., 2013), was found to participate in genetic epigenetic modification through the deactivation of acetylated lysine on histones, and to play a carcinogenic role in ESCC (Figure 1). In addition, the occurrence of malignant conjunctival melanoma (Larsen, 2016) in the Danish population was epidemiologically associated with the upregulation of miR-30d-5p. In MDS samples, the expression of miR-30d-5p was significantly reduced, and the target was concentrated in the AML



pathway (Ozdogan et al., 2017), indicating that miR-30d-5p is connected with tumor development, but the specific mechanism has not been clarified. LAMC3 might be regulated by 15 miRNAs, including miR-30d-5p, to affect the motility of cancer cells (He et al., 2019). What's more, miR-30d-5p reduced the expression of ADAM12 promoted by LINC00284, which significantly inhibited the progression of thyroid malignant disease (Hu C. et al., 2021). In lung cancer, miR-30d-5p also had the function of reducing tumor occurrence and development, which depended on the ceRNA regulatory mechanism of circCD151/miR-30d-5p/GLI2 (Zhao et al., 2021). Taken together, researches into miR-30d-5p in tumorigenesis have progressed, while the specific mechanism needs further investigations.

### Effect of Drugs

In the cell experiments of non-small cell lung cancer, the application of cryptotanshinone increased miR-30d-5p expression and limited the metastatic ability of lung cancer cells, suggesting that it might be a therapeutic direction to delay the progression of the disease, but more researches were still needed (Wang et al., 2019a). In addition to the above effects of miR-30d-5p, we summarized the related impacts of different drugs on cell activities involving miR-30d-5p and the participating pathways. Resveratrol (Res) is a polyphenol whose food sources include wine, berries and peanuts. This compound has many beneficial properties, such as anticancer and anti-aging effects (Galiniak et al., 2019). The protective role of Res was established in H9C2

cells through the miR-30d-5p/SIRT1/NF- $\kappa$ B axis in hypoxia-induced apoptosis (Han et al., 2020b). But this mechanism was just reported in cardiac protection, and it would be interesting to explore whether it would be present in malignancies.

### CONCLUSION

Many scholars have suggested that miRNA imbalance had a significant influence on the occurrence and progression of human cancers. This review summarized the key effects of miR-30d-5p in different types of tumors, such as lung cancer, colorectal cancer, prostate cancer, and esophageal cancer, emphasizing the vital roles of this potential biomarker of human cancers. MiR-30d-5p has been proven to be associated with clinical indications. When the expression of miR-30d-5p is detected to be significantly different from that of healthy tissue, it may indicate that miR-30d-5p is a significant factor in the diagnosis of tumorigenesis. Meanwhile, the up-regulation or down-regulation of miR-30d-5p during tumor development may also reflect the prognosis of cancer. Even though the relationship between miR-30d-5p and clinical indications is helpful to the prognosis of cancer, the specific mechanism of miR-30d-5p in tumors still deserves to be clarified. In particular, the relationship between miR-30d-5p in some cancers, such as ovarian cancer, uterine cancer and thyroid cancer, needs more researches to explore. In summary, miR-30d-5p is considered an important target for further studies.

We highlight its potentially significant role in other cancers, as well as its ability to become a target and biomarker in the diagnosis and treatment of a variety of human cancers. To this end, research efforts targeting the miR-30d-5p-related mechanisms are expected.

## AUTHOR CONTRIBUTIONS

QZ and XY selected references and completed the main part of the manuscript. QZ sort out the tables. LZ draw the figures and correct

the preliminary manuscript. MX provided the idea and reviewed the article.

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# Noncoding RNAs in Drug Resistance of Gastrointestinal Stromal Tumor

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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the gastrointestinal tracts and a model for the targeted therapy of solid tumors because of the oncogenic driver mutations in KIT and PDGFRA genes, which could be effectively inhibited by the very first targeted agent, imatinib mesylate. Most of the GIST patients could benefit a lot from the targeted treatment of this receptor tyrosine kinase inhibitor. However, more than 50% of the patients developed resistance within 2 years after imatinib administration, limiting the long-term effect of imatinib. Noncoding RNAs (ncRNAs), the non-protein coding transcripts of human, were demonstrated to play pivotal roles in the resistance of various chemotherapy drugs. In this review, we summarized the mechanisms of how ncRNAs functioning on the drug resistance in GIST. During the drug resistance of GIST, there were five regulating mechanisms where the functions of ncRNAs concentrated: oxidative phosphorylation, autophagy, apoptosis, drug target changes, and some signaling pathways. Also, these effects of ncRNAs in drug resistance were divided into two aspects. How ncRNAs regulate drug resistance in GIST was further summarized according to ncRNA types, different drugs and categories of resistance. Moreover, clinical applications of these ncRNAs in GIST chemotherapies concentrated on the prognostic biomarkers and novel therapeutic targets.

**Keywords:** GIST, drug resistance, noncoding RNAs, targeted therapies, imatinib mesylate

## INTRODUCTION

Gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumor in the gastrointestinal (GI) tracts, are malignancies generated from the interstitial cells of Cajal (ICCs) or their undifferentiated precursors (Sircar et al., 1999; Min and Leabu, 2006). GIST, which occurs most commonly in the stomach and small intestine at middle and old age (Joensuu et al., 2001; Miettinen et al., 2002; von Mehren and Joensuu, 2018), has an incidence of 10 per million populations per year generally and comprises around 20% of the soft tissue sarcomas (Ducimetiere et al., 2011; Joensuu et al., 2013). Standard therapies for GIST confirmed by immunohistology are as the following: for the resectable GISTs without metastasis, surgical resection is the first choice; and for the unresectable, metastatic, or recurrent GISTs, administration of tyrosine kinase inhibitors (TKIs) is the primary approach (Nishida et al., 2008a; Li J. et al., 2017; Casali et al., 2022).

Gain-of-function mutations of the genes of KIT proto-oncogene receptor tyrosine kinase (KIT) or the platelet-derived growth factor receptor alpha (PDGFRA) resulted in the constitutive activation of these receptors, thus leading to the induction of GIST (Hirota et al., 1998; Rubin et al., 2001; Heinrich

et al., 2003b) and imatinib mesylate (Gleevec), one receptor tyrosine kinase inhibitor, was found to competitively and effectively inhibit KIT and PDGFRA at submicromolar concentrations (Joensuu et al., 2002). Thus, imatinib mesylate is used and regarded as the first-line targeted therapy for GIST patients because of its durable responses in most tumors (Tuveson et al., 2001; Demetri et al., 2002). And the second- and third-line therapies for GIST patients consist of sunitinib malate (Sutent) and regorafenib (Stivarga), which are TKIs with activities against KIT, PDGFR and other targets like Raf-1 proto-oncogene serine/threonine kinase (RAF1), v-raf murine viral oncogene homolog B1 (BRAF) and fibroblast growth factor (FGFR) (Rock et al., 2007; Demetri et al., 2009; Demetri et al., 2013).

However, the resistance of these drugs widely exists in the therapy of patients with GISTs. There are about 14% of the patients with GISTs initially resistant to imatinib (Demetri et al., 2002). Additionally, within 2 years after the inception of the therapy based on imatinib in GIST, over 50% of the advanced and metastatic tumor patients developed secondary resistance (Gramza et al., 2009). When the resistance of imatinib occurred, second-line therapy including imatinib dose doubling and sunitinib administration was performed and third-line therapy of regorafenib was carried after the failure of second-line therapy (Serrano and George, 2020). And the novel TKIs, like sunitinib and regorafenib, showed a drug resistance trend, too (Heinrich et al., 2008a; Gajiwala et al., 2009; Garner et al., 2014; Ou B. et al., 2019). Therefore, it is of great significance to find the mechanisms involved in the drug resistance of GIST for further intervention to overcome the resistance.

Various genomic approaches have been used to understand the initiation, progression and drug resistance of multiple cancers (Hayes et al., 2014; Wang W.-T. et al., 2019; Yu et al., 2019). The genomic mutation landscapes have revealed that myriad mutations or copy number changes were frequently located in the noncoding regions of DNA when cancers were taken place (Guttman and Rinn, 2012). Functional noncoding RNAs (ncRNAs), like microRNAs (miRNAs), circular RNAs (circRNAs); small interfering RNAs (siRNAs), antisense RNAs (asRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), PIWI-interacting RNAs (piRNAs) and long noncoding RNAs (lncRNAs), are transcribed from noncoding DNAs which cover 95% of DNA sequences in the human genome (Esteller, 2011; Cech and Steitz, 2014). Furthermore, various studies showed that ncRNAs might play essential roles in anti-tumor drug resistances by changing drug efflux, regulating the tumor microenvironment and activating the signaling pathways (Bester et al., 2018; Wang W.-T. et al., 2019; Guo et al., 2020).

In GIST therapies, ncRNAs were also found to act in the process of drug resistance towards GISTs. For example, downregulation of lncRNA CCDC26 (Yan et al., 2019b) and miRNA-30a (Chen et al., 2020) contributes to imatinib resistance in GISTs. Thus, investigating how ncRNAs affect drug resistance might provide us with a new sight for GIST therapy. Here, we summarized the alterations of ncRNAs and different types of ncRNAs, drugs and resistance in the drug resistance process of

GIST. We also discussed the functions and the potential clinical applications of ncRNAs during drug resistance in GIST.

## AN OVERVIEW OF NONCODING RNAS

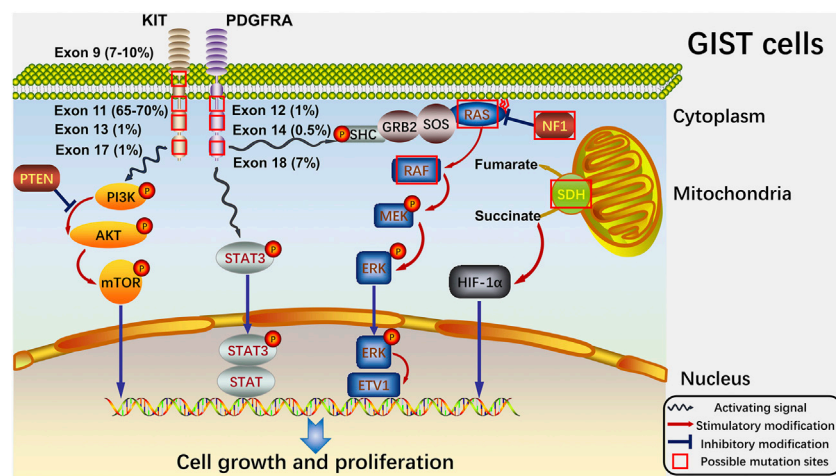
The ncRNA family, which the majority of human genes are transcribed into, is a large and diverse class of RNAs with multiple functions (Dunham et al., 2012; Wong et al., 2018). These ncRNA molecules are simply classified relying on the cutoff of 200 nucleotides, which divides small ncRNAs from lncRNAs (Brandenburger et al., 2018). Amidst the different types of ncRNAs, miRNAs, circRNAs and lncRNAs are studied broadly nowadays.

MiRNAs, a group of single-stranded ncRNAs, are about 18–22 nt in length and act as post-transcriptional regulators of gene expression (Krol et al., 2010; Ameres and Zamore, 2013; Stepień et al., 2017). The mature miRNA strand is integrated into the RNA-induced silencing complex (RISC), and this protein complex has the ability to bind the complementary sequence in the 3' untranslated regions (3'-UTR), which is dictated by the miRNA, and later silence its target genes by degrading the mRNA or blocking the mRNA translation (Sheu-Gruttadauria and MacRae, 2018). Additionally, a great many studies focused on miRNAs because of their crucial roles in the biological process (Shenouda and Alahari, 2009; Sun et al., 2018). Increasing evidence showed that miRNAs had functions in the cancer cell resistance towards chemotherapy and targeted therapies (Leonetti et al., 2019).

Unlike linear RNAs who have 5' caps, 3' tails and 5' to 3' polarity, circRNAs are circular, possessing covalently closed loop structures without either 5' caps, 3' tails or 5' to 3' polarity (Chen and Yang, 2015; Li et al., 2020). Functions of circRNAs in biological process are mainly mediated by regulating the alternative splicing (AS) and *cis*-transcription of RNAs, sequestering or scaffolding of macromolecules to act as competing endogenous RNAs (ceRNAs), and serving as translation templates (Lasda and Parker, 2014; Chen, 2020). Importantly, in the research of tumors, circRNAs have been found to involve and play essential roles in the proliferation, apoptosis, invasion and drug resistance (Zhang L. et al., 2019; Li et al., 2019). Moreover, circRNAs have unique functions and potential applications in the drug resistance through acting as a microRNA sponge and activating signaling pathways (Chen et al., 2019; Xu et al., 2020).

lncRNAs are longer than 200 nucleotides and comprise 80–90% of all mammalian noncoding transcriptome with low expression levels, poor interspecies conservation and high variance expression coefficient (Mercer et al., 2009; Yuan et al., 2020). Recent studies found that lncRNAs had the ability to regulate gene expression through various mechanisms like moderating the transcription of protein-coding genes, binding to proteins modulating their functions and conducting protein synthesis and RNA maturation (Wang and Chang, 2011; Carrieri et al., 2012). lncRNAs also participated widely in diverse physiological and pathological processes in human (Westra et al., 2018; Dallner et al., 2019). As important regulators of chromatin dynamics and gene





**FIGURE 1 |** Signaling pathways of GISTs and potential sites where mutations always happen. At cellular level, the four main pathways (PTEN/PI3K/AKT/mTOR, STAT3, RAS/RAF/MEK/ERK and SDH/succinate/HIF-1 $\alpha$ ) and the significant proteins in the pathways are shown. And the possible mutation sites with their own mutation rates are also exhibited. The red box indicates the possible mutation sites. The blue wave-like arrow shows the activating signals from the receptor tyrosine kinase. And the red arrow shows the stimulatory modification between the proteins, while the blue "T" symbol shows the inhibitory modification.

regulation, lncRNAs correlated with countless cell signaling pathways and could influence multifarious factors including hormones, nutrients and age (Alvarez-Dominguez et al., 2014a; Alvarez-Dominguez et al., 2014b; Alvarez-Dominguez et al., 2015; Knoll et al., 2015). And with the studying of lncRNAs' functions going on in recent years, mechanisms of lncRNAs on cancer drug resistance have been found a lot: they could affect the metabolizing enzymes, such as phase I and phase II enzymes, associate with oxidative stress and alter the epithelial-mesenchymal transition (EMT) in the process of drug resistance (Jiang et al., 2020; Liu et al., 2020). NcRNAs have played pivotal roles on drug resistance of multiple cancers which might be conducive to the research focusing on the chemotherapy and targeted therapy resistance.

## MOLECULAR CHARACTERISTICS OF GASTROINTESTINAL STROMAL TUMORS

Around 95% of GISTs stain positive for KIT in immunohistochemistry (Medeiros et al., 2004), and the sequencing data have shown that approximately 75% and 15% of the tumors harbored a gain-of-function mutation in KIT and PDGFRA, respectively. The activating mutations of KIT and PDGFRA are recognized as the major oncogenic drivers of GISTs (Hirota et al., 1998; Heinrich et al., 2003a; Corless et al., 2011; Wozniak et al., 2012). There are other gene mutations rarely occurring in GISTs, including mutations of set domain containing 2 (SETD2), succinate dehydrogenase (SDH), BRAF, RAS, neurofibromatosis type 1 (NF1), TP53, multiple endocrine neoplasia type 1 (MEN1) and retinoblastoma susceptibility gene (Rb1) (Huang et al., 2016; Merten et al., 2016; Shi et al., 2016a; von Mehren and Joensuu, 2018) (Figure 1).

KIT is a 145 kDa transmembrane glycoprotein from the receptor tyrosine kinase family and composes an extracellular

domain, a transmembrane domain, a juxtamembrane domain and a tyrosine kinase domain encoded by exon 1-9, exon 10, exon 11 and exon 13-21, respectively (Lennartsson and Ronnstrand, 2012; Hemming et al., 2019). Stem cell factor (SCF), the ligand of KIT, induces the dimerization and activation of KIT, and then downstream signaling pathways of KIT, including phosphoinositide 3-kinase (PI3K) pathway, are stimulated to act in the process of differentiation, proliferation and survival (Zsebo et al., 1990; Hemesath et al., 1998; Timokhina et al., 1998; Palam et al., 2018). And mutations in KIT usually take place at exon 11 (90%) and exon 9 (8%) but seldom appear in exon 13 (1%) or exon 17 (1%) in GISTs (Joensuu et al., 2015; Joensuu et al., 2017). PDGFRA, whose mutations at exon 18 (D842V) and exon 12 respectively encode the activation loop and juxtamembrane domain of the tyrosine kinase (Cassier et al., 2012), is the other receptor tyrosine kinase and has a tight correlation with KIT at the molecular structure and chromosomal gene location (Heinrich et al., 2003b; Antonescu et al., 2005). Additionally, there are no significant differences between KIT and PDGFRA-mutant GISTs in the signaling pathways downstream, revealing that oncogenic signals presented by PDGFRA mutations in GIST are the same as KIT mutations (Heinrich et al., 2003a; Hirota et al., 2003; Corless et al., 2005). The remaining group of GISTs without KIT or PDGFRA mutations (5–10% of adult GISTs and 85% of pediatric GISTs) was termed "wild-type" in the past (Pappo and Janeway, 2009; Alkhuziem et al., 2017). And such GISTs have been recently found to harbor mutations concentrated on the NF1 gene or genes composed of SDH complex (Boikos et al., 2016; Ricci, 2016).

Besides, GISTs harboring mutations without KIT or PDGFRA would not be inhibited by the targeted therapy of imatinib (von Mehren and Joensuu, 2018). And when GIST contains the PDGFRA mutation at exon 18, imatinib or other permitted TKIs will not work, while Avapritinib (BLU-285), one type I



expression of mitochondrial respiratory Complex II, thus increasing the cell death induced by imatinib in GIST cells (Huang et al., 2021). OXPHOS played a pivotal role in the drug resistance of GIST and the detailed mechanisms between cell metabolism and ncRNAs in the drug resistance need further investigation.

## NoncodingRNAs Moderate Autophagy in Drug Resistance of Gastrointestinal Stromal Tumors

Autophagy, whose major acting procedures include activation, vesicle nucleation and elongation, fusion and degradation, is a self-degradative system and plays pivotal roles in the metabolism of cells and the body (Kim and Lee, 2014). Enormous structures in cells would be degraded by autophagy, which benefits the cells' survival via recycling their composing metabolites (Smith and Macleod, 2019). Autophagy widely exists in the progression of tumor. It supports nutrient reuse and metabolic balance, thus affecting the tumor genesis and development (Bu et al., 2021). And for the tumor chemotherapy, studies showed that constructive autophagy prevented cell death from tumor chemotherapy and brought about drug resistance and recurrence of tumors (Buchser et al., 2012; Li Y.-J. et al., 2017). Moreover, studies have demonstrated that autophagy was a stress response to avoid the imatinib-induced starvation in GISTs and chronic myeloid leukemia (Gupta et al., 2010; Ravegnini et al., 2017; Mitchell et al., 2018; Han et al., 2019; Zheng et al., 2020; Zhang et al., 2021). And autophagy inhibition by lysosomotropic agents could reduce the imatinib resistance of GISTs *in vitro* and *in vivo* (Rubin and Debnath, 2010; Burger et al., 2015). It could be concluded from these studies that autophagy plays protective roles in the imatinib-induced cell death of GISTs, while the inhibition of autophagy could overcome the resistance, to a certain extent.

Accumulating evidence illustrated that ncRNAs, including miRNAs, lncRNAs and circRNAs, could engage in not only the transcriptional but the post-transcriptional regulation of genes relevant to autophagy through the regulatory networks of autophagy (Frankel et al., 2017; Yao et al., 2019). Different expression levels of ncRNAs determine the levels of autophagy at various physiological and pathological stages, including cellular senescence, cancer genesis and drug resistance (Zhang et al., 2017; Wang J. et al., 2019; Bermúdez et al., 2019; Xu et al., 2019).

In the imatinib resistance of GISTs, a research concentrating on the functions of miRNA-30a has found that autophagy caused by imatinib exposure in GIST cell lines was correlated with miRNA-30a (Chen et al., 2020). Beclin-1, the mammalian autophagy gene, which has also been proved as a tumor suppressor, was found to be directly targeted by miRNA-30a and served as a link between miRNA-30a and autophagy in GISTs (Liang et al., 1999; Chen et al., 2020). Moreover, miRNA-30a increased the sensitivity of imatinib in GIST cells by down-regulating the expression of Beclin-1. Additionally, studies of Zhang et al. reported that lncRNA-HOTAIR activated autophagy by the miRNA-130a/autophagy-related protein 2 homolog B (ATG2B) pathway, thus promoting the imatinib resistance of

GIST cells (Zhang et al., 2021). Generally speaking, ncRNAs could adjust the process of drug resistance in GISTs by regulating the expression levels of proteins or pathways related to autophagy.

## NoncodingRNAs Adjust Apoptosis in Drug Resistance of Gastrointestinal Stromal Tumors

Diverse studies revealed that cell death through apoptosis pathways could be generated by the larger part of cancer chemotherapy drugs. And once the apoptosis is disorganized, drug resistance and increased cancer cell survival appear (Mohammad et al., 2015; Das et al., 2018; Mou et al., 2018). In the drug resistance of GISTs, there are a great many studies exhibiting that functions of ncRNAs on the resistance are associated with apoptosis.

lncRNA CCDC26 knocking down decreased the apoptosis of GIST cells treated with imatinib through upregulating insulin-like growth factor 1 receptor (IGF-1R), which acted in the apoptosis pathways (Li et al., 2018; Yan et al., 2019b; Zhang Y. et al., 2019). Additionally, lncRNA CCDC26 was also reported to interact with c-KIT, thus affecting the rates of apoptotic cells caused by imatinib (Cao K. et al., 2018). BCL-2, whose gene families play pivotal roles in the programmed cell death regulations, induces apoptosis evasion and drug resistance evolution in cancers (Ashkenazi et al., 2017; Maji et al., 2018). In GISTs, BCL-2 was down-regulated by miRNA-21, miRNA-221 and miRNA-222, whose mimics were transfected in GIST cells thus significantly aggravating the apoptosis motivated by imatinib (Ihle et al., 2015; Cao et al., 2016). The overexpression of miRNA-518a-5p increased the proportion of apoptotic GIST cells treated with imatinib, which advocated that miRNA-518a-5p acted a part in interfering with the imatinib resistance (Shi Y. et al., 2016). When treated with imatinib, the resistance generated by inhibiting the apoptosis of GISTs could be attenuated by multiple ncRNAs through targeting different apoptosis-related proteins.

## NoncodingRNAs Affect Drug Targets in Drug Resistance of Gastrointestinal Stromal Tumors

Drug resistance in GISTs mainly concentrated on the primary and secondary resistance of imatinib (Joensuu et al., 2013). And the primary resistance is correlated with several genotypes of GISTs, such as mutations of KIT exon 9 whose receptor dimerization without ligand hinders the binding of imatinib structurally (Heinrich et al., 2003a) and mutations of PDGFRA D842V located in exon 18 (Lasota et al., 2004). The secondary resistance mainly happens via the acquisitions of secondary mutations on KIT under the imatinib pressure and leads to the pathological activation of downstream signaling pathways like the PI3K/protein kinase B (PKB, AKT) pathway (Liegler et al., 2008; Pierotti et al., 2011).

Recent research revealed that ncRNAs in GISTs could alter the resistance of imatinib by directly targeting and reducing the



activation of KIT. At protein expression levels, exogenous miRNA-221 and miRNA-222 reduced phosphorylated KIT and total KIT levels in GISTs (Ihle et al., 2015). And phosphorothioation and 2'-O-methylation of miRNA-221 and miRNA-222 in GIST cells were reported to effectively inhibit KIT expression, thus affecting various cellular processes mediated by KIT, including resistance of imatinib (Durso et al., 2016). Another research found the direct regulation relationship between miRNA-222 and KIT in imatinib-resistant GIST cells whose proliferation ability was inhibited by miRNA-222 (Gits et al., 2013). In GISTs, KIT was also targeted directly by miRNA-218 and miRNA-148b-3p, which acted as tumor suppressors and sensitized GIST cells to imatinib therapy (Fan et al., 2014; Wang Y. et al., 2018). Moreover, through collaborating with c-KIT, knocking down lncRNA CCDC26 and overexpressing lncRNA RP11-616M22.7 increased the resistance of GIST cells to imatinib (Cao K. et al., 2018; Shao et al., 2021). The microarrays which screened tumour-specific circRNA profiles of GIST, identified a GIST-specific circRNA-miRNA-mRNA regulatory network related to KIT, which showed the potentials of circRNAs in the drug resistance of GIST by targeting KIT (Jia et al., 2019). By directly affecting the drug targets in GISTs, ncRNAs play essential roles in drug resistance.

## NoncodingRNAs Activate Signaling Pathways in Drug Resistance of Gastrointestinal Stromal Tumors

As a critical signaling pathway adjusting various biological processes, phosphatase and tensin homolog (PTEN)/PI3K/AKT signaling conducts cell proliferation, apoptosis and invasion in GI tract tumors, especially in GISTs (Carnero et al., 2008; Long et al., 2018; Hu et al., 2019). Abnormal expression of PTEN/PI3K/AKT signaling was exhibited as being conducive to the drug resistance of targeted therapies caused by stimulating mutations of PI3K-related genes, including PIK3C2A, to improve the cell proliferation regulated by the growth factors, invasion and metastasis (Hafsi et al., 2012). PTEN/PI3K/AKT pathway was also found to regulate and participate in the drug resistance process in GISTs. Serving as the gene-specific target of PIK3C2A, miRNA-518a-5p downregulated PIK3C2A in GISTs to alter the cellular response towards imatinib, thus lessening the resistance (Shi Y. et al., 2016). And miRNA-218, miRNA-148b-3p, miRNA-221 and miRNA-222 were found to increase the sensitivity of GISTs towards imatinib through targeting PI3K/AKT pathway (Fan et al., 2015; Ihle et al., 2015; Wang Y. et al., 2018). Moreover, in the resistance process of cisplatin in GISTs, PTEN/PI3K/AKT pathway also played pivotal roles. MiRNA-22-3p could increase the chemosensitivity of cisplatin in GIST cell lines by targeting the PTEN/PI3K/AKT pathway (Xu et al., 2018).

Also, the integrin-mediated signaling transduction pathway of focal adhesion kinase (FAK) was identified by the Gene Expression Omnibus (GEO) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis as the pathway where the significantly different ncRNAs between the imatinib treated GIST patients with and without resistance were enriched in (Zhang et al., 2018). Moreover, FAK is verified as the downstream

pathway of PTPN18 contributing to the imatinib resistance in GISTs, which is regulated by miRNA-125a-5p (Akcakeya et al., 2014; Huang et al., 2018). MiRNA-125a-5p decreased the expression levels of PTPN18, thus increasing the phosphorylation of FAK in the imatinib-resistant GIST cell lines (Huang et al., 2018).

The Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling pathway, which was activated abnormally in various cancers to increase the proliferation, survival, drug resistance and metastasis of cancer cells (Wang T. et al., 2018; Johnson et al., 2018), was identified from the KEGG pathway analysis, based on ncRNAs with significantly different expression levels selected by the genome-scale clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 knockout screening in GISTs with imatinib resistance (Cao J. et al., 2018). STAT3 was also found to be downregulated by miRNA-148b-3p in GISTs, thus reducing the imatinib resistance of GIST cells (Wang Y. et al., 2018). Gene set enrichment analysis (GSEA) also identified JAK-STAT3 signaling pathway as one of the interfered signaling pathways of lncRNA RP11-616M22.7, which was increased in the imatinib-resistant GIST samples (Shao et al., 2021).

As a critical signaling pathway involving in a broad range of cancers, Hippo pathway controls multiple cellular functions and tissue-level processes crucial to the cancer progression and therapy, like cell proliferation, cell survival, drug resistance, stem cell phenotype, planar and apicobasal cell polarity, cell-cell adhesion, contact inhibition, metastasis and so on (Harvey et al., 2013). And in GISTs, deregulation of the Hippo pathway also occurs (Ou W.-B. et al., 2019; Guerin et al., 2020). Hippo pathway acted as an oncogenic regulator in KIT-independent GISTs by inducing the expression of Cyclin D1, which was the oncogenic mediator for primary imatinib resistant and untreatable GISTs (Ou W.-B. et al., 2019; Chen et al., 2021). NcRNAs targeting the Hippo pathway also involved in the malignant transformation of GISTs (Yin et al., 2021). In the drug resistance of GISTs, lncRNA RP11-616M22.7 promoted GIST resistance to imatinib by interacting with the Ras association domain family protein1 isoform A (RASSF1A), which is an important upstream regulator of the Hippo pathway (Shao et al., 2021). And through specifically binding to RASSF1A thus inhibiting its function, lncRNA RP11-616M22.7 decreased the expression of mammalian STE20-like kinase (MST)-1, MST2, large tumor suppressor kinase (LATS)-1, and LATS2, and increased the expression of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), thus promoting the cell proliferation and inhibiting the apoptosis under imatinib treatment (Shao et al., 2021). More key signaling pathways relevant to ncRNAs would be found in future studies on drug resistance of GISTs.

## DIFFERENT TYPES OF NONCODINGRNAs ON THE DRUG RESISTANCE OF GASTROINTESTINAL STROMAL TUMORS MicroRNAs

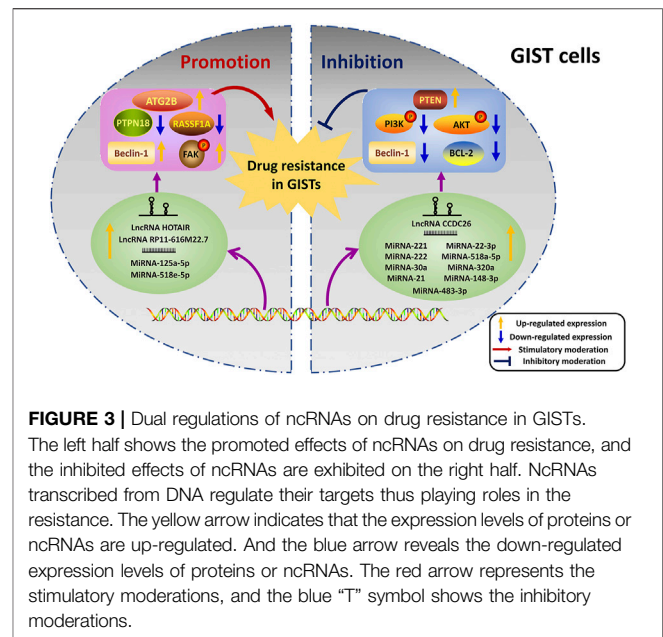
Among the different kinds of ncRNAs, miRNAs are studied most broadly in the resistance of targeted therapies (Corra et al., 2018;



Wang W.-T. et al., 2019). In GISTs, there are also a great deal of miRNAs relating to drug resistance. Recently, miRNA-221 and miRNA-222, whose chemical modifications affected the proliferation of imatinib-resistant GIST cells (Durso et al., 2016), were found to target KIT and promoted apoptosis of GIST cells through the KIT/AKT pathway during the imatinib resistance (Gits et al., 2013; Ihle et al., 2015). Another miRNA, miRNA-125a-5p, was also an important ncRNA in the drug resistance of GISTs. Studies by Lui et al. revealed that overexpression of miRNA-125a-5p suppressed the expression of PTPN18 and increased the phosphorylation of FAK, thus contributing to the re-sensitization of GIST cells towards imatinib (Akcaakaya et al., 2014; Huang et al., 2018). One research based on the GEO database (GSE63159 and GSE45901) also identified miRNA-125a-5p as a critical ncRNA in patients with GISTs treated by the targeted therapy of imatinib (Zhang et al., 2018). Moreover, reducing influence of miRNA-218 on imatinib resistance was proved by Fan et al. (Fan et al., 2014; 2015). There were also other miRNAs, such as miRNA-483-3p (Huang et al., 2021), miRNA-518e-5p (Kou et al., 2018), miRNA-518a-5p (Shi Y. et al., 2016), miRNA-148b-3p (Wang Y. et al., 2018), miRNA-17-92 (Gits et al., 2013), miRNA-21 (Cao et al., 2016), miRNA-23b (Cao J. et al., 2018), miRNA-30a (Zheng et al., 2015), miRNA-320a (Gao et al., 2014), miRNA-505 (Cao J. et al., 2018), miRNA-92a-3p (Amirnasr et al., 2019) and miRNA-99a-5p (Amirnasr et al., 2019), being identified or demonstrated that they played important roles in the process of imatinib resistance in GISTs.

## Long NoncodingRNAs

LncRNA, another kind of ncRNA, has been considered as a new mechanism of drug resistance nowadays and attracted full attention for researching various cancers. Through upregulating IGF-1R and c-KIT to affect the cell viability, proliferation, and apoptosis of imatinib treated GIST cells, downregulation of lncRNA CCDC26 promoted the process of imatinib resistance which could be used to develop potential strategies overcoming the imatinib resistance of GIST patients (Cao K. et al., 2018; Yan et al., 2019b). Multidrug resistance protein (MRP)-1 is an ATP-binding cassette transporter that participated in the resistance of many drugs like anti-cancer drugs, opiates and antibiotics (Johnson and Chen, 2017) and also played essential roles in the chemotherapy of GISTs (Plaat et al., 2000; Theou et al., 2005). The lncRNA HOTAIR, which considerably reduced the MRP1 expression, was found to have the possibility of inactivating the PI3K/AKT pathway, thus decreasing the resistance of imatinib treatment (Dai et al., 2017; Wang et al., 2017). However, the lncRNA HOTAIR whose distribution was transferred from nucleus to cytoplasm after imatinib treatments was reported to increase imatinib resistance through activating autophagy by the miRNA-130a/ATG2B pathway in GISTs by Zhang et al. (Zhang et al., 2021). The exact function of lncRNA HOTAIR needs to be further explored in the drug resistance in GIST. Additionally, one study based on the microarray identified lncRNA-DNAJC6-2 together with the hypoxia-inducible factor-1 (HIF-1) pathway as drug targets. And lncRNA-DNAJC6-2 probably played a part in the secondary imatinib-resistant GISTs by targeting the translational factors, including TBP, TAF1, NRF1, MAX, STAT3



**FIGURE 3 |** Dual regulations of ncRNAs on drug resistance in GISTs.

The left half shows the promoted effects of ncRNAs on drug resistance, and the inhibited effects of ncRNAs are exhibited on the right half. ncRNAs transcribed from DNA regulate their targets thus playing roles in the resistance. The yellow arrow indicates that the expression levels of proteins or ncRNAs are up-regulated. And the blue arrow reveals the down-regulated expression levels of proteins or ncRNAs. The red arrow represents the stimulatory modulations, and the blue "T" symbol shows the inhibitory modulations.

and E2F6, which regulate the translation process of mRNA (Yan et al., 2019a). LncRNA RP11-616M22.7 was also found to regulate the imatinib resistance of GISTs through the Hippo pathway (Shao et al., 2021). These researches showed that lncRNA, as a noncoding RNA, is closely related to drug resistance in GISTs, which might be applied as novel therapeutic strategies against GISTs.

## DUAL REGULATIONS OF NONCODINGRNAs ON DRUG RESISTANCE IN GASTROINTESTINAL STROMAL TUMORSS

The functions of ncRNAs mainly concentrated on the promotion and inhibition aspects during the regulation of drug resistance in GISTs (Figure 3).

### Promotion of NoncodingRNAs on Drug Resistance

As positive regulators of drug resistance, various ncRNAs were proved to promote drug resistance in numerous cancer types (Loewen et al., 2014; Qu et al., 2016; Ren et al., 2018). And in GISTs, one study of miRNA-125a-5p focused on its promoting effects in the imatinib resistance (Akcaakaya et al., 2014). In this study, the overexpression of miRNA-125a-5p silenced the expression of PTPN18, whose expression level was negatively correlated with cell viability in GIST cells under the exposure of imatinib. Additionally, miRNA-125a-5p was demonstrated to correlate with metastasis and mutational status of KIT in GISTs by Kaplan Meier survival and log-rank analyses. Moreover, the promoted effects of miRNA-125a-5p and its downstream regulations were ulteriorly studied (Huang et al.,

2018). Expression of phosphorylated FAK (pFAK), which was higher in the imatinib-resistant GIST subclones, could be increased by miRNA-125a-5p and PTPN18 silencing in GIST cells. Suppression of pFAK by FAK inhibitor 14 was shown to rescue the imatinib resistance caused by miRNA-125a-5p overexpression. Up-regulation of miRNA-125a-5p and pFAK promoted drug resistance in GISTs. Moreover, expression levels of miRNA-518e-5p in the serum were positively correlated with imatinib resistance in GISTs (Kou et al., 2018). The lncRNA HOTAIR and RP11-616M22.7 were also proved to increase the resistance of imatinib in GISTs (Shao et al., 2021; Zhang et al., 2021). NcRNAs might promote drug resistance which could be applied as therapy targets against drug resistance in GISTs.

## Inhibition of NoncodingRNAs on Drug Resistance

NcRNAs are also served as negative regulators in the resistance of chemotherapies (Fanini and Fabbri, 2016; Jin et al., 2019). Inhibited functions of ncRNAs on drug resistance are universal in GISTs. It is reported that GIST cells which possessed high expression levels of lncRNA CCDC26 were more sensitive to imatinib than those GIST cells with the lower levels, and knockdown of lncRNA CCDC26 obviously increased imatinib resistance. Further studies showed that the downregulation of lncRNA CCDC26 helped to the imatinib resistance in GISTs by upregulating IGF-1R and interacting with c-KIT (Cao K. et al., 2018; Yan et al., 2019b). MiRNA-221 and miRNA-222 targeted KIT and restrained the KIT/AKT signaling pathway to inhibit cell proliferation, modify the progression of the cell cycle, induce apoptosis and reduce resistance in imatinib-resistant GIST cells (Gits et al., 2013; Ihle et al., 2015). In addition, imatinib treatment was found to reduce the expression levels of miRNA-30a, which was found to inhibit the imatinib resistance of GIST cells by targeting Beclin-1, thus lessening the imatinib resistance related autophagy (Chen et al., 2020). Moreover, ncRNAs like miRNA-483-3p, miRNA-21, miRNA-22-3p, miRNA-518a-5p, miRNA-320a, miRNA-218, and miRNA-148b-3p acted as negative regulators to enhance the drug sensitivity and played significant roles in the process of GIST drug resistance (Gao et al., 2014; Shi Y. et al., 2016; Cao et al., 2016; Wang Y. et al., 2018; Xu et al., 2018; Huang et al., 2021). These drug resistance-inhibiting ncRNAs could be served as small molecular drugs against drug resistance in GISTs in the future.

## EFFECT OF NONCODINGRNAs ON RESISTANCE OF VARIOUS DRUGS FOR GASTROINTESTINAL STROMAL TUMORS Resistance of Imatinib

Imatinib mesylate has become the first-line agent for GISTs from its approval in 2002 as a TKI against KIT, PDGFRA, Abelson tyrosine kinase (ABL), FMS-like tyrosine kinase-3 (FLT-3) and colony stimulating factor-1 receptor (CSF1R) (Demetri et al., 2002; Bauer and Joensuu, 2015). The usage of imatinib improved

the outcome of patients with untreatable advanced and metastatic GIST extensively. Nevertheless, primary resistance occurs in 14% of the patients, and over 50% of the remaining patients would develop secondary resistance within 2 years (Demetri et al., 2002; Gramza et al., 2009). A great deal of studies reported the influence of ncRNAs in the process of imatinib resistance.

In the imatinib-resistant cell line GIST48, miRNA-221 mimic transfection markedly reduced cell proliferation and induced apoptosis related to imatinib (Gits et al., 2013; Ihle et al., 2015). Interestingly, modified miRNA-221 was also found to inhibit the expression of the KIT gene effectively, which might be used in the solution to overcome imatinib resistance in GIST (Durso et al., 2016). Additionally, high expression of miRNA-148b-3p suppressed cell proliferation synergistically with the effect of imatinib, causing sensitivity to imatinib treatment in GIST cells (Wang Y. et al., 2018). In parallel, Chen et al. demonstrated that miRNA-30a could decrease the autophagy level to sensitize GIST cells to imatinib mesylate by targeting Beclin-1 (Chen et al., 2020). A study by Cao et al. showed the potential role of miRNA-23b and miRNA-505 during imatinib resistance by performing the genome-scale CRISPR-Cas9 knockout screening in the GIST cell lines with and without imatinib resistance (Cao J. et al., 2018). It can be concluded that ncRNAs played essential roles in the imatinib resistance of GISTs, and the clinical applications need to be further explored.

## Resistance of Cisplatin

Cisplatin, the best-known platinum-based drug, is used in the chemotherapy of multiple solid tumors, including reproductive system cancers, head and neck cancers, lung cancers and cancers in the GI tracts (Prestayko et al., 1979; Galluzzi et al., 2012). The most widely studied mechanism of how cisplatin acting on cancers is activating the DNA damage response of cancer cells (Galluzzi et al., 2012). Treated with cisplatin, DNAs in cancer cells form adducts to inhibit transcription and synthesis. This process sets off a cascade of intracellular signaling transduction to eliminate the disease (Siddik, 2003). However, cisplatin resistance, including intrinsic and acquired resistance, introduces itself rapidly, thus causing therapeutic failure and recurrence. Cancer cells resist the cell death caused by cisplatin by reducing the uptake of cisplatin, strengthening the efflux of cisplatin and invalidating cisplatin through covalent binding with glutathione or metalloproteins (Zisowsky et al., 2007; Galluzzi et al., 2012; Rocha et al., 2014).

In GIST cells, the chemosensitivity of cisplatin was found to be increased by miRNA-22-3p through activating PTEN/PI3K/AKT pathway (Xu et al., 2018). The research of Xu et al. pointed out that GIST cells administrated with both miRNA-22-3p mimics and cisplatin would have lower survival rate and BCL-2/BCL-2 associated X (Bax) ratio, and higher apoptosis rate and Caspase-3 level than those treated with cisplatin merely (Xu et al., 2018). Though cisplatin was not used as the first-line agent for GIST therapy in the clinic, analyzing and probing into the functions of ncRNAs on the cisplatin resistance in GIST provided us with more information about the chemotherapy of GIST and new strategies to overcome the drug resistance dilemma in GIST.

## EFFECT OF NONCODINGRNAS ON DIFFERENT TYPES OF DRUG RESISTANCE IN GASTROINTESTINAL STROMAL TUMORSS

Primary resistance symbolizes the initial resistance of the drugs when the drugs are first applied for the treatment, while the secondary resistance represents that the resistance gradually takes place after a period of successful drug treatment.

### Primary Resistance

There are 10–15% of GIST patients initially failing to react to the imatinib exposure, and the early progression always occurs in 3–6 months after the initial exposure (Sleijfer et al., 2007; Blanke et al., 2008). It was widely considered that patients with primary resistance to imatinib harbored drug target mutations such as KIT mutation in exon 9, wild-type KIT mutations and PDGFRA point mutation D842V (Heinrich et al., 2003a; Heinrich et al., 2008b). To overcome the primary resistance, one should target KIT, which is the target of imatinib, but in a different way rather than imatinib does.

And in GISTs, ncRNAs, whose functions concentrated on the KIT, played essential roles in the primary resistance. It was reported that miRNA-17-92, miRNA-221, miRNA-222 and miRNA-148b-3p induced apoptosis and cell cycle regulation of primary imatinib-resistant GIST cells by directly targeting KIT (Gits et al., 2013; Ihle et al., 2015; Wang Y. et al., 2018). One study based on the microRNA profiles of 53 GIST samples was conducted to identify the differentially expressed miRNAs among the imatinib-sensitive and primary imatinib-resistant GISTs, and miRNAs-mRNAs interaction networks were drawn to show the biochemical pathways and gene regulations in the process of resistance (Amirnaser et al., 2019). LncRNA CCDC26 also played pivotal roles in the primary resistance of imatinib by regulating IGF-1R in GISTs, which could help the targeted treatment (Yan et al., 2019b). In other researches which focused on primary drug resistance in GISTs, lncRNA RP11-616M22.7, miRNA-22-3p, miRNA-218, and microRNA-30a showed potential functions, including changing drug targets, activating relevant pathways and inactivating autophagy in the primary resistance (Fan et al., 2015; Xu et al., 2018; Chen et al., 2020; Shao et al., 2021).

### Secondary Resistance

Secondary resistance of imatinib happens more frequently than the primary resistance in GIST, with over 50% of the imatinib administrated GIST patients developing secondary resistance within 2 years after the beginning of the imatinib therapy (Demetri et al., 2002; Gramza et al., 2009; Wang et al., 2011). A second mutation in KIT, the same allele as primary resistance happening, is the most universal reason for the secondary resistance, and such a mutation has not been observed in the tumors with primary resistance yet (Chen et al., 2004; Antonescu et al., 2005). Secondary mutations usually appeared at exons 13 and 17 of KIT, and these mutations changed the tyrosine kinase domains to inhibit the binding of imatinib and made the catalytic

site related to imatinib insensitive (Chen et al., 2004; Nishida et al., 2008b). In addition, multiple secondary mutations have been found to appear on KIT along with the tumor progression, even in the same tumor nodule. The intra- and inter-lesional heterogeneity of the secondary mutations in GIST are extensive, which lead to the therapeutic difficulties of imatinib. And this is where the selection of other more target-specific TKIs comes in (Heinrich et al., 2006; Wardelmann et al., 2006; Liegl et al., 2008).

Nowadays, functions of ncRNAs in the secondary resistance on imatinib in GISTs have attracted full attention. A series of studies based on the microarray and qRT-PCR analysis identified the differential expression levels of miRNAs between imatinib treated GIST patients with and without secondary resistance and highlighted the functional role of miRNA-125a-5p on secondary imatinib resistance by regulating PTPN18 to affect the phosphorylation of FAK (Akcakaya et al., 2014; Huang et al., 2018). MiRNA-483-3p inhibited the mitochondrial respiratory complexes to restrain the OXPHOS caused by imatinib resistance in GIST cells (Huang et al., 2021). MiRNA-518 family members miRNA-518a-5p and miRNA-518e-5p were also proved to play an important part in the secondary imatinib resistance through targeting PIK3C2A and affecting the cellular response to imatinib in GISTs (Shi Y. et al., 2016; Kou et al., 2018). Moreover, lncRNA CCDC26, whose expression levels time-dependently reduced by the imatinib exposure, sensitized the secondary resistant GIST cells to imatinib by inhibiting c-KIT (Cao K. et al., 2018). Additionally, significant ncRNAs in the secondary resistance in GISTs could also be identified by techniques like genome-scale CRISPR-Cas9 knockout screening (Cao J. et al., 2018), databases like the GEO database (Zhang et al., 2018) and gene functions analyses like KEGG pathway analysis and Gene Ontology enrichment analysis (Yan et al., 2019a). NcRNAs have definite functions in the secondary resistance of GIST, which provides us with a novel insight into the targeted therapies for GISTs.

## CLINICAL APPLICATIONS AND POSSIBLE RESEARCH DIRECTIONS OF NONCODINGRNAS IN GASTROINTESTINAL STROMAL TUMORSS

### Act as Potential Prognostic Biomarkers

Aside from the functions and mechanisms of ncRNAs in the chemotherapy resistance as discussed above, prognostic roles of ncRNAs in GISTs are also shown. Numerous ncRNAs have been revealed as prognostic biomarkers in multiple kinds of tumors such as gastric cancer (Wei et al., 2020), non-small cell lung cancer (NSCLC) (Leonetti et al., 2019), hepatocellular carcinoma (Wei et al., 2019), myeloma (Rastgoo et al., 2017) and leukemia (Bhat et al., 2020). And in GISTs, ncRNAs might be considered as a biomarker for recurrence, metastasis and drug resistance as well. Kou et al. analyzed the expression profiles of miRNA in the serum for figuring out the differential expressed miRNAs. Serum samples from GIST patients with and without imatinib resistance and healthy controls were collected for qRT-PCR analyzing and



the results showed that the content of miRNA-518e-5p in serum could distinguish imatinib-resistant GIST patients from imatinib-sensitive patients and healthy controls, which might be used as a possible biomarker for the early detection and diagnosis of GISTs and imatinib resistance by measuring miRNA-518e-5p content in serum (Kou et al., 2018). Besides, by conducting Kaplan-Meier survival analysis and log-rank analysis, expression levels of miRNA-1915 and miRNA-148b-3p were found to associate with disease-free survival (DFS) rates and overall survival (OS), and they could be regarded as biomarkers for the prognosis of GIST (Akçakaya et al., 2014; Wang Y. et al., 2018). These elementary results revealed that ncRNAs might be used as potential biomarkers to sort GIST patients in line with the prognosis for better cures.

## Therapeutic Targets

Diverse researches revealed that cell death through apoptosis pathways could be generated by the larger part of cancer chemotherapy drugs. And once the apoptosis is disorganized, drug resistance and increased cancer cell survival appear (Mohammad et al., 2015; Das et al., 2018; Mou et al., 2018). In the drug resistance of GISTs, there are a great many studies exhibiting that functions of ncRNAs on the resistance are associated with apoptosis.

LncRNA CCDC26 knocking down decreased the apoptosis of GIST cells treated with imatinib through upregulating IGF-1R, which acted in the apoptosis pathways (Li et al., 2018; Yan et al., 2019b; Zhang Y. et al., 2019). Furthermore, lncRNA CCDC26 was also reported to interact with c-KIT, thus affecting the rates of apoptotic cells caused by imatinib (Cao K. et al., 2018). BCL-2, whose gene families played pivotal roles in the programmed cell death regulations, induces apoptosis evasion and drug resistance evolution in cancers (Ashkenazi et al., 2017; Maji et al., 2018). In GISTs, BCL-2 was down-regulated by miRNA-21, miRNA-221 and miRNA-222, whose mimics were transfected in GIST cells and could significantly aggravate the apoptosis motivated by imatinib (Ihle et al., 2015; Cao et al., 2016). The overexpression of miRNA-518a-5p increased the proportion of apoptotic GIST cells treated with imatinib, which advocated that miRNA-518a-5p acted a part in interfering with the imatinib resistance (Shi Y. et al., 2016). When treated with imatinib, the resistance generated by inhibiting the apoptosis of GISTs could be attenuated by multiple ncRNAs through targeting different apoptosis-related proteins.

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## CONCLUSION

With the researches of ncRNAs going on, an increasing number of ncRNAs in GIST have been found to play significant roles in drug resistance. This review discussed the mechanisms concerning the functions of ncRNAs in the drug resistance of GIST from five aspects: restraining the OXPHOS increased by the drugs, regulating autophagy against drug resistance, affecting the apoptosis relevant to drug resistance, changing targets of the drugs and activating relevant signaling pathways. Different types of ncRNAs on the drug resistance of GISTs were summarized. And we divided the regulating functions of ncRNAs during the drug resistance in GISTs into promotion and inhibition. Roles of ncRNAs related to different drug types and drug resistance types of GISTs were also summed up. In the end, we evaluated the potential clinical applications for ncRNAs as prognostic biomarkers and therapeutic targets. However, it remains challenging to identify the most pivotal ncRNAs from the various candidates. Identifying ncRNA that really matter in the clinical setting is of great significance for the future research, and more experiments relevant to clinic are required. Moreover, the delivery and applications of ncRNAs are difficult due to the complex internal microenvironment. Further studies on the precision and efficiency of drug delivery and translations from the experimental results into the clinical trials are urgently needed.

## AUTHOR CONTRIBUTIONS

JL and SG performed the literature review, wrote the manuscript and prepared the figures. YF and ZS critically reviewed and edited the manuscript. All authors read and approved the final manuscript.

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## GLOSSARY

**ABL** Abelson tyrosine kinase

**AS** alternative splicing

**asRNAs** antisense RNAs

**ATG2B** autophagy-related protein 2 homolog B

**Bax** BCL-2 associated X

**BCL-2** B-cell lymphoma-2

**BRAF** v-raf murine viral oncogene homolog B1

**ceRNAs** competing endogenous RNAs

**circRNAs** circular RNAs

**CRISPR** clustered regularly interspaced short palindromic repeats

**CSF1R** colony stimulating factor-1 receptor

**DFS** disease-free survival

**EMT** epithelial-mesenchymal transition

**FAK** focal adhesion kinase

**FGFR** fibroblast growth factor

**FLT-3** Fms-like tyrosine kinase-3

**GEO** Gene Expression Omnibus

**GIST** gastrointestinal stromal tumor

**GI tract** gastrointestinal tract

**GSEA** gene set enrichment analysis

**HIF-1** hypoxia-inducible factor-1

**ICCs** the interstitial cells of Cajal

**IGF-1R** insulin-like growth factor 1 receptor

**JAK** Janus kinase

**KEGG** Kyoto Encyclopedia of Genes and Genomes

**KIT** KIT proto-oncogene receptor tyrosine kinase

**LATS** large tumor suppressor kinase

**lncRNAs** long noncoding RNAs

**MEN1** multiple endocrine neoplasia type 1

**miRNAs** microRNAs

**MRP** multidrug resistance protein

**MST** mammalian STE20-like kinase

**ncRNAs** noncoding RNAs

**NF1** neurofibromatosis type 1

**NSCLC** non-small cell lung cancer

**OS** overall survival

**OXPHOS** oxidative phosphorylation

**PDGFRA** platelet-derived growth factor receptor alpha

**pFAK** phosphorylated FAK

**piRNAs** PIWI-interacting RNAs

**PI3K** phosphoinositide 3-kinase

**PKB** protein kinase B

**PTEN** phosphatase and tensin homolog

**RAF1** raf-1 proto-oncogene serine/threonine kinase

**RASSF1A** ras association domain family protein1 isoform A

**Rb1** retinoblastoma susceptibility gene

**RISC** RNA-induced silencing complex

**rRNAs** ribosomal RNAs

**SCF** stem cell factor

**SDH** succinate dehydrogenase

**SETD2** set domain containing 2

**siRNAs** small interfering RNAs

**snoRNAs** small nucleolar RNAs

**STAT3** signal transducer and activator of transcription 3

**TAZ** transcriptional coactivator with PDZ-binding motif

**TKIs** tyrosine kinase inhibitors

**tRNAs** transfer RNAs

**YAP** Yes-associated protein

**3'-UTR** 3' untranslated regions



# Prediction of Clinical Outcome in Endometrial Carcinoma Based on a 3-lncRNA Signature

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Endometrial carcinoma (EC) is one of the common gynecological cancers with increasing incidence and revived mortality recently. Given the heterogeneity of tumors and the complexity of lncRNAs, a panel of lncRNA biomarkers might be more precise and stable for prognosis. In the present study, we developed a new lncRNA model to predict the prognosis of patients with EC. EC-associated differentially expressed long noncoding RNAs (lncRNAs) were identified from The Cancer Genome Atlas (TCGA). Univariate COX regression and least absolute shrinkage and selection operator (LASSO) model were selected to find the 8-independent prognostic lncRNAs of EC patient. Furthermore, the risk score of the 3-lncRNA signature for overall survival (OS) was identified as CTD-2377D24.6 expression  $\times$  0.206 + RP4-616B8.5  $\times$  0.341 + RP11-389G6.3  $\times$  0.343 by multivariate Cox regression analysis. According to the median cutoff value of this prognostic signature, the EC samples were divided into two groups, high-risk set (3-lncRNAs at high levels) and low-risk set (3-lncRNAs at low levels), and the Kaplan–Meier survival curves demonstrated that the low-risk set had a higher survival rate than the high-risk set. In addition, the 3-lncRNA signature was closely linked with histological subtype ( $p = 0.0001$ ), advanced clinical stage ( $p = 0.011$ ), and clinical grade ( $p < 0.0001$ ) in EC patients. Our clinical samples also confirmed that RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 levels were increased in tumor tissues by qRT-PCR and *in situ* hybridization. Intriguingly, the  $p$ -value of combined 3-lncRNAs was lower than that of each lncRNA, indicating that the 3-lncRNA signature also showed higher performance in EC tissue than paracancerous. Functional analysis revealed that cortactin might be involved in the mechanism of 3-lncRNA signatures. These findings provide the first hint that a panel of lncRNAs may play a critical role in the initiation and metastasis of EC, indicating a new signature for early diagnosis and therapeutic strategy of uterine corpus endometrial carcinoma.

**Keywords:** endometrial carcinoma, lncRNA signature, prognosis, diagnostic, biomarker

## INTRODUCTION

Globally, the incidence for uterine corpus endometrial carcinoma (UCEC) persistently increased with 1.3% per year from 2007–2016, in part due to continued declines in the fertility rate as well as increased obesity (Siegel et al., 2020). In China, the incidence of EC was also increasing from 2014, which ranked second in female reproductive malignancies on account of the increased risk factors such as diabetes and obesity (Chen et al., 2019). Although EC has a good prognosis with 5-year overall survival (OS) of 74–91%, the advanced or metastatic EC patients still have a poor prognosis due to tumor metastasis and poor differentiation (Piulats et al., 2017). Histological classification and the International Federation of Gynecology and Obstetrics (FIGO) staging system are the traditional treatment guideline and prognostic indicators (Pecorelli, 2009; Morice et al., 2016). However, distinct molecular characteristics have been demonstrated in the same stage and histology of cancers (Murali et al., 2014; Yang et al., 2016). With the development of precision medicine, a new therapeutic approach according to molecular profiling has been provided. In 2021, to improve outcomes of EC patients, molecular classification was recommended to select appropriate treatment regimens by the National Comprehensive Cancer Network (NCCN). Four molecular subgroups have been classified in 2013 based on the integrated genomic data of 373 endometrial carcinomas (Levine et al., 2013). Nevertheless, the integrated classification had limited application due to high expense and complex procedures. Therefore, identifying an efficient prognostic and diagnostic signature to guide clinical practice for EC is urgent.

Noncoding RNA was initially recognized as simply leaky transcription noise because they are not translated into proteins. However, numerous noncoding RNAs showed specific functions in cellular processes, as well as the dysregulation in human pathologies. Long noncoding RNA (lncRNA) is a class of noncoding transcripts with more than 200 nucleotides in length. Compelling studies reported that lncRNAs were associated with various human diseases including cancer by participating in biological processes widely (Schmitt and Chang, 2016; Peng et al., 2017; Yang et al., 2019). Meanwhile, accumulating evidence supported the potential ability of lncRNAs as cancer biomarkers (Lim et al., 2019; Li et al., 2020; Xie et al., 2020) and the prognostic value of lncRNAs (He et al., 2014; Sun et al., 2021). For example, Liu et al. systematically discussed the EC-related lncRNAs and their roles in different cancer hallmarks, including tumor growth, metastasis, maintenance of cancer stem cells, and chemoresistance (Liu H et al., 2019). Until now, some biomarkers for EC have been identified using gene expression profile data. However, these models are limited to a specific stage or grade of EC. For example, one study identified a prognostic model for patients with early-stage EC using reverse-phase protein arrays (Yang et al., 2016). Others found a prognostic value of immune, metabolic, or autophagy-related coding and noncoding lncRNAs for EC (Ouyang et al., 2019; Gao et al., 2020; Li and Wan, 2020; Wang et al., 2021). However, given the heterogeneity of EC and the complexity of lncRNAs, a panel of lncRNA biomarkers might be more precise and stable for predicting prognosis rather than one lncRNA. Therefore, it is timely to investigate the new lncRNA

biomarkers by combining The Cancer Genome Atlas (TCGA) data with UCEC-specific data.

In the present study, we obtained the lncRNA expression profile and clinical information of UCEC patients from the datasets of TCGA project. By bioinformatic approaches, a potential 3-lncRNA signature was identified in EC, and the association between the signature and clinical characteristics was confirmed. Furthermore, clinical samples were used to demonstrate that 3-lncRNA signature has a much better performance than independent 3 lncRNAs, providing a new signature for early diagnosis and therapeutic strategy of EC.

## RESULTS

### Identification of Differentially Expressed Long Noncoding RNAs Associated with Uterine Corpus Endometrial Carcinoma from The Cancer Genome Atlas

We obtained lncRNA expression profiles in 548 UCEC tissues and 35 normal tissues from TCGA datasets to screen DElncRs. To obtain reliable and stable results, lncRNA expression data were downloaded and performed using “DEseq,” “edgeR,” and “limma” R package separately in the R software (Figures 1A–C), and the intersections were acquired. Among the acquired lncRNAs, a set of 233 lncRNAs, including 93 upregulated and 140 downregulated, was abundantly expressed in all the uterine corpus endometrial carcinoma (Figures 1D–F, Supplementary Table S1). These results indicated the role of differentially expressed lncRNAs in the initiation and progression of uterine corpus endometrial carcinoma.

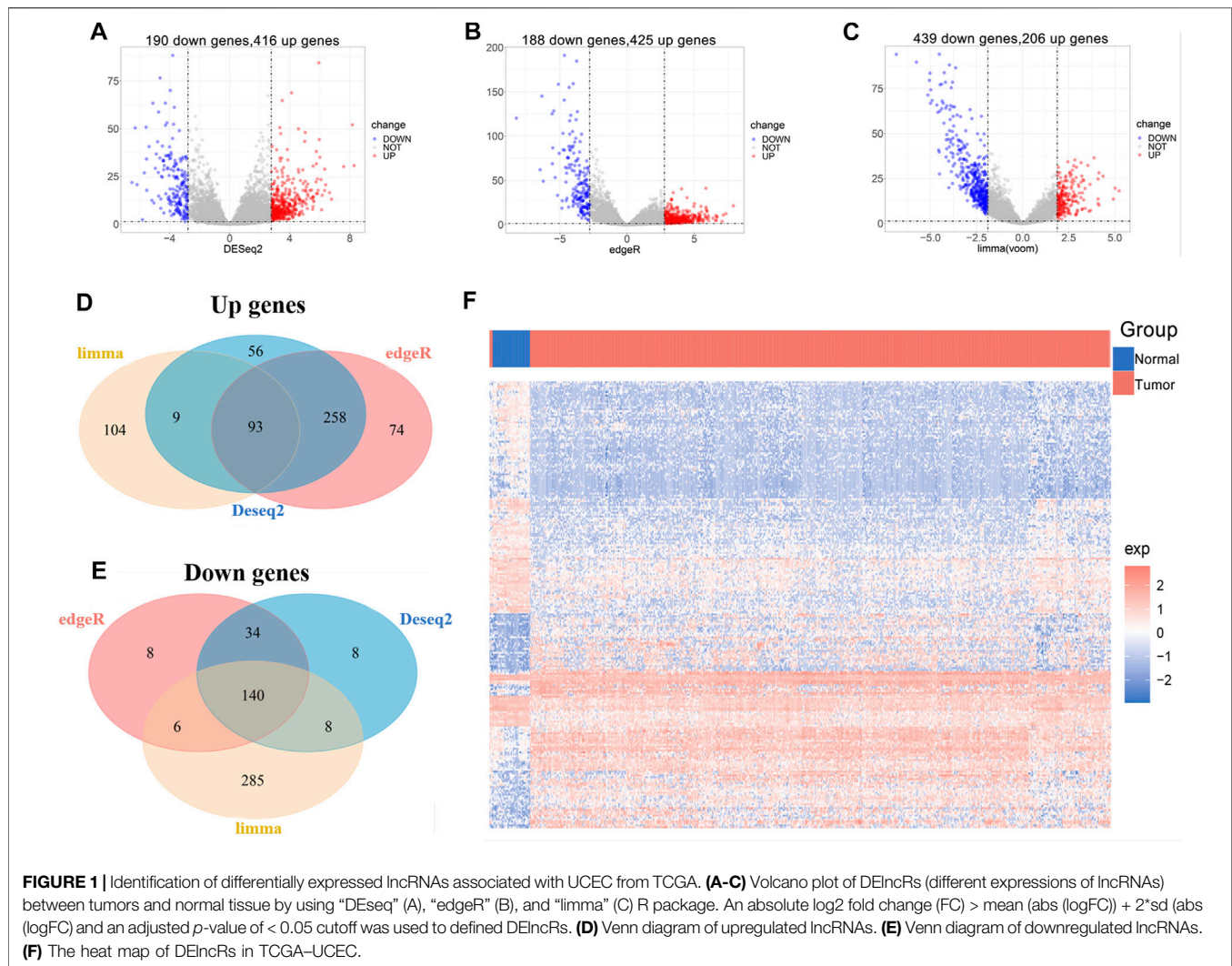
### Validation of Prognostic Long Noncoding RNA Signature

Subsequently, univariate Cox regression analysis was conducted to estimate the prognostic relationship between DElncRs and EC patient OS, and 31 prognostic lncRNAs were obtained with a  $p < 0.05$  (Figure 2A). Furthermore, to minimize prediction errors, 9 lncRNAs were screened out using the LASSO regression method. Kaplan–Meier survival curves were used to further analyze the relationship between the 9 lncRNAs and the OS of EC patients. Ultimately, 8 lncRNAs were identified to be related with OS (Figures 2B–I). Multivariable Cox regression analysis revealed the hazard ratios of 8 lncRNAs for OS of endometrium carcinoma (Figure 2J). The area under the ROC curve (AUC) for OS was 0.71 (Figure 2K). These results implied that the 8-lncRNA model could efficiently identify the risk of EC prognosis.

### Assessment of Prognostic Risk in Uterine Corpus Endometrial Carcinoma Patients Using a 3-Long Noncoding RNA Model

To better identify the prognostic signature for UCEC, 3 lncRNAs (hazard ratios for CTD-2377D24.6 = 1.229, RP4-

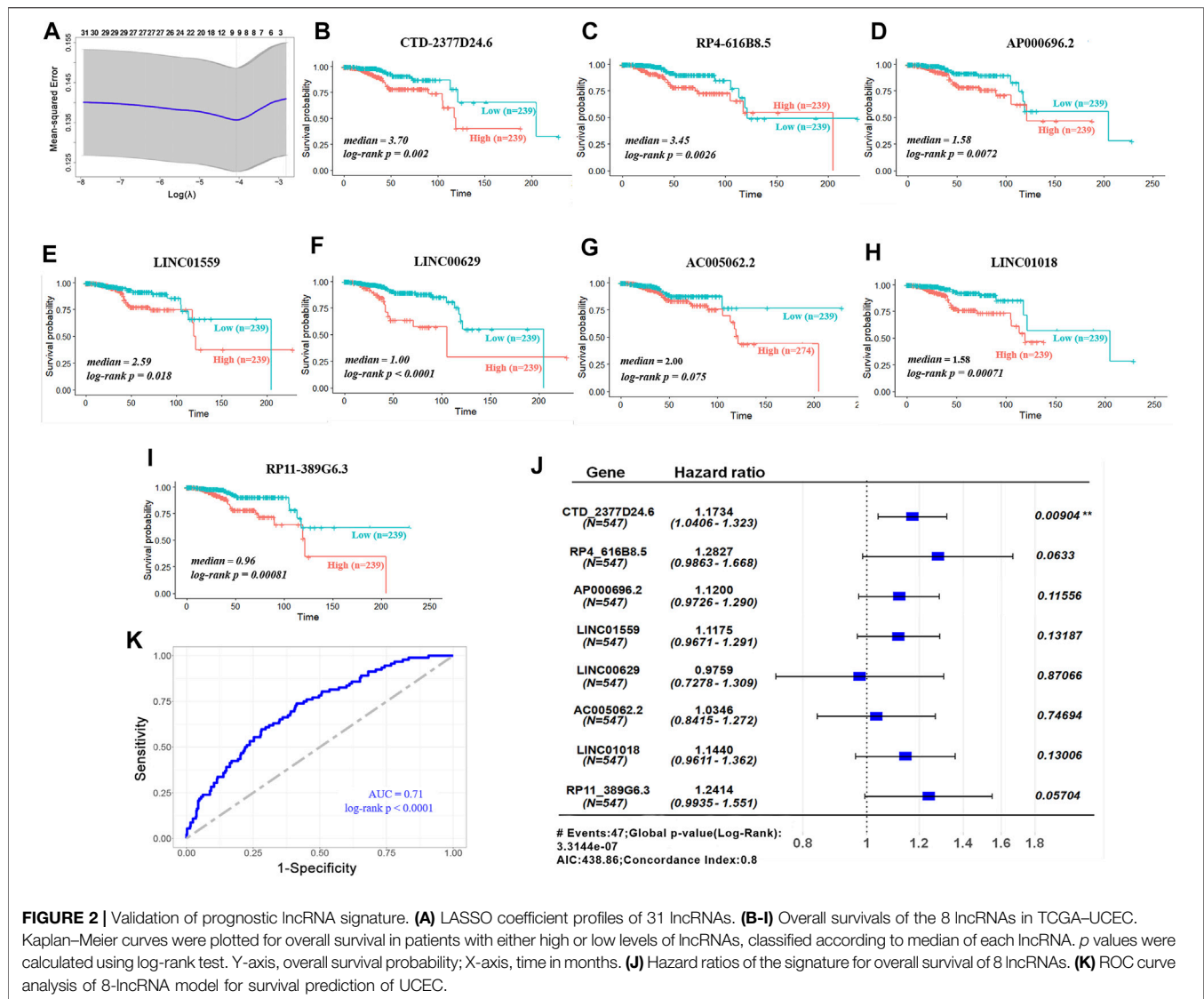




616B8.5 = 1.407, and RP11-389G6.3 = 1.409) with the lowest *p*-value ( $p < 0.1$ ) were picked out for further investigation (**Figure 3A**). Based on the coefficients of 3 prognostic lncRNAs from multivariate Cox regression analysis (Liu Y et al., 2019; Jiang et al., 2021), the risk score of the 3-lncRNA signature for OS was identified as  $\text{CTD-2377D24.6 expression} \times 0.206 + \text{RP4-616B8.5} \times 0.341 + \text{RP11-389G6.3} \times 0.343$ . According to the median cutoff value of this prognostic signature, patients were divided into low-risk and high-risk sets. The survival results demonstrated that the low-risk set had a higher survival rate than that of the high-risk set ( $p < 0.0001$ , **Figure 3B**). To assess the potential prediction of 3-lncRNAs for overall survival of UCEC patients, the AUC analysis was performed to test the 3-lncRNA signature compared with each lncRNA. The results showed that the 3-lncRNA signature insignificantly showed an excellent performance than that of each lncRNA and two lncRNAs (**Supplementary Figure S1, S2**).

### Correlation Between the 3-Long Noncoding RNA Signature and Clinical Characteristics of The Cancer Genome Atlas–Uterine Corpus Endometrial Carcinoma

To better understand the prognostic value of the 3-lncRNA signature, we further evaluated the relationships between the 3-lncRNA signature and traditional clinical characteristics. According to the median expressions of CTD-2377D24.6, RP4-616B8.5, RP11-389G6.3, and the 3-lncRNA signature risk score, the UCEC samples were divided into two sets. Pearson chi-square or Fisher's exact tests revealed that the 3-lncRNA signature was closely linked with histological subtype ( $p < 0.0001$ ), advanced clinical stage ( $p = 0.011$ ), and clinical-grade ( $p < 0.0001$ ) (**Table 1**). Compared to low-risk sets, the high-risk set tended to be serous adenocarcinoma (SAC), a histopathological type with worse differentiation and distant metastasis. These results demonstrated that 3-lncRNA



signature was closely related to the conventional prognostic indicators.

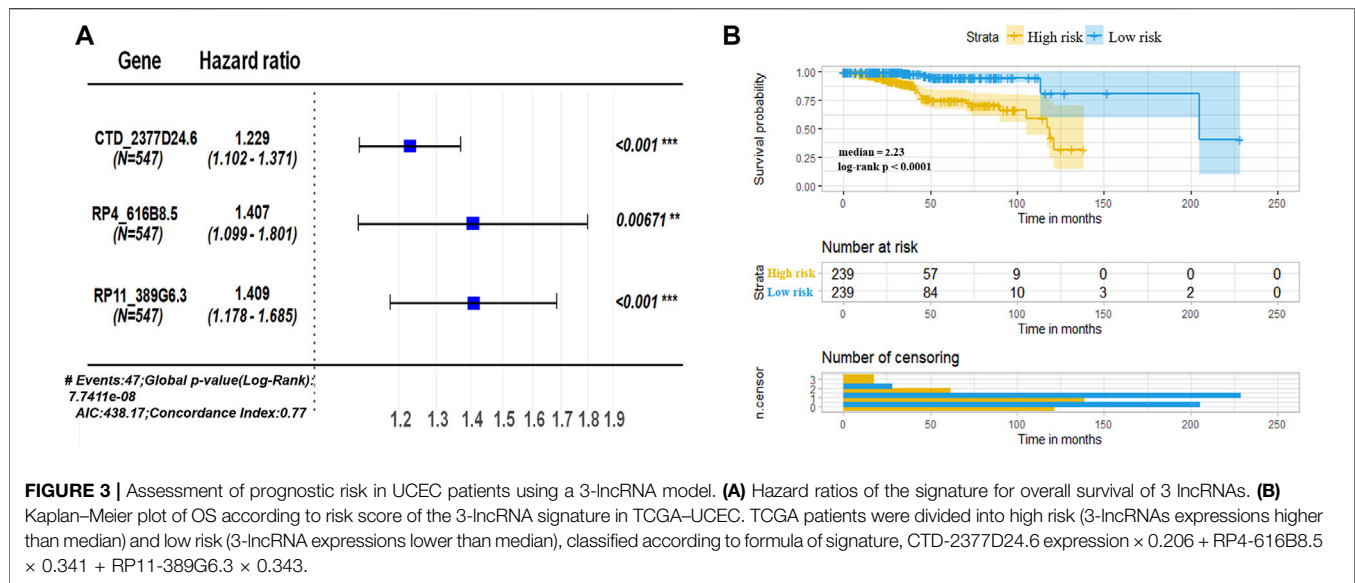
## Expressions of 3 Long Noncoding RNAs in Paracancerous and Tumor Tissues of Uterine Corpus Endometrial Carcinoma Patients

In addition, we validated the expressions of 3 lncRNAs in 30 paired paracancerous and tumor tissues of UCEC patients. First, the transcript abundances of RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 were evaluated by qRT-PCR, and the results indicated that the expressions of RP11-389G6.3 and CTD-2377D24.6 were significantly higher in tumor tissues with *p*-values of 0.023 and 0.002, respectively, while the expression of RP4-616B8.5 did not show significant difference between tumor and paracancerous tissues with a *p*-value of 0.087 (Figure 4A), and the *p*-value of combined 3-lncRNAs was 0.027 using Hotelling  $T^2$  test ( $F = 3.56$ ) (Figure 4B).

Furthermore, *in situ* hybridization assay was also utilized to confirm the expressions of lncRNAs in paracancerous and tumor tissues of UCEC patients (Figure 4C). The staining scores of RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 in EC tissues were significantly higher than those in paracancerous tissues with *p*-values of 0.042, 0.005, and 0.011, respectively (Figure 4D), and the *p*-value of combined 3-lncRNAs was 0.0002 using the Fisher's methods ( $\chi^2 = 25.84$ ) (Figure 4E). These results revealed that 3-lncRNA signature exhibited a better performance than the independent 3 lncRNAs for EC diagnosis.

## Functional Analysis of 3-Long Noncoding RNA Signature in Uterine Corpus Endometrial Carcinoma

To explore the potential roles of 3-lncRNA signature in UCEC, differentially expressed mRNAs (DeRNAs) between the high-risk (3-lncRNAs at high levels) and low-risk (3-lncRNAs at low levels)



**TABLE 1 |** Relationships between 3-lncRNA set with histological subtype, clinical stage, and grade of EC patients.

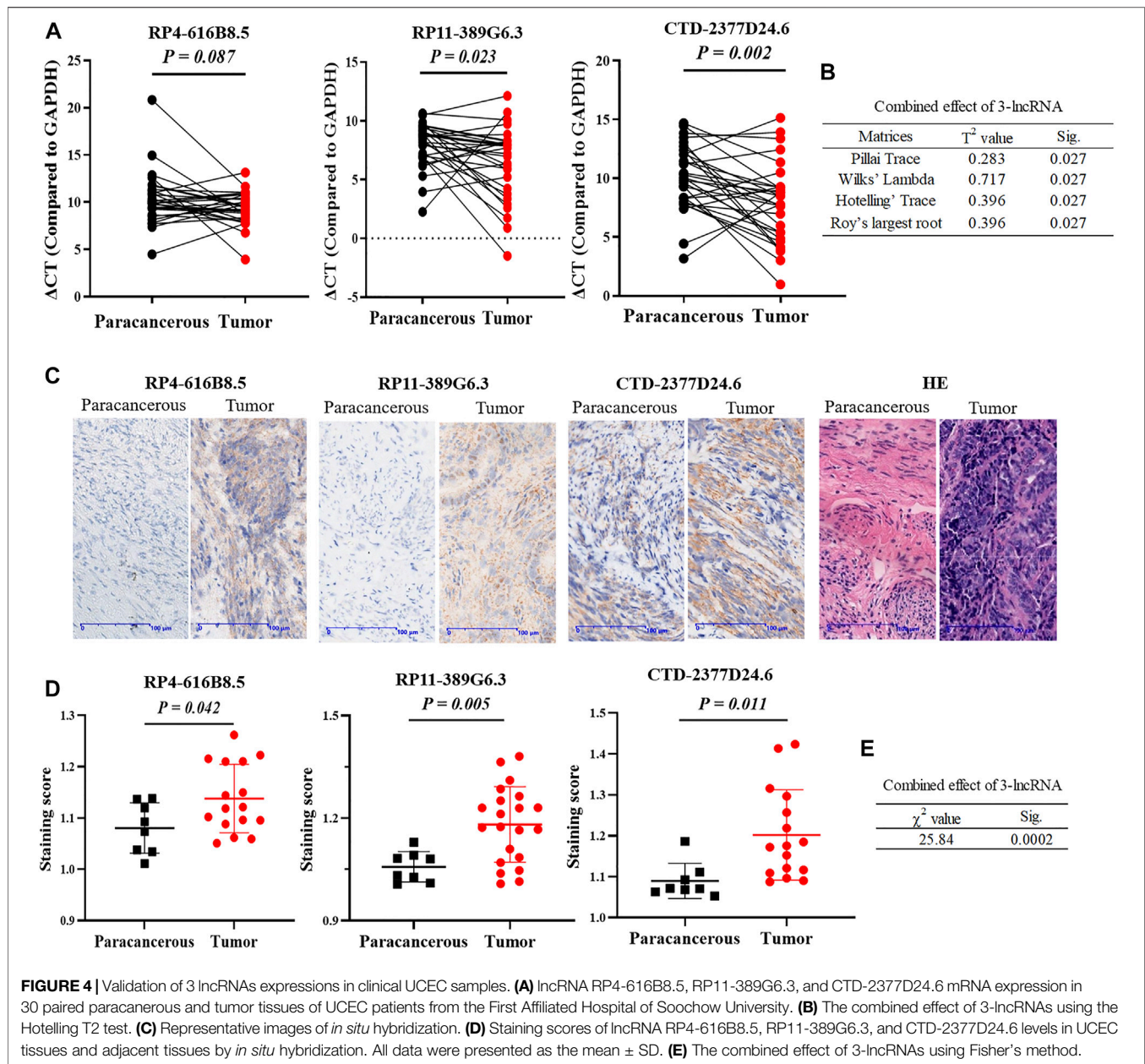
Characteristics	RP11-389G6.3			RP4-616B8.5			CTD-2377D24.6			3-lncRNA signature		
	Low	High	p value	Low	High	p value	Low	High	p value	Low	High	p value
Histological subtype	—	—	—	—	—	—	—	—	—	—	—	—
EAC	235	174	0.0013	233	176	<0.0001	246	163	<0.0001	242	167	<0.0001
SAC	46	70	—	30	86	—	25	91	—	25	91	—
Other	9	14	—	11	12	—	9	14	—	7	16	—
Menopause status	—	—	—	—	—	—	—	—	—	—	—	—
Pre	40	29	0.4108	48	21	0.001	48	21	0.001	53	16	<0.0001
Post	237	213	—	217	233	—	217	233	—	213	237	—
Clinical stage	—	—	—	—	—	—	—	—	—	—	—	—
I	192	149	0.1841	198	143	<0.0001	187	154	0.0242	188	153	0.0111
II	26	25	—	15	36	—	26	25	—	21	30	—
III	60	67	—	50	77	—	59	68	—	56	71	—
IV	12	17	—	11	18	—	8	21	—	9	20	—
Grade	—	—	—	—	—	—	—	—	—	—	—	—
G1	62	36	0.0002	76	22	<0.0001	62	36	<0.0001	73	25	<0.0001
G2	77	43	—	83	37	—	78	42	—	79	41	—
G3 and high grade	151	179	—	115	215	—	140	190	—	122	208	—

EAC, endometrioid adenocarcinoma; SAC, serous adenocarcinoma.

groups were identified in endometrium carcinoma patients (**Supplementary Table S2**), and KEGG and GO analysis were conducted. Functional enrichment analysis revealed that these DeRNAs were significantly enriched in 5 KEGG pathways, including carcinogenesis, drug metabolism and resistance, fluid shear stress, and steroid hormone biosynthesis (**Figure 5A**), 20 GO terms in biological processes, 10 GO terms in cellular components, and 10 GO terms in molecular functions (**Figure 5B**), indicating that drug metabolism, chemical carcinogenesis, and cell motility-related pathways might be involved. Given the importance of cortactin for invadopodia formation, cancer cell migration, and metastasis (Ji et al., 2020), we examined the mRNA expression levels and location

of cortactin by qRT-PCR and immunohistochemical staining. The results showed that two cortactin-encoding genes, CTTN and HCLS1, were markedly increased in tumor tissues (**Figures 5C,D**). Interestingly, immunohistochemical staining revealed that cortactin exhibited in gland duct cells, but not in supporting cells (**Figure 5I**). In addition, the greatest differentially expressed mRNAs between the high-risk (3-lncRNA signature at high levels) and low-risk (3-lncRNA signature at low levels) groups were also determined. DNAH5, LTF, and Ezrin were significantly increased in tumor tissues ( $p < 0.05$ , **Figures 5E–G**), and WNT7A displayed a slight increase ( $p = 0.0669$ , **Figure 5H**). These results indicated that cortactin might be associated with the function of 3-lncRNA signature.





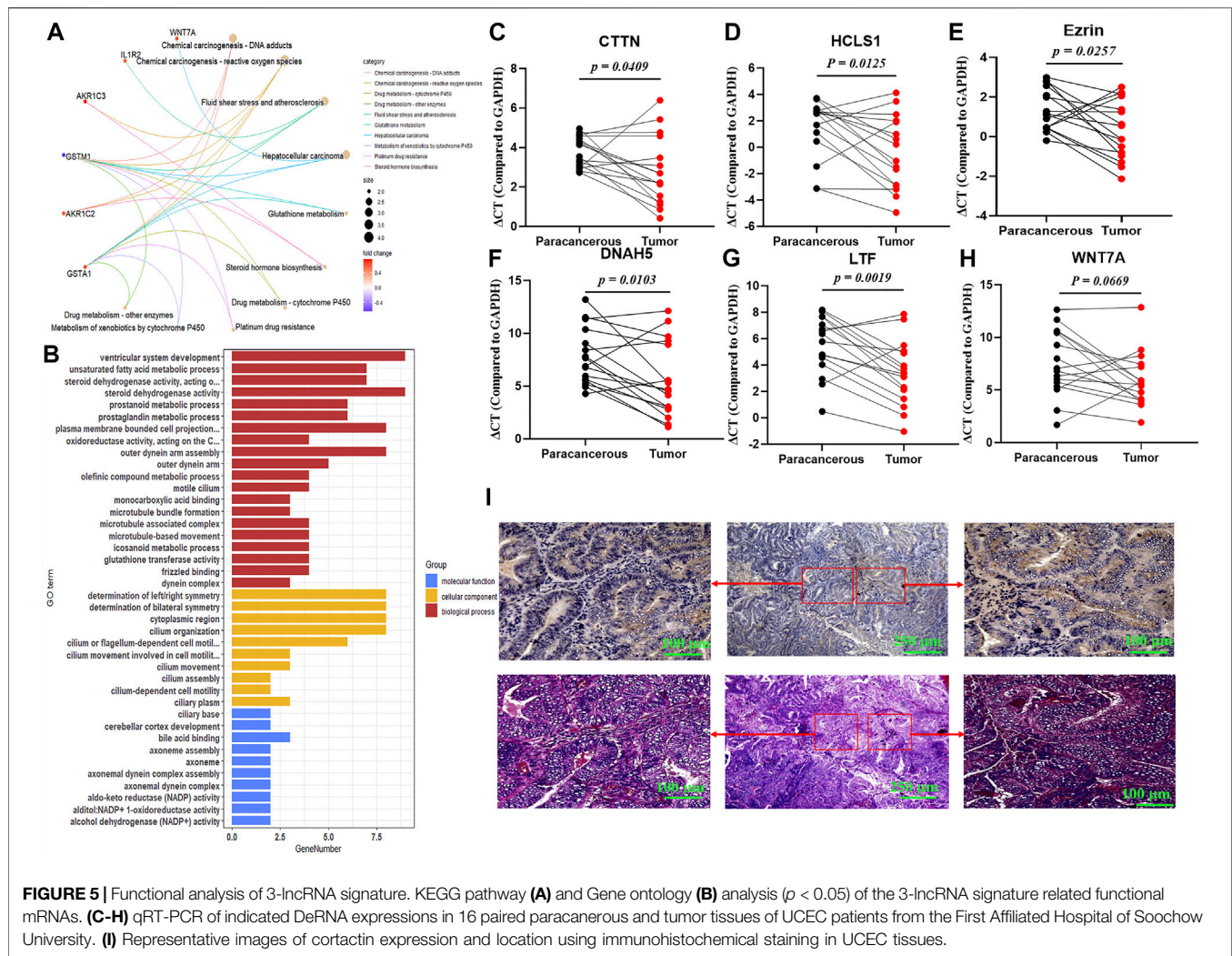
## DISCUSSION

Currently, a growing number of literatures demonstrated that dysregulated lncRNAs were involved in various diseases, as well as cancers (Evans et al., 2016). lncRNA might be a promising biomarker for cancer diagnosis, treatment, and prognosis prediction. Due to the heterogeneity of the tumor, a panel of lncRNA signature was more precise than a single lncRNA. In the present study, using Cox and LASSO regression, a 3-lncRNA signature was identified for predicting OS of EC patients. According to the median cutoff value of 3-lncRNA model, we demonstrated that the high-risk set displayed a poor survival, a higher clinical stage, and clinical grade and tended to be serous adenocarcinoma, a histopathological type with worse differentiation and distant metastasis. Our clinical samples also

confirmed that 3-lncRNA, RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 levels were increased in EC tissues than in paracancerous tissues by qRT-PCR and *in situ* hybridization. These findings provide an important hint that the 3-lncRNA signature has the potential performance for EC diagnosis and prognosis.

Mechanically, lncRNAs play a crucial role in EC progression by multiple patterns such as signaling, decoying, scaffolding, and guidance (Liu H et al., 2019). For example, Dong et al. reported that DLEU2 potentially interacted with miR-455 and miR-181a to promote epithelial-to-mesenchymal transition (EMT) and aerobic glycolysis (Dong et al., 2021). NEAT1 initiates a miR-361-mediated network to drive aggressive EC progression (Dong et al., 2019). lncRNA SOCS2-AS1 was found to suppress EC progression by





promoting Aurora kinase A (AURKA) degradation via the ubiquitin-proteasome pathway (Jian et al., 2021). Additionally, Zhao et al. revealed that DLX6-AS1 promoted EC progression by recruiting p300/E2F1 in the DLX6 promoter region (Zhao and Xu, 2020). However, we found that little was known about the roles of RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 in tumorigenesis and progression. One study reported that CTD-2377D24.6 was significantly induced by heat shock (Kim et al., 2020). Another study showed that CTD-2377D24.6 was a predictive factor in HCC patients with cirrhosis (Ye et al., 2020). No public study reported other two lncRNAs (RP4-616B8.5 and RP11-389G6.3). In the present study, we verified that the expressions of RP4-616B8.5, RP11-389G6.3, and CTD-2377D24.6 were higher in EC tissues than paracancerous tissues by qRT-PCR and *in situ* hybridization assays. According to the median cutoff value of 3-lncRNA signature, low-risk and high-risk sets were divided, and DeRNAs were identified. KEGG and GO analysis found that drug metabolism, chemical carcinogenesis, and cell motility-related pathway were enriched, indicating the potential roles of a panel of lncRNAs in initiation, metastasis, and chemoresistance of EC. The extensive quantity of published reports suggested that cell motility at

an early stage in cancer correlated with metastasis (Lambert et al., 2017). In particular, the importance of cortactin for invadopodia formation, cancer cell migration, and metastasis has been proven (Schnoor et al., 2018; Ji et al., 2020). However, the link between lncRNAs and cortactin in endometrial carcinoma remains unclear. Here, we demonstrated that cortactin was markedly increased in UCEC tumor tissues, and especially exhibited in gland duct cells. In addition, the greatest differentially expressed mRNAs between the high-risk and low-risk groups, such as DNAH5, LTF, and Ezrin, were significantly increased in tumors. These hinted that more comprehensive studies about the molecular mechanism of 3-lncRNAs will remain to be elucidated.

Traditional therapeutic strategies and risk stratification for EC patients are based on clinical and histological characteristics. However, the conventional classification does not adequately depict tumor biology owing to the high heterogeneity of EC. Recently, molecular or genomic classification has drawn attention as a promising approach to predict cancer prognosis. Levine et al. assessed the genome, transcriptome, and proteome of 373 endometrial carcinomas. Based on integrated genomic data, they were classified into four subgroups: POLE ultramutated,

microsatellite instability hypermutated, copy-number low, and copy-number high. Subsequently, another molecular classification for EC termed “ProMisE” was identified (Talhok et al., 2015). A similar integrated risk profile was established by the TransPORTEC international consortium (Stelloo et al., 2015; Stelloo et al., 2016). However, compared with genome sequencing, our 3-lncRNA signature was more suitable for clinical diagnosis and classification due to its higher stability and lower cost.

By bioinformatic approaches and verification of clinical samples, we demonstrated that the 3-lncRNA signature might be a reliable prognostic biomarker. However, there are several limitations in our study. First, the 3-lncRNA signature was constructed by the TCGA–UCEC datasets, in which the Caucasian race was the main patient. So, the prognostic value in other races is needed to be validated. Second, we detected the independent difference of 3 lncRNAs between the paracancerous tissues and UCEC tissues in our clinical samples, while the prognostic value of the signature was not analyzed due to insufficient prognostic data. Third, the 3 lncRNAs were rarely reported, and their potential function was unclear. Although functional enrichment analysis based on the DeRNAs in high- and low-risk signatures was performed, the potential mechanisms should be further experimentally investigated.

In conclusion, we revealed a potential 3-lncRNA signature that could accurately predict outcomes for UCEC patients. Meanwhile, we found that the 3-lncRNA signature was closely associated with clinical characteristics. Furthermore, we validated the different expressions of the 3 lncRNAs in our clinical samples, indicating that a panel of 3-lncRNAs exhibited better performance for EC diagnosis. These findings provide the first hint that the set of lncRNAs may play a critical role in the initiation and metastasis of EC, indicating a new signature for early diagnosis and therapeutic strategy of uterine corpus endometrial carcinoma.

## MATERIALS AND METHODS

### Data Source and Differentially Expressed Long Noncoding RNA Screening

The lncRNA expression data and corresponding clinical information of UCEC patients were open-accessed from TCGA data portal (<https://www.cancer.gov/tcga>), including 548 UCEC tissues and 35 normal tissues. The differentially expressed lncRNAs (DElncRs) were identified by using the R package “DEseq,” “edgeR,” and “limma” with  $|\text{Log}_2 \text{fold-change (FC)}| > \text{mean}|\text{Log}_2 \text{FC}| \pm 2 \times \text{sd}|\text{Log}_2 \text{FC}|$  and adjusted  $p < 0.05$ . A volcano plot of DElncRs was obtained by using the R package (Anders and Huber, 2010; Robinson et al., 2010; Ritchie et al., 2015).

### Construction and Assessment of Long Noncoding RNA-Based Prognostic Signature

First, univariate COX regression and least absolute shrinkage and selection operator (LASSO) model were selected to find the independent prognostic lncRNAs. The LASSO method was

**TABLE 2 |** Primer sequences for qRT-PCR.

Primers	Sequence (5'to 3')
CTD-2377D24.6-F	TTCCGGTGTCCAGATGTTCA
CTD-2377D24.6-R	AAGGTGAGTTGGGGAGGATG
RP4-616B8.5-F	ATGAGTGTGGCAGCCTATGT
RP4-616B8.5-R	AACTCCTGACCTCGTGATCC
RP11-389G6.3-F	GGCCTTGAGAGATAGAGGGG
RP11-389G6.3-R	ATACGTCCTTCCCATCCTCG
DNAH5-F	GAGGCAGAGTCACTGACGAC
DNAH5-R	TCTCATCCCTCCACCAGAG
WNT7A-F	CTGGGCATGGTCTACCTCCG
WNT7A-R	GGCCATTGCGGAACGAAAC
LTF-F	GTCCCTTCTCATGCCGTTGT
LTF-R	CCTTTCAGCACCCAGGGCGA
Ezrin-F	GGATAAGAAGGTGTCTGCCCA
Ezrin-R	TCCCACTGGTCCCTGGTAAG
CTTN-F	ATGTCACCCAGGTGTCTCT
CTTN-R	AAGCCGCATCCTCATAGACG
HCLS1-F	TGAGTATGTTGCCGAGGTGG
HCLS1-R	CTCGTGTCTCTCCGCTCTCC
GAPDH-F	GCACAGTCAAGGCTGAGAATG
GAPDH-R	ATGGTGGTGAAGACGCCAGTA

performed by the package “glmnet” in the R software. Subsequently, multivariate Cox regression analysis was used to construct the prognostic signature. A risk score formula was constructed as follows:  $\text{gene 1} \times b_1 + \text{gene 2} \times b_2 + \text{gene 3} \times b_3 + \dots + \text{gene n} \times b_n$ , in which  $b$  represented the respective coefficient of genes. Gene represented the expression level of each gene. Subsequently, the risk score of prognostic signature formula was calculated as follows:  $\text{CTD-2377D24.6 expression} \times 0.206 + \text{RP4-616B8.5} \times 0.341 + \text{RP11-389G6.3} \times 0.343$ . According to the median of risk score, the TCGA–UCEC patients were divided into the high-risk set and the low-risk set. To evaluate the prognostic signature of lncRNAs, the Kaplan–Meier and time-dependent receiver operating characteristic (ROC) curve analysis were performed.

### Functional Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology analysis were performed with the “clusterProfiler” R package to identify the function of lncRNA-based signature (Yu et al., 2012). Significant functional categories were filtered into the meaning of  $p$ -value and false discovery rate (FDR) values  $< 0.05$ .

### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Life Technologies, NY, United States). The concentration and integrity of RNA were verified by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Afterward, the total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China). The expressions of 3 lncRNAs were measured by qRT-PCR using the Hif qPCR SYBR Green Master Mix (Yeasen Biotech Co., Shanghai, China) in the QuantStudio 6 system (Applied Biosystems, Waltham, MA). The primers synthesized are listed in **Table 2**.

**TABLE 3 |** Probes sequences for *in situ* hybridization.

Probe	Sequence (5'to 3')
CTD-2377D24.6	CCC UUACCCACGGGUGACAGCCAUUUUGAG
RP11-389G6.3	GAGACAGGAGUUUGGGCUGAUGGGCUUGG
RP4-616B8.5	GAAGAGCAGGCAGUUUUUCUGUUUUUGAGGUUAG

## Ethics Statement

Publicly available TCGA datasets were analyzed in this study, and approval from a local Ethics Committee was not necessary. For human subjects, all procedures were carried out according to Helsinki Declaration and institutional guidelines and were approved by the Ethics Committee at the First Affiliated Hospital of Soochow University.

## In situ Hybridization Assay

The paraffin embedded UCEC and adjacent normal tissues were stained to detect the lncRNA expression. The lncRNA probes were designed and produced by SimaifuBio (Suzhou, Jiangsu, China). The probe sequences are presented in **Table 3**. In brief, sections were deparaffinized, digested, and blocked with 3% methanol-H<sub>2</sub>O<sub>2</sub>; then, the sections were dropped with prehybridization solution and incubated for 1 h in the incubator at 37°C. With the absorption of the excess liquid, the hybridization solution containing indicated lncRNA probes was added and then incubated in the incubator at 42°C overnight. Next day, after washing, the samples were dropped with block solution and incubated for 30 min at room temperature. After that, digoxigenin-labeled peroxidase antibody was added to incubate for 40 min in the incubator at 37°C. Afterward, the sections were added with DAB coloration, and the positive signal appeared brown–yellow. Hematoxylin staining solution was used to stain the nucleus. CaseViewer 2.2.1 (3DHISTECH Ltd.) and Image Pro Plus 6 were used for image capture and analysis, respectively.

## Statistical Analysis

All of the expression profiles and clinical information were obtained from TCGA by R software. All statistical analyses were carried out using SPSS23.0 (SPSS, Chicago, IL, United States) or R software. For continuous variables, Student's t-test was used to compare the difference between the two groups. For categorical variables,  $\chi^2$  test was used to compare the differences among groups. Fisher's method and Hotelling T2 test were used to combine *p* value. *p* < 0.05 was considered to be statistically significant.

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## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee at the First Affiliated Hospital of Soochow University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceptualization: BL and YC; methodology: HD, FJ, LD, WS, and JW; software: FJ and PW; validation: HD, LD, and JW; formal analysis: HD and LD; investigation: HD, FJ, and JL; resources: LD and JW; data curation: LD and JW; writing—original draft preparation: HD, FJ, LD, and JW; writing—review and editing: LC, JL, YP, YZ, and ZZ; visualization: PW and MJ; supervision: BL and YC; project administration: BL and YC; funding acquisition: BL, JL, YP, and FJ.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.814456/full#supplementary-material>

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# The LncRNA DUXAP10 Could Function as a Promising Oncogene in Human Cancer

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Cancer is one of the most prevalent and deadliest diseases globally, with an increasing morbidity of approximately 14 million new cancer cases per year. Identifying novel diagnostic and prognostic biomarkers for cancers is important for developing cancer therapeutic strategies and lowering mortality rates. Long noncoding RNAs (lncRNAs) represent a group of noncoding RNAs of more than 200 nucleotides that have been shown to participate in the development of human cancers. The novel lncRNA DUXAP10 was newly reported to be abnormally overexpressed in several cancers and positively correlated with poor clinical characteristics of cancer patients. Multiple studies have found that DUXAP10 widely regulates vital biological functions related to the development and progression of cancers, including cell proliferation, apoptosis, invasion, migration, and stemness, through different molecular mechanisms. The aim of this review was to recapitulate current findings regarding the roles of DUXAP10 in cancers and evaluate the potential of DUXAP10 as a novel biomarker for cancer diagnosis, treatment, and prognostic assessment.

**Keywords:** DUXAP10, lncRNAs, cancer, function, clinical applications

## INTRODUCTION

Despite significant advances in clinical diagnostic and treatment options, many diseases still have high mortality rates, high health care costs. And poor quality of life, especially cancer (Lee et al., 2018; Moliner et al., 2019; Sidney et al., 2019; Mulder et al., 2021; Su et al., 2021). Cancer (Mohsen et al., 2014; Christensen et al., 2018; Kannan et al., 2021; Mokgautsi et al., 2021) has become one of the most common causes of death worldwide, and the identification of cancer-related targets and relevant carcinogenesis mechanisms for patients at the early stage of the disease are urgently needed (Saha et al., 2019; Jiang et al., 2020; Brennan and Smith, 2021; Hua et al., 2021; Jones et al., 2021).

Long noncoding RNAs (Zhu et al., 2013; Rathinasamy and Velmurugan, 2018; Zheng et al., 2021; Ma et al., 2022) (lncRNAs) represent a special type of non-coding RNA with more than 200 nucleotides that are not translated into proteins. The aberrant expression of lncRNAs has been frequently observed in a variety of diseases, including human malignancies (Isin and Dalay, 2015; Bhan et al., 2017; Bian et al., 2021; Homayoonfal et al., 2021; Liu and Lei, 2021). Moreover, an increasing number of studies have shown that lncRNAs are involved in the pathogenesis and development of many cancers and are associated with different clinicopathological features (Wang et al., 2021b; Dong et al., 2021; Ma et al., 2021; Zhou et al., 2021). For example,

**TABLE 1 |** DUXAP10 expression and clinicopathological features in cancers.

Disease type	Expression	Clinical characteristics	Refs
liver cancer	upregulation	overall survival rate and progression-free survival rate	(Zhu et al., 2018; Han et al., 2019; Sun et al., 2019)
kidney cancer	upregulation	male sex, tumor size, TNM stage, lymph node metastasis, pathologic stage, and overall survival rate	Chen et al. (2020)
lung cancer	upregulation	tumor size, pathological stage, lymph node metastasis, overall survival rate, relapse-free survival rate, and poor prognosis	(Wei et al., 2017; Lin et al., 2021)
glioma	upregulation		Wu et al. (2021)
thyroid carcinoma	upregulation		Li et al. (2020)
prostate cancer	upregulation		Wang et al. (2019a)
chronic myelogenous leukemia	upregulation	clinical stage	Yao et al. (2018)
ovarian cancer	upregulation	tumor size and FIGO stage	Zhang et al. (2018)
gastric cancer	upregulation	pathological stage, lymph node metastasis, and poor prognosis	Xu et al. (2018)
pancreatic cancer	upregulation	TNM stage, lymph node metastasis, and poor prognosis	Lian et al. (2018)
bladder cancer	upregulation		Lv et al. (2018)
colorectal cancer	upregulation	pathological stage, tumor size, lymph node metastasis, and poor prognosis	Lian et al. (2017)
esophageal squamous cell carcinoma	upregulation	TNM stage, lymph node metastasis, and survival time	Wang et al. (2018)

overexpression of the lncRNA MCM3AP-AS1 (Wang et al., 2019b) has been shown to modulate hepatocellular carcinoma (HCC) occurrence and progression and is strongly correlated with advanced tumor grade and stage, large tumor size, and poor prognosis. Additionally, a high expression level of the lncRNA AK023391 (Huang et al., 2017) was found to exert a pivotal role in gastric cancer (GC) oncogenesis and development and was significantly linked to decreased survival rates. Functionally, lncRNAs have been demonstrated to frequently participate in the regulation of multiple crucial biological processes (Gao et al., 2020; Le et al., 2021; Yi et al., 2021; Zheng et al., 2021; Zhu et al., 2021), such as cell proliferation, apoptosis, and invasion. Given the complex functions of lncRNAs in cancers, lncRNAs exhibit tremendous potential for use in tumor diagnosis, prognosis, and treatment.

Recently, the pseudogene lncRNA DUXAP10 (DUXAP10) (Booth HA, and Holland PW., 2007; Zhu et al., 2018), localized on chromosome 14q11.2, was found to be overexpressed in multiple cancers, including HCC, bladder cancer (BC), non-small-cell lung cancer (NSCLC), glioma, renal cell carcinoma (RCC), papillary thyroid carcinoma (PTC), prostate cancer (PCa), chronic myelogenous leukemia (CML), ovarian cancer (OC), pancreatic cancer (PC), GC, colorectal cancer (CRC), esophageal squamous cell carcinoma (ESCC) and oral squamous cell carcinoma (OSCC). Its aberrant expression level was predominantly in line with many poor clinical characteristics. Studies of the biological functions of DUXAP10 showed that DUXAP10 can exhibit protumor effects on regulating cell processes, such as cell proliferation, apoptosis, migration, and invasion, by targeting specific genes or through a variety of different specific pathways. Therefore, the above evidence implicates the high potential of DUXAP10 as a biomarker for cancer diagnosis prognosis and therapy.

In this review, we summarize the roles of DUXAP10 in different cancers, including dysregulated expression, related clinical characteristics, biological functions underlying molecular mechanisms, and potential clinical applications.

## THE ROLE OF THE LNCRNA DUXAP10 IN DIFFERENT CANCERS

Several studies have reported that DUXAP10 is aberrantly expressed in numerous human cancers, such as HCC, BC, NSCLC, glioma, RCC, PTC, PCa, CML, OC, PC, GC, CRC, ESCC, and OSCC. In addition, it was demonstrated that a high DUXAP10 expression level was positively related to advanced clinicopathological features (Table 1). The diverse regulatory functions and underlying mechanisms of DUXAP10 during tumor progression are shown in Table 2.

We next discuss the DUXAP10 expression level, relevant clinical characteristics, and biological functions in different cancer types.

### Liver Cancer

Liver cancer is one of the leading causes of cancer death worldwide (Sun et al., 2013; Zhao et al., 2015; Kido et al., 2020). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer (Ahn et al., 2018; Xu et al., 2021). Numerous studies have recently revealed that DUXAP10 was overexpressed in HCC cell lines (including Hep3B, HepG2, SMMC7721, HuH7, MHCC-97L, MHCC-97H, HCC-LM, and SK-Hep-1 cells) and tissues (Zhu et al., 2018; Han et al., 2019; Sun et al., 2019). And its level was positively related to the severity of HCC, higher DUXAP10 expression was observed in advanced HCC patients. In addition, a high expression level of DUXAP10 was significantly correlated with poor overall survival (OS) and

**TABLE 2 |** Functions and mechanisms of DUXAP10 in cancers.

Disease type	Cell lines	Related mechanisms		Functions	Refs
		Molecule	Pathway		
liver cancer	Hep3B, Hep G2, SMMC7721, HuH7, MHCC-97L, MHCC-97H, HCC-LM, and SK-Hep-1	microRNA-1914, and GPR39	PI3K/AKT/mTOR pathway, and Wnt/ $\beta$ -catenin pathway	cell cycle, colony formation, proliferation, epithelial-mesenchymal transition, metastasis, and apoptosis	(Zhu et al., 2018; Han et al., 2019; Sun et al., 2019)
kidney cancer	786-O and A498			cell cycle, proliferation, apoptosis, migration, and invasion	Chen et al. (2020)
lung cancer	A549, H1975, SPC-A1, H1299, and BEAS-2B	Cd, Pax6, GLI1, LSD1, LATS2, and RRAD	Hedgehog pathway	cell cycle, proliferation, migration and invasion, and cancer stem cell transformation	(Wei et al., 2017; Lin et al., 2021)
glioma	HS683, U251, U373, U87, T98G LN-319 and SW1783	HuR, CD133, Oct4, and Sox12		cell stemness	Wu et al. (2021)
thyroid carcinoma	TPC-1, BCPAP, K1, and IHH-4		Akt/mTOR pathway	cell proliferation, apoptosis, invasion, and migration	Li et al. (2020)
prostate cancer	PC3, 22RV1, and DU145			cell cycle, proliferation, and metastasis	Wang et al. (2019a)
chronic myelogenous leukemia	THP-1, KG-1, and K562	PTEN		cell proliferation, apoptosis, and cell cycle	Yao et al. (2018)
ovarian cancer	HO8910 and A2780			cell proliferation	Zhang et al. (2018)
gastric cancer	BGC823, SGC7901, MGC803, AGS, HGC27, and MKN45	PRC2, LSD1, HuR, KLF2, LATS1, and $\beta$ -catenin		cell proliferation, cell cycle, invasion, and migration	Xu et al. (2018)
pancreatic cancer	AsPC-1, BxPC-3, and PANC-1	EZH2, and LSD1		cell cycle, proliferation, and apoptosis	Lian et al. (2018)
bladder cancer	5.637, T24, E-j, TCCSUP, UM-UC-3, and RT4		PI3K/Akt/mTOR pathway	cell cycle, proliferation, and apoptosis	Lv et al. (2018)
colorectal cancer	HCT116, SW620, and SW480	LSD1, PTEN, and p21		cell proliferation, apoptosis, and cell cycle	Lian et al. (2017)
esophageal squamous cell carcinoma	KYSE30, KYSE510, KYSE180, and KYSE150	EZH2, and p21		cell cycle, proliferation, metastasis, and apoptosis	Wang et al. (2018)

progression-free survival (PFS) rates (Han et al., 2019). It has also been proven to exert pivotal pro-oncogenic functions in the regulation of cell cycle progression, colony formation, proliferation, epithelial-mesenchymal transition (EMT), invasion, migration, and cell apoptosis in SMMC-7721, Hep G2, Hep3B, and MHCC-97L cells.

## Kidney Cancer

Renal cell carcinoma (RCC), arising from the renal epithelium (Wang et al., 2020b; Singh et al., 2020), is the most common kidney tumor (Wang et al., 2017; Chen et al., 2018). High expression of DUXAP10 was observed in 18 RCC specimens collected from the Urology Department of Peking University Shougang Hospital and 786-O and A498 cell lines in comparison with adjacent normal tissues and normal kidney epithelial cells (HKCs). Its level was positively correlated with male sex, tumor size, TNM stage, lymph node metastasis, and advanced pathologic stage. Additionally, Kaplan–Meier analysis further verified the strong link between DUXAP10 and poor overall patient survival. DUXAP10 has been proven to have pro-oncogenic functions in the regulation of cell proliferation, cell cycle transition to the S phase, cell apoptosis, cell migration, and invasion.

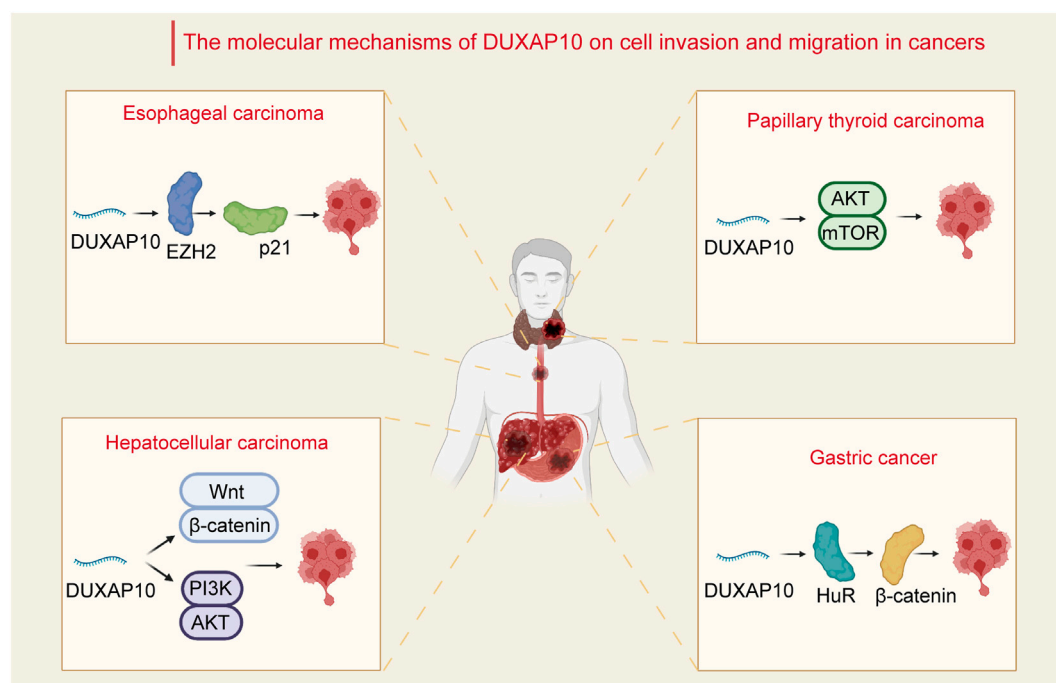
## Lung Cancer

Lung cancer (Molina et al., 2008; Zhang et al., 2019; Chen et al., 2021b) remains the leading cause of cancer-related mortality

worldwide, with approximately 1.8 million (Chen et al., 2021a; Sung et al., 2021) deaths per year. While there has been a modest improvement in lung cancer (Ślawińska-Brych et al., 2021) OS in recent decades, further studies are needed to improve patients' clinical outcomes. Several studies have reported that DUXAP10 is significantly upregulated in 93 human cancer tissues obtained from The First and Second Affiliated Hospital of Nanjing Medical University, non-small-cell lung cancer (NSCLC) cell lines (A549, H1975, SPC-A1, and H1299 cells) and the chronic cadmium (Cd)-induced human bronchial epithelial BEAS-2B cell line. A high level of DUXAP10 expression in lung cancer patients was linked to not only lower OS and relapse-free survival (RFS) rates but also larger tumor sizes, advanced tumor stages, lymph node metastasis, and even poor prognosis (Wei et al., 2017; Lin et al., 2021). Moreover, *in vitro* functional assays and *in vivo* tumor models have demonstrated that DUXAP10 is mainly implicated in eliciting the transformation of Cd-exposed (Cd-T) cells to cancer stem cells (CSCs), promoting the cell cycle progression, proliferation, migration, and invasion of A549 or H1975 cells, and thus accelerating tumorigenesis and progression in lung cancer.

## Glioma

Gliomas are the most common primary malignancy of the central nervous system (Briancçon-Marjollet et al., 2010; Deluche et al., 2019; Shi et al., 2020a). DUXAP10 has been indicated to be highly expressed in glioma cell lines (HS683, U251, U373, U87, T98G



**FIGURE 1 |** Relevant molecular mechanisms of DUXAP10 in the process of cell invasion and migration in cancers. In hepatocellular carcinoma, DUXAP10 enhances cell invasion and migration through the Wnt/ $\beta$ -catenin and PI3K/Akt signaling pathways. In papillary thyroid carcinoma, DUXAP10 mediates the activity of the Akt/mTOR pathway to regulate cancer cell invasion and migration. In gastric cancer, DUXAP10 is involved in the regulation of invasion and migration via combination with the RNA-binding protein HuR and subsequently increases the stability of  $\beta$ -catenin. In esophageal carcinoma, DUXAP10 regulates cell metastasis by binding with EZH2 and inhibiting p21 expression.

LN-319, and SW1783 cells) and tissues gained from patients under surgery at the First Affiliated Hospital of Jinan University. Notably, it was found that DUXAP10 (Wu et al., 2021) was involved in facilitating the stem cell-like properties of glioma U251 and T98G cells by increasing the expression of Sox2, CD133, Oct4 stemness markers, the ability of tumorsphere formation, and the activity of ALDH, which closely aligns with the CSC induction mentioned in the above chronic Cd exposure study on lung cancer.

## Thyroid Carcinoma

Thyroid carcinoma can be divided into four types: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, medullary thyroid carcinoma, and anaplastic thyroid carcinoma (Wen et al., 2019; Zhang et al., 2021). PTC is the most common type of thyroid carcinoma (Li et al., 2018; Hu et al., 2019; Wang et al., 2021a). DUXAP10 was first confirmed by Li et al. (2020) to be highly expressed in PTC tissues and TPC-1, BCPAP, K1, and IHH-4 cells compared with adjacent normal thyroid tissues. In addition, DUXAP10 has been demonstrated to contribute to protumorigenic effects by promoting BCPAP and K1 (Li et al., 2020) cell proliferation and invasion in addition to inhibiting cell apoptosis.

## Prostate Cancer

Prostate cancer (Chang et al., 2014; Siegel et al., 2021) (PCa) is one of the most frequently diagnosed malignancies in the male

genitourinary system. In PCa (Luo et al., 2019; Shang et al., 2019; Wen et al., 2020), lncRNAs have been found to play increasingly vital roles in tumorigenesis and development in recent years. Previously, X-F Wang indicated that DUXAP10 was highly expressed in PCa tissues and PC3, 22RV1, and DU145 cell lines. Downregulation of DUXAP10 was able to suppress tumor development by inactivating the processes of cell cycle progression, cell proliferation, and metastasis in PC3 and DU145 cells (Wang et al., 2019b).

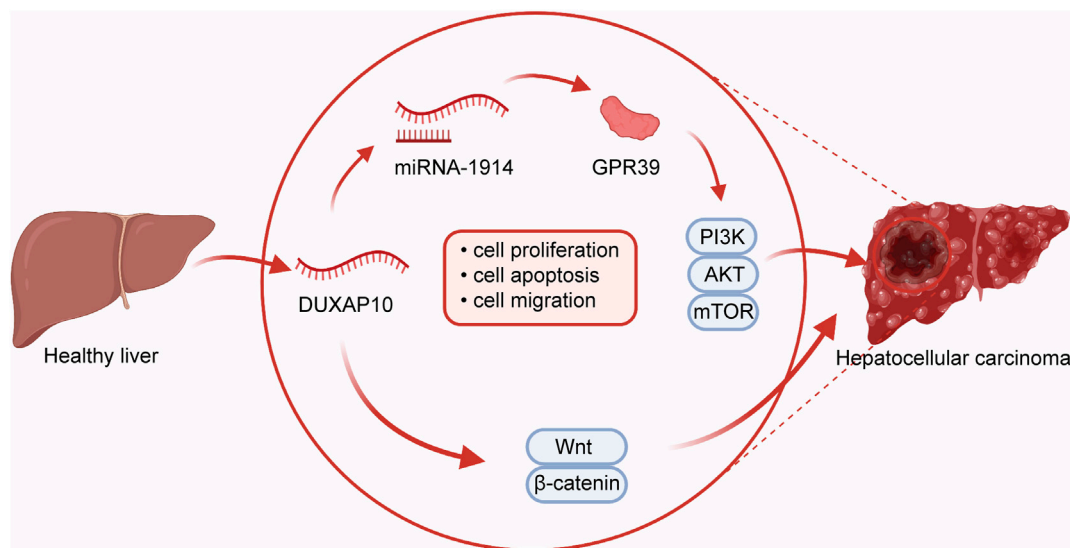
## Chronic Myelogenous Leukemia

DUXAP10 (Yao et al., 2018) was upregulated in chronic myelogenous leukemia (CML) THP-1, KG-1, and K562 cells, and its expression level was observed to gradually increase in response to clinical upstaging of CML (chronic phase, acceleration phase, and blast phase). Furthermore, *in vitro* functional assays showed that knockdown of DUXAP10 notably weakened cell proliferation and enhanced cell apoptosis and cell cycle arrest in K562 and KG-1 cells.

## Ovarian Cancer

Ovarian cancer (OC) has the highest mortality rate among gynecological cancers (Chen et al., 2013; Gurunathan et al., 2019; Yu et al., 2019). Studies have indicated that DUXAP10 (Zhang et al., 2018) is upregulated in OC tissues and cell lines. A high expression level of DUXAP10 was remarkably connected with tumor size and the FIGO stage. More importantly,





**FIGURE 2 |** In hepatocellular carcinoma, DUXAP10 plays an oncogenic role in regulating the processes of cell proliferation, apoptosis, and migration by combining with microRNA-1914 and further activating the GPR39-dependent PI3K/AKT/mTOR pathway or Wnt/β-catenin pathway.

DUXAP10 was involved in the momentous modulation of tumor progression by stimulating the proliferation of HO8910 and A2780 cells.

### Gastric Cancer

Gastric cancer (GC) is the fourth most common cancer and the third most common cause of cancer-related death worldwide (Sun et al., 2017; Zhao et al., 2017; Shin et al., 2021). DUXAP10 has been recently reported to show higher expression in GC (Xu et al., 2018) tissues and cell lines (including BGC823, SGC7901, MGC803, AGS, HGC27, and MKN45 cells) and was strongly correlated with deteriorating pathological stage, lymph node metastasis and even worse prognosis. In AGS, BGC823, SGC7901, and MGC803 cell lines, DUXAP10 was found to exert cancer-promoting functions through the activation of cell proliferation, cell cycle progression, invasion, and migration. In an *in vivo* BALB/c nude mouse tumor formation study, the mice exhibited larger tumor weights and sizes, which further verified the tumorigenic ability of DUXAP10.

### Pancreatic Cancer

In recent years, it has been found that DUXAP10 is excessively expressed in pancreatic (Lian et al., 2018) cancer (PC) tissues and cell lines (AsPC-1, BxPC-3, and PANC-1 cells), and a close correlation was observed between the overexpression of DUXAP10 and unfavorable clinicopathological characteristics, such as poor prognosis, aggressive TNM stage and lymph node metastasis. Functional analyses in BxPC-3 and PANC-1 cell lines provided powerful evidence that DUXAP10 accelerated cell cycle progression and proliferation and suppressed cell apoptosis. Additionally, *in vivo* xenograft tumor model experiments validated the tumor-promoting role of DUXAP10 in accelerating tumor growth and increasing tumor volumes.

### Bladder Cancer

Bladder cancer (BC) is the most common cancer of the urinary tract (Gan et al., 2016; Loras et al., 2019). Lv et al. (2018) proposed for the first time that DUXAP10 was overexpressed in BC tissues and 5,637, T24, E-j, TCCSUP, UM-UC-3, and RT4 cells. Additionally, DUXAP10 contributed to cancer progression through its involvement in several cellular functions in T24 and 5,637 cells, including cell cycle progression, proliferation, and apoptosis.

### Colorectal Cancer

Over the past decade, DUXAP10 was highly expressed in colorectal cancer (CRC) (Lian et al., 2017) tissues and HCT116, SW620, and SW480 cell lines. DUXAP10 was also found to be positively correlated with advanced pathological stages, larger tumor sizes, lymph node metastasis, and poor prognosis. Knocking down DUXAP10 attenuated the proliferative ability, accelerated the apoptotic process, and blocked the cell cycle progression of HCT116 and SW480 cells. Subsequently, the increased tumor volumes and weights in experimental tumor models further demonstrated the carcinogenicity of DUXAP10.

### Esophageal Squamous Cell Carcinoma

Esophageal squamous cell carcinoma (ESCC) (Pan et al., 2019; Wang et al., 2020a; Li et al., 2021) is the predominant subtype of esophageal carcinoma, with a poor 5-years survival (De Angelis et al., 2014) rate of less than 21%. DUXAP10 has been found to be expressed at high levels in ESCC tissues and cells (KYSE30, KYSE510, KYSE180, and KYSE150 cells), whereas overexpression of DUXAP10 was closely correlated with short survival time, TNM stage, and lymph node metastasis. In addition, DUXAP10 essentially participates in ESCC development and progression by enhancing cell proliferation

and metastasis, accelerating cell cycle progression, and hindering cell apoptosis in KYSE30 and KYSE180 cells.

## Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) (Meehan et al., 2020) represents a heterogeneous mucosal malignancy derived from the oral, oropharyngeal, hypopharyngeal, and laryngeal cavities. Oral squamous cell carcinoma (Feng et al., 2017; Jia et al., 2020) (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) are the most frequent types of HNSCC, accounting for 377,713 new cases and 177,757 (Sung et al., 2021) deaths worldwide. Recent studies have also demonstrated that DUXAP10 is differentially expressed in OSCC and OPSCC tissues.

## REGULATORY MECHANISMS OF THE LNCRNA DUXAP10

As a newfound oncogene, DUXAP10 has been reported to be widely involved in the mediation of several crucial biological processes, such as cell proliferation, apoptosis, and metastasis, in diverse cancer types. Here, we mainly provide a current understanding of the major biological functions and corresponding molecular mechanisms of DUXAP10 (**Figure 1**).

### Cell Proliferation and Apoptosis

Cell proliferation and apoptosis are fundamental for normal cell growth and development. Abnormal cell growth is a key marker for cancer (Shi et al., 2020a; Go et al., 2021; Hu et al., 2021). It has been shown that microRNA-1914 could increase the effect of DUXAP10 on cell proliferation and apoptosis through activation of the GPR39-mediated PI3K/AKT/mTOR pathway in HCC Hep3B and MHCC-97L (Sun et al., 2019) cells (**Figure 2**). In addition, the proliferative mechanism of DUXAP10 in HCC SMMC-7721 and HepG2 (Han et al., 2019) cells was observed via the Wnt/ $\beta$ -catenin and PI3K/Akt signaling pathways. Likewise, one study has shown that DUXAP10 can enhance cell cycle progression and subsequent cell proliferation in NSCLC (Wei et al., 2017) A549 or H1975 cells by specifically binding to LSD1 and increasing the levels of LATS2 and RRAD. In PTC (Li et al., 2020), there was experimental evidence that DUXAP10 restrained cell apoptosis via inactivation of Caspase-3, in turn resulting in the promotion of proliferative ability in BCPAP and K1 cells. Moreover, DUXAP10 was found to facilitate cell proliferation and inhibit the apoptosis of CML (Yao et al., 2018) K652 and KG-1 cells by inhibiting PTEN expression. Functional studies in GC (Xu et al., 2018) BGC823, SGC7901, and MGC803 cells also suggest a critical regulatory role for DUXAP10 in cell proliferation by directly interacting with PRC2 and LSD1, thus repressing the expression of LATS1. Similarly, DUXAP10 has been indicated to induce PC BxPC-3 and PANC-1 cell proliferation and suppress apoptosis through combination with the RNA-binding proteins EZH2 and LSD1. Mechanistic research in T24 and 5,637 (Lv et al., 2018) BC cells has also proven that DUXAP10 regulates cell proliferation and apoptosis by modulating the PI3K/Akt/mTOR signaling

pathway. In CRC (Lian et al., 2017), DUXAP10 regulates the expression of p21 and phosphatase and tensin homolog (PTEN) by binding to the histone demethylase lysine-specific demethylase 1 (LSD1), enhancing CRC cell proliferation and reducing apoptosis. As shown in KYSE30 and KYSE180 ESCC (Wang et al., 2018) cells, DUXAP10 has been similarly confirmed to modulate the processes of cell proliferation and apoptosis by negatively modulating p21 expression by interacting with zeste homolog 2 (EZH2).

### Cell Invasion and Migration

Cancer metastasis remains one of the biggest challenges in cancer therapy (Gou et al., 2020; Wang et al., 2021c). Migration and invasion are prerequisites for cancer cell metastasis (Zacharias et al., 2018). The mechanisms of cell migration and invasion have been a focus of research. Current studies have revealed that DUXAP10 mainly affects the processes of tumor cell EMT, thus enhancing tumor cell migratory properties in several cancers. By modulating the Wnt/ $\beta$ -catenin and PI3K/Akt signaling pathways, DUXAP10 has been demonstrated to influence the invasion and migration of HCC (Han et al., 2019) SMMC-7721 and HepG2 cells through the regulation of EMT. A previous study of PTC also demonstrated that DUXAP10 was involved in increasing cell invasion and migration and regulating EMT via activation of the Akt/mTOR (Li et al., 2020) pathway. Additionally, DUXAP10 was found to play a crucial role in GC (Xu et al., 2018) cell invasiveness and migration by interacting with the RNA-binding protein HuR and subsequently stabilizing  $\beta$ -catenin mRNA. It was also demonstrated that in ESCC KYSE30 and KYSE180 cells, DUXAP10 regulated cell metastasis by binding with EZH2 and downregulating p21 (Wang et al., 2018) expression.

### Cell Stemness

The CSC hypothesis suggests that CSCs (Tang, 2012; Eun et al., 2017) are a subpopulation of tumor cells with the characteristics of powerful self-renewal and aberrant differentiation potential. Interestingly, an emerging role of DUXAP10 in promoting the transition of CSCs has been observed, which is essential for the tumorigenicity of cancer cells.

4 A recent study using a post-chronic Cd-exposed human bronchial epithelium BEAS-2B cell model demonstrated that DUXAP10 induces CSC-like (Lin et al., 2021) properties by improving GLI1 protein stability and ultimately regulating the Hedgehog signaling pathway. In Cd-exposed transformed cells, Hedgehog signaling pathway was activated and subsequently involved in mediating CSC-like characteristics. At the same time, Pax6 was upregulated and significantly increase the duxap10 level and CSC like characteristics. It was also found that DUXAP10 promotes the stemness of glioma (Wu et al., 2021) U251 and T98G cells by binding to HuR and thus upregulating the expression of Sox12. DUXAP10 knockdown remarkably reduced the activity of ALDH and the expression of stemness markers (Sox2, CD133, Oct4) in glioma cells. Thus, these results have shown DUXAP10 had vital effects on glioma cell stemness. Promising Clinical Applications of DUXAP10.

Accumulating studies have shown that dysregulated lncRNA (Bhan et al., 2017; Gao et al., 2021; Tan et al., 2021) expression contributes to the development of tumors and can be used as a promising marker for lncRNA-based applications in cancer management. Aberrantly expressed DUXAP10 was recently revealed to be involved in a wide range of biological functions and pathological characteristics, and its vital clinical applications in several cancer types are valuable for clinical diagnosis, prognosis, and treatment management. In this section, we describe the meaningful medicinal applications of DUXAP10 in numerous tumor types.

## DUXAP10 as a Diagnostic and Prognostic Biomarker

It is now widely accepted that the early diagnosis (An et al., 2021; Kannan et al., 2021) of tumors is essential for achieving a better prognosis and a lower mortality rate. Accurate diagnostic biomarkers (Ott et al., 2009; Wardle et al., 2015; Dragani et al., 2020) for detecting early-stage tumors are of great clinical significance. An increasing number of oncology studies have reported that the overexpression of DUXAP10 in diverse tumor tissues (such as NSCLC (Wei et al., 2017), glioma (Wu et al., 2021), and ESCC (Wang et al., 2018)) could be used to distinguish normal from tumor tissues, making it highly promising for the early diagnosis of tumors.

In addition, high DUXAP10 expression was closely associated with more advanced tumor stage or grade, earlier lymph node metastasis, and unfavorable OS, PFS, and RFS rates, which provides powerful evidence for the prognostic ability of DUXAP10 in various cancers, such as HCC (Zhu et al., 2018), NSCLC (Wei et al., 2017), RCC (Chen et al., 2020), OC (Zhang et al., 2018), GC (Xu et al., 2018), CRC (Lian et al., 2017), and ESCC (Wang et al., 2018). Therefore, DUXAP10 in combination with relevant clinicopathological features can function as an independent prognostic indicator in diverse cancer types.

## DUXAP10 as a Treatment Target

In recent decades, several reports have shown that lncRNAs (Yin et al., 2018; Diniz et al., 2021; Homayoonfal et al., 2021) play important roles in tumor progression and could be biomarkers for clinical treatments. Increasing studies have demonstrated that DUXAP10 is involved in the tumorigenesis and development of tumors through the modulation of diverse cellular processes, comprising cell colony formation, cell cycle progression, cell proliferation, apoptosis, metastasis, and even CSC-like properties. Moreover, numerous molecular mechanism experiments have confirmed that DUXAP10 plays a tumor promoter role by regulating key target gene activity and affecting multiple important signaling pathways, making it a possible molecular factor that can be used as a therapeutic target in HCC (Han et al., 2019; Sun et al., 2019), NSCLC

(Wei et al., 2017), glioma (Wu et al., 2021), RCC (Chen et al., 2020), PTC (Li et al., 2020), OC (Zhang et al., 2018), GC (Xu et al., 2018), PC (Lian et al., 2018), BC (Lv et al., 2018), CRC (Lian et al., 2017) and ESCC (Wang et al., 2018). Therefore, a further in-depth understanding of the pro-oncogenic mechanisms of DUXAP10 for cancer treatment is needed.

## CONCLUSION

With the advent of novel genomic approaches as well as technological breakthroughs, an unprecedented understanding of the development of cancer has provided novel insight for establishing more effective cancer management strategies. Mounting evidence indicates that DUXAP10 is abnormally highly expressed in a variety of cancers, including HCC, lung cancer, glioma, RCC, PTC, PCa, CML, OC, GC, PC, BC, CRC, and ESCC. The higher expression of DUXAP10 in tumor tissues compared with adjacent normal tissues indicates the diagnostic potential of using DUXAP10 to successfully distinguish cancerous tissues from normal tissues. However, the detection of DUXAP10 in tissue is an invasive and complicated method, and further studies are needed to search for possible noninvasive methods for the diagnosis of cancers using DUXAP10.

In addition, DUXAP10 overexpression was positively correlated with the adverse clinicopathological features and aggressive outcomes of several cancer types, including tumor size, TNM staging, histological grading, lymph node metastasis, and survival rates. These associations revealed the potential for DUXAP10 to be used as a prognostic biomarker to predict cancer prognosis and provide guiding recommendations for the future treatment of tumors. More importantly, DUXAP10 has been found to play an oncogenic role and participate in the biological processes of cellular proliferation, apoptosis, invasion, migration, and stemness through the regulation of various target genes or signaling pathways. Thus, the molecular mechanisms of DUXAP10 involved in tumor progression should be further explored, contributing to new hopes in tumor treatment.

## AUTHOR CONTRIBUTIONS

JX designed the study. LZ and ZG drafted the manuscript. LZ and MX analyzed the data. All authors read and approved the final manuscript.

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# Targeting MALT1 Suppresses the Malignant Progression of Colorectal Cancer *via* miR-375/miR-365a-3p/NF- $\kappa$ B Axis

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Colorectal cancer (CRC) is a malignant tumor with the second highest morbidity and the third highest mortality in the world, while the therapeutic options of targeted agents remain limited. Here, mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), known as the upstream of the NF- $\kappa$ B signaling pathway, was identified to be highly upregulated in CRC tumors and cell lines. Furthermore, the downregulation of MALT1 or inhibition of its proteolytic function by MI-2 suppressed the cell proliferation and migration of CRC cells. *In vivo*, suppressing the MALT1 expression or its proteasome activity effectively reduced the size of the subcutaneous tumor in nude mice. Mechanistically, miR-375 and miR-365a-3p were identified to inhibit NF- $\kappa$ B activation *via* targeting MALT1. Overall, our results highlight that a novel regulatory axis, miRNA-MALT1-NF- $\kappa$ B, plays a vital role in the progression of CRC and provides novel and hopeful therapeutic targets for clinical treatment.

**Keywords:** MALT1, CRC, NF- $\kappa$ B, MI-2, miRNA

## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. In 2020, there were surprisingly about 1.9 million new cases of CRC and more than 935,000 deaths all over the world. Moreover, the incidence rate of early-onset CRC is rising by 1%–4% per year (Sung et al., 2021), which is paralleled by the increasing mortality rate (Siegel et al., 2020). However, there is still a shortage of effective treatments in clinics. Traditional therapies, like endoscopic treatment and surgery, are limited for local and early-stage patients. Emerging immunotherapies still have not shown an increase of overall survival compared to chemotherapy alone (Dekker et al., 2019). Thus, it is necessary and imperative to put forward new treatment strategies to improve the outcomes of CRC and reduce its increasing mortality.

The NF- $\kappa$ B pathway has a broad role in various cellular responses involving immunity, inflammation, and cancer. Its activation is closely associated with all cancer hallmarks, especially in enhancing cell proliferation and metastasis. Targeting inhibitors, such as proteasome inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs), have been applied in CRC for chemotherapeutic resistance and the prevention of recurrence (Richardson et al., 2003; Patel et al., 2018), whereas they are accompanied by



inevitable side events due to their extensive effects (Katona and Weiss, 2020). Thus, it is supposed that target therapies with strong specificity would represent a safer and more beneficial efficacy. MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1, is known as a key upstream molecule in the NF- $\kappa$ B pathway, which was identified as a human paracaspase. Its deficiency causes infantile combined immunodeficiency and immune dysregulation (Punwani et al., 2015). In almost all different types of lymphoma, MALT1 is required to cleave NF- $\kappa$ B negative regulators A20 and RelB (Coornaert et al., 2008), while it is less studied in solid tumors (Fontan et al., 2012; Di Pilato et al., 2019; Liu et al., 2020).

MicroRNAs (miRNAs) are a kind of small non-coding RNAs with 18–25 nucleotides in length. They function as post-transcriptional repressors by binding to the 3' untranslated region (UTR) of target mRNAs. In various types of cancers, miRNA expressions are altered to promote or suppress tumorigenesis and tumor development (Lin and Gregory, 2015). So far, there are several clinical trials of miRNA drugs undergoing to expect an application for tumors. MRX34, a liposomal miR-34a mimic, reached phase-I clinical trials showing that one of three CRC patients had a stable disease (Beg et al., 2017). The ongoing multicenter phase-I clinical trials of miR-16 mimic already showed a 68% stable disease in NSCLC treatment (van Zandwijk et al., 2017). Following the recent advances in miRNA chemistry and delivery technologies, the miRNA-based agents moving into the clinic become more available and a deeper exploration of miRNA drugs in CRC is more imperative (Rupaimoole and Slack, 2017).

In this study, we discovered that MALT1 was highly expressed in CRC and promoted its malignant progression. Our study revealed that downregulated MALT1 or the inhibition of its protease activity with MI-2 inhibited cell proliferation and migration through activating the NF- $\kappa$ B pathway. Meanwhile, miR-365a-3p and miR-375 were identified as the upstream regulators of MALT1. Taken together, these results demonstrated that MALT1 acted as an oncogene in CRC, which would be a promising therapeutic target *via* epigenetic regulation or its activity.

## METHODS AND MATERIALS

### Patients and Tissue Samples

The CRC tissue samples were obtained from 58 patients who were diagnosed and underwent surgery from January 2010 to December 2011 in Jiangmen Central Hospital, Affiliated Jiangmen Hospital of Sun Yat-sen University. A written informed consent to use the surgical samples has been signed by all the patients, and the study was approved by the Institutional Review Board of Nanfang Hospital affiliated to Southern Medical University. Collected samples were fixed by paraformaldehyde and embedded in paraffin.

### Immunohistochemistry Assay

Paraffin-embedded tissue was cut into 5  $\mu$ m thick slices, dewaxed with xylene, and hydrated with ethanol. Then, the slides were

heated at 95°C in a 0.01 M citrate buffer ( $pH = 6.0$ ) and quenched for peroxidase activity with 3% hydrogen peroxide for 20 min to retrieve antigens. After being treated with 10% goat serum, the slides were incubated overnight with antibodies at 4°C. PBS washing was followed by incubation with goat anti-rabbit IgG for 1 h and then stained with 3,3-diaminobenzidine. When all sections were dehydrated and sealed, we selected images with an inverted microscope (ZEISS). Immunohistochemistry was performed with the following antibodies: Ki67 (27309-1-AP; Proteintech), MALT1 (66225-1-Ig; Proteintech), p-p65 (AB11014, phosphor-Ser536), fibronectin (66042-1-Ig; Proteintech), N-cadherin (22018-1-AP; Proteintech), E-cadherin (20874-1-AP; Proteintech), vimentin (10366-1-AP; Proteintech) Snail1 (26183-1-AP; Proteintech), and Snail2 (12129-1-AP; Proteintech).

### Bioinformatics Analysis

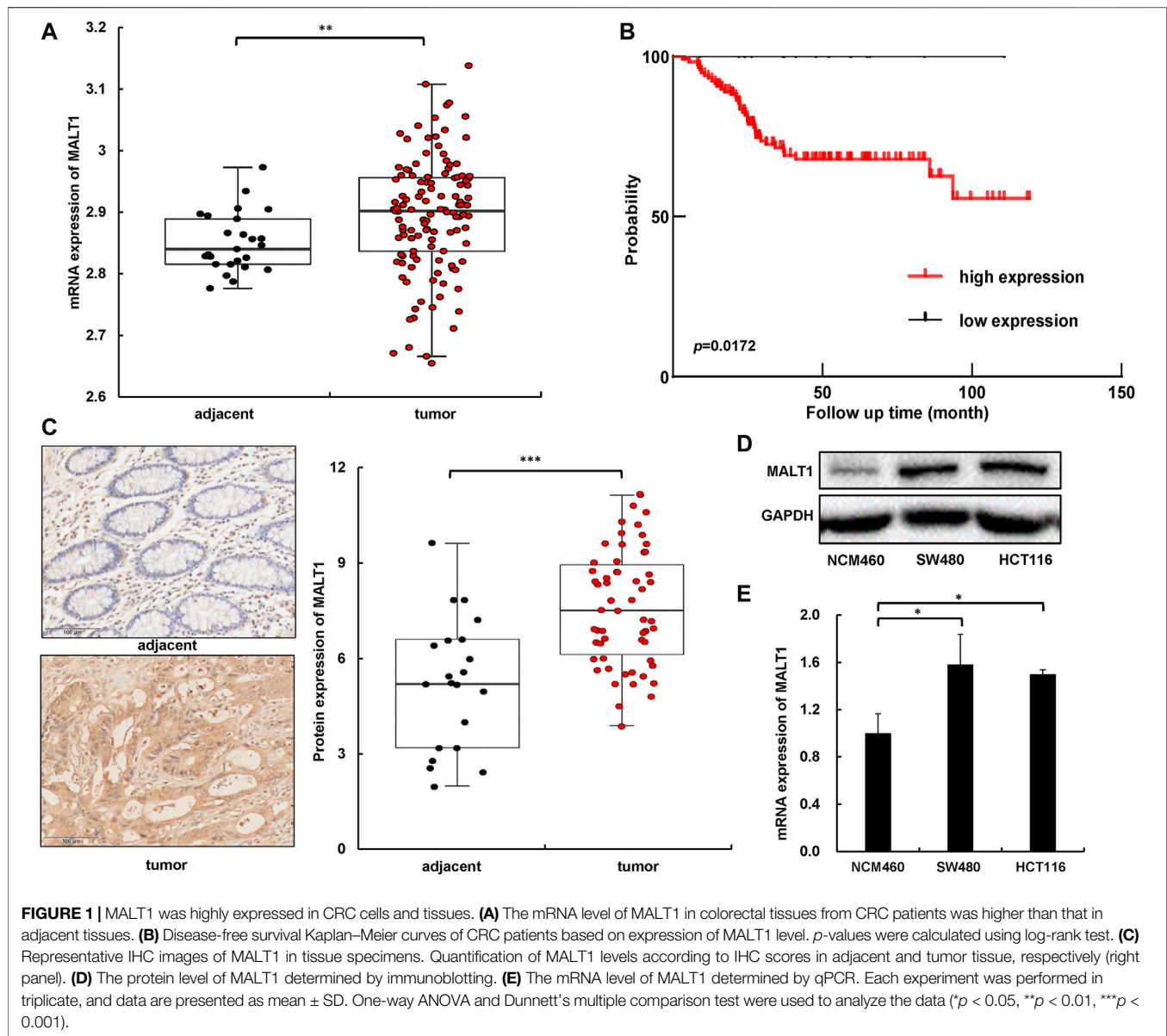
The mRNA microarray datasets (GSE21510, GSE17536) and miRNA microarray datasets (GSE38389, GSE41655, GSE30454, GSE18392) were obtained from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). In GSE21510, patients were divided into the normal group and tumor group according to the type of tissue resected in the surgeries. The expression of MALT1 is compared by the content of corresponding mRNA. Meanwhile, in GSE17536, patients were divided into high expression group (85%) and low expression group (15%) depending on the protein expression of MALT1, so that we were able to judge the relationship between MALT1 and prognosis by comparing the time of disease-free survival.

### Cell Lines and Culture Conditions

The human CRC cell lines HCT116 and SW480 and the human normal colorectal epithelial cell line NCM460 were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States). All cell lines were cultured in Dulbecco's modified eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Beyotime Biotechnology, Shanghai, China), 100  $\mu$ g/ml streptomycin, and 100 units/ml penicillin. They were all placed in a humidified incubator with 5% CO<sub>2</sub> at 37°C and a mild atmosphere.

### Reverse Transcription and qPCR

Total RNA was extracted with TRIzol Reagent (ambin, United States). After gDNA was removed, 500 ng of total RNA was reversed with Evo M-MLV RT Kit with gDNA Clean for qPCR II AG11711 (Accurate Biology) according to the manufacturer's instructions. Real-time PCR was performed using PerfectStart Green qPCR SuperMix (TransGen Biotech) on a LightCycler 96 Detection System (Roche). GAPDH was served as an internal reference gene, and the primers used for qPCR in this study were as follows: MALT1: F, TGGAAGCCCTATTCCTCACTACC; R, CATGACACCAG-TAGGTTCCTTGG, GAPDH: F, GATATT GTTGCCATCAATGACC; R, AGCC-TTCTCCATGGTGGTG AAGA, miR-218-5p: F, TTGTGCTTGATCTAACCATGT; miR-338-3p: F, TCCAGCATCAGTGATTTTGTGTG; miR-365-3p: F, TAATG-CCCCTAAAAATCCTTAT; miR-375-3p: F,



TTTGTTTCGTTTCGGCTCGCGTGA; and U6: F, CGTTCACGA ATTTGCGTGTTCAT. The reverse primer used in the qPCR of miRNA was the mRQ 3' primer supplied with the microRNA first-strand synthesis and miRNA quantitation kits (Takara).

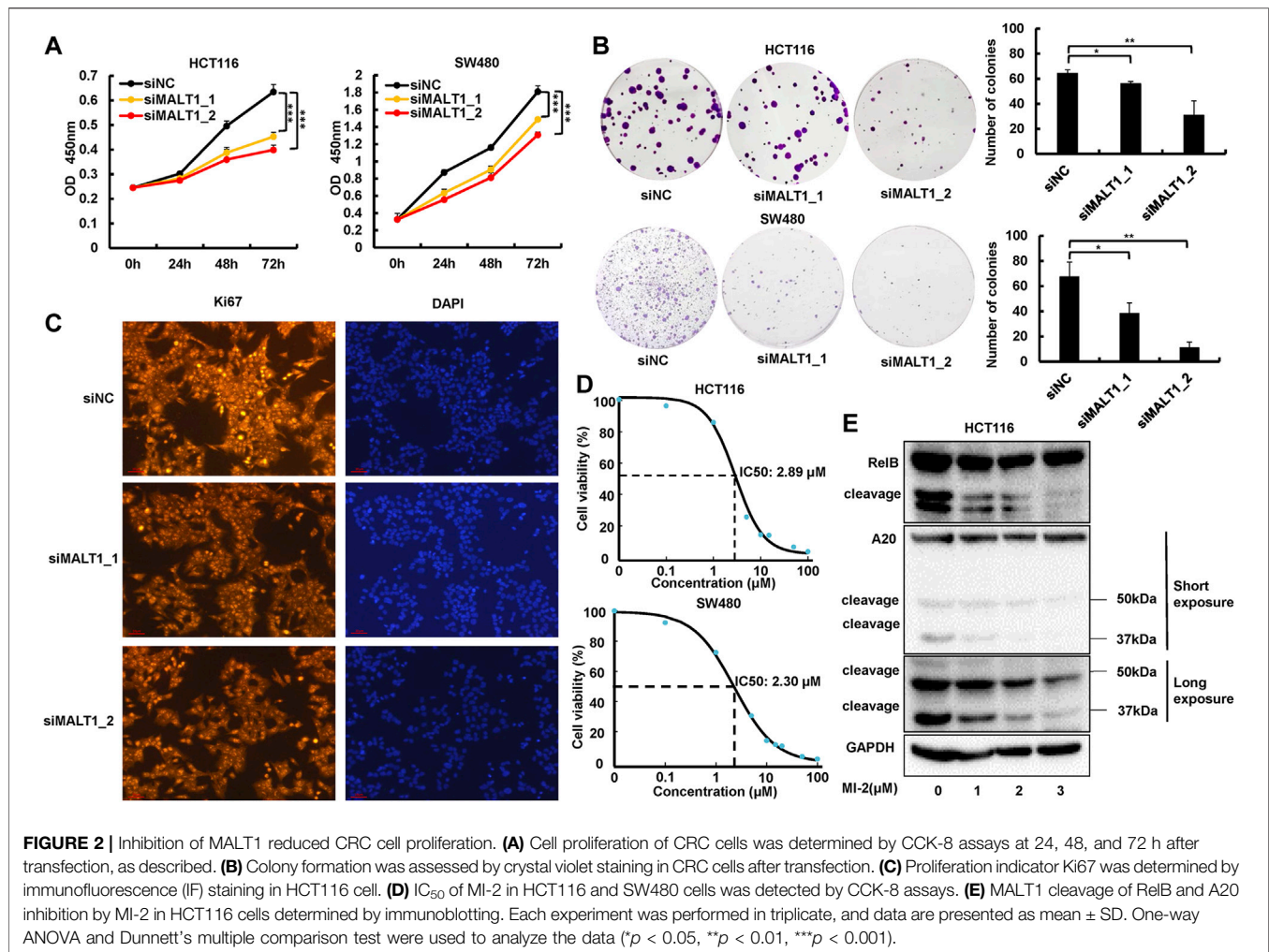
## Western Blot

Total protein was extracted using a cell lysis buffer (Beyotime, Shanghai, China) mixed with a protease inhibitor cocktail on ice, and protein concentration was determined by the Bradford method. SDS-polyacrylamide gel electrophoresis was used to separate the desired proteins. Subsequently, proteins were transferred to PVDF membranes and blocked with 10% milk powder solution. Membranes were placed in a primary antibody overnight at 4°C and then incubated with a secondary antibody at room temperature for 2 h. Immunoblotting was performed with the following antibodies:  $\beta$ -actin (47,778; Santa Cruz

Biotechnology), GAPDH (47,724; Santa Cruz Biotechnology), p-p65 (AB11014, phosphor-Ser536), MALT1 (2494S; Cell Signaling Technology), A20 (WL00820; Wanleibio), RELB (WL02922; Wanleibio), Fibronectin (66042-1-Ig; Proteintech), N-cadherin (WL01047; Wanleibio), E-cadherin (20874-1-AP, Proteintech), Vimentin (10366-1-AP; Proteintech) Snail (WL01863; Wanleibio), secondary antibodies: anti-mouse IgG-HRP (VC297958; Invitrogen), and anti-rabbit IgG-HRP (VC297287; Invitrogen). The data were acquired by Tanon 5200 system (Tanon Science) with Luminata Forte Western HRP substrate (Millipore).

## Cell Viability and Colony Formation Assays

HCT116 and SW480 were seeded into six-well plates at a number of  $1 \times 10^6$  cells per well, and the cells were transfected with plasmid (MALT1-pcDNA3.1-3xFlag-C; Hunan Fenghui



Biotechnology Co.) and siRNA for 6 h. After transfection, cells were seeded into 96-well plates at 1,000 cells per well. CCK-8 was added to each well according to the instructions, and after waiting for 2 h of reaction, the OD value at 450 nm was measured with a microplate reader.

For the colony formation assay, transfected HCT116 were seeded into 6-well plates at 500 cells per well and transfected SW480 were seeded into 6-well plates at 500 cells per well. All cells were cultured in DMEM supplemented with 10% FBS for 14 days. After 14 days, the colonies were fixed with 3.7% formaldehyde permeabilized with pure methanol and stained with 0.1% crystal violet. The ability of colony formation was determined by counting the number of stained colonies.

The siRNA (RIBOBIO) used in this study were as follows: siMALT1\_1: sense, CCGGAGAUAAUAAUGUGdTT; antisense, CACACAUAAUUAUCUCCG-GdTdT, siMALT1\_2: sense, CUACGAUGAUACCAUCCAdTT; and antisense, UG-GAAUGGUAUCAUCGUAGdTdT. The miRNA mimic and inhibitor (RIBOBIO) used in this study were as follows: miRNA-365-3p mimic: sense, AGGGACUUUUGGGGGCA-GAUGUG; antisense, CACAUCUGCCCCAAAAGUCCCU,

miRNA-365-3p inhibitor: sense, CACAUCUGCCCCAAAAGUCCCU, miRNA-375-3p mimic: sense, UUUGUUCGUUCG GCUCGCGUGA; and antisense, UCACGCGAGCCGAACG-AACAAA; miRNA-375-3p inhibitor: sense, UCACGCGAG CCGAACGAACAAA.

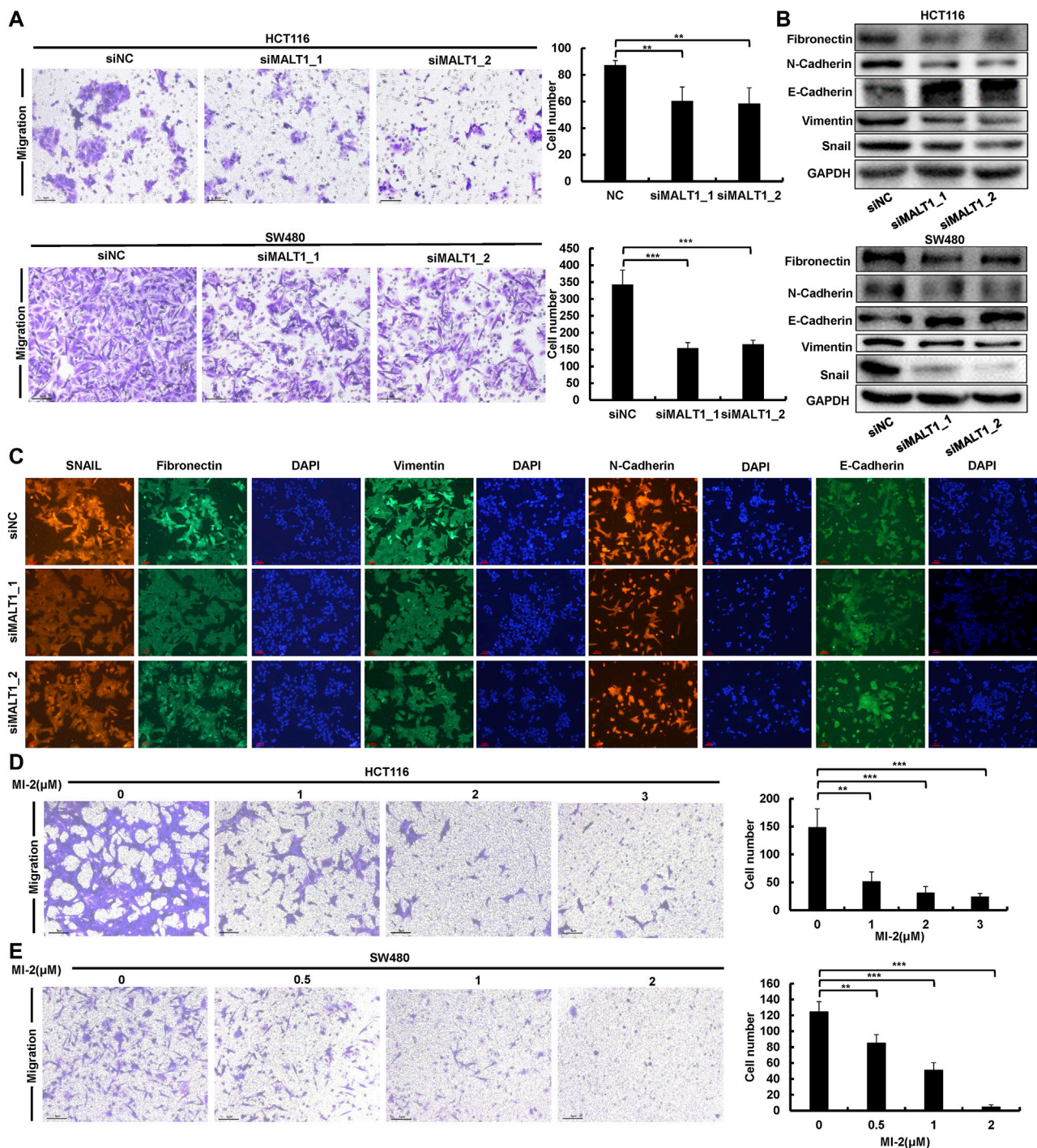
## Cell Migration Assay

HCT116 and SW480 cells ( $2.5 \times 10^6$ ) transfected with MALT1 or siMALT1 were seeded to the top migration chambers (Millipore) with 200  $\mu$ l FBS-free DMEM, and 750  $\mu$ l of DMEM with 10% FBS was added to the lower chamber. After 30 h, the upper chambers were washed with PBS, fixed with 3.7% formaldehyde, permeabilized with pure methanol, and stained with 0.1% crystal violet. Photographs were recorded under an inverted microscope (ZEISS), and the number of cells was then counted with ImageJ.

## Immunofluorescence Assay

For the immunocytochemistry assay, transfected HCT116 were seeded on glass coverslips in 12-well plates at  $2.4 \times 10^5$  cells per well. About 24 h later, the cells on glass coverslips were washed by PBS twice, fixed with 4% paraformaldehyde for 30 min, and then



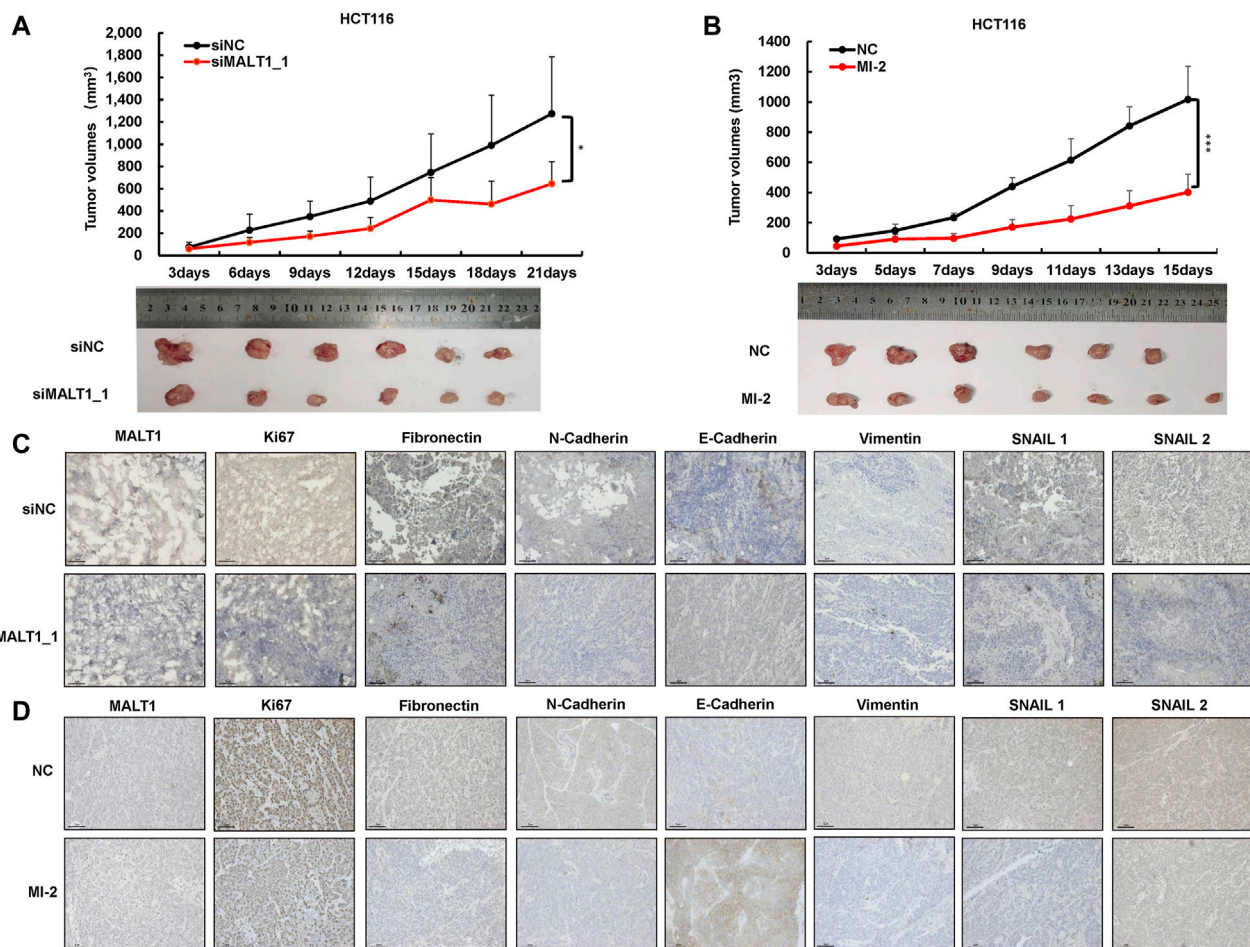


**FIGURE 3 |** Downregulation of MALT1 inhibited CRC cell migration. **(A)** Cell migration of CRC cells transfected, as described, was determined by transwell assays. **(B)** EMT-associated protein level was examined by immunoblotting in CRC cells after transfection. **(C)** The expression of EMT-associated protein was determined by IF in HCT116 cell after transfection. MI-2 inhibited HCT116 **(D)** and SW480 **(E)** cell migration was detected by transwell assays. Each experiment was performed in triplicate and data are presented as mean  $\pm$  SD. One-way ANOVA and Dunnett's multiple comparison test were used to analyze the data ( $p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ).

permeabilized by 0.5% NP-40 for 20 min at room temperature. After being blocked with 3% BSA (bovine serum albumin; Biotopped), glass coverslips were placed in primary antibody

dissolved in 3% BSA overnight at 4°C and then incubated for 1 h with specific fluorescence-conjugated secondary antibodies in the dark. 4',6-diamidino-2-phenylindole was used to stain the cells





**FIGURE 4 |** Knockdown of MALT1 inhibited CRC progression *in vivo*. **(A)** HCT116 cell transfected with siMALT1\_1 was used to establish subcutaneous tumor growth in a mouse xenograft model. Growth curves of tumor volumes were determined every 3 days. Representative photographs of tumors were shown below. **(B)** HCT116 cell was used to establish subcutaneous mouse model. After 3 days, mice were randomly divided into NC (5% DMSO) and MI-2 (25 mg/kg) group. Growth curves of tumor volumes were determined every 3 days. Representative photographs of tumors were shown below. **(C,D)** Representative IHC images of MALT1, Ki67, and on EMT-associated proteins' tumor sections. Each experiment was performed in triplicate, and data are presented as mean  $\pm$  SD. One-way ANOVA and Dunnett's multiple comparison test were used to analyze the data (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

on glass coverslips. Photographs were collected under an inverted microscope (ZEISS).

### Dual-Luciferase Reporter Assay

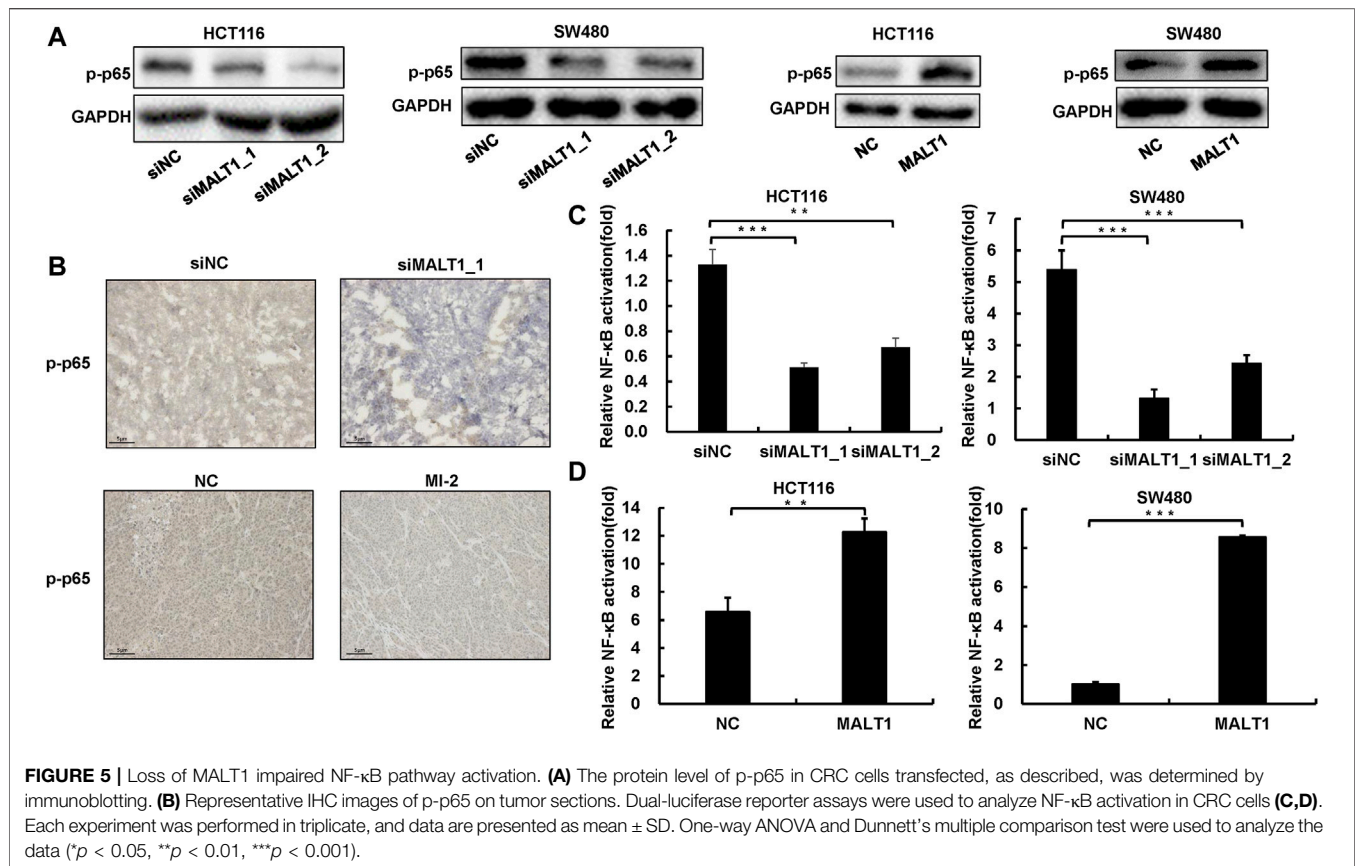
HCT116 and SW480 ( $8 \times 10^4$ ) was seeded into 24-well plates, co-transfected with NF- $\kappa$ B-Luc reporter vector (Beyotime Biotechnology, Shanghai, China), MALT1 and siMALT1. About 48 h later, luciferase intensity was measured using a dual-luciferase reporter assay kit (Beyotime Biotechnology, Shanghai, China), and the expression of each component was analyzed according to the instructions.

### Subcutaneous Xenograft Model

For the xenograft experiments, BALB/c nude mice (6 weeks old, female) were adopted from Guangdong Medical Laboratory Animal Center. As mentioned earlier, HCT116 cells ( $5 \times 10^6$

cells in 50  $\mu$ l DMEM) transfected with siNC or siMALT1 were subcutaneously inoculated into the scapular region of nude mice. The body weight and tumor volume [largest diameter  $\times$  (smallest diameter) $^2/2$ ] were measured daily, and after 7 days, a mixture of DMEM (50  $\mu$ l) with siNC or siMALT1 (12  $\mu$ l) and LIPO 2000 (12  $\mu$ l) was injected into the tumor location every 2 days. Tumors were removed immediately after the mice were sacrificed on Day 21 and frozen at  $-80^\circ\text{C}$  to prepare for the following experiments.

For the *in vivo* suppression studies of MI-2, HCT116 cells ( $5 \times 10^6$  cells in 50  $\mu$ l DMEM) were subcutaneously inoculated into the scapular region of nude mice (6 weeks old, male). Three days later, MI-2 and dimethyl sulfoxide (DMSO) dissolved in PBS were injected intraperitoneally at a dose of 25 mg/kg body weight in a volume of 10  $\mu$ l/g body weight every 2 days. Mice were killed and the tumors were removed after 14 days. All procedures were



approved by the Southern Medical University Animal Care and Use Committee. All animal studies were conducted in accordance with institutional guidelines.

## Statistical Analysis

The experimental results were expressed as mean values ± SD. A two-tailed Student's *t*-test, paired *t*-test, log-rank test, and one-way ANOVA were used for the analysis of statistical difference between two groups in IBM SPSS statistics 25.  $p < 0.05$  was considered statistically significant.

## RESULTS

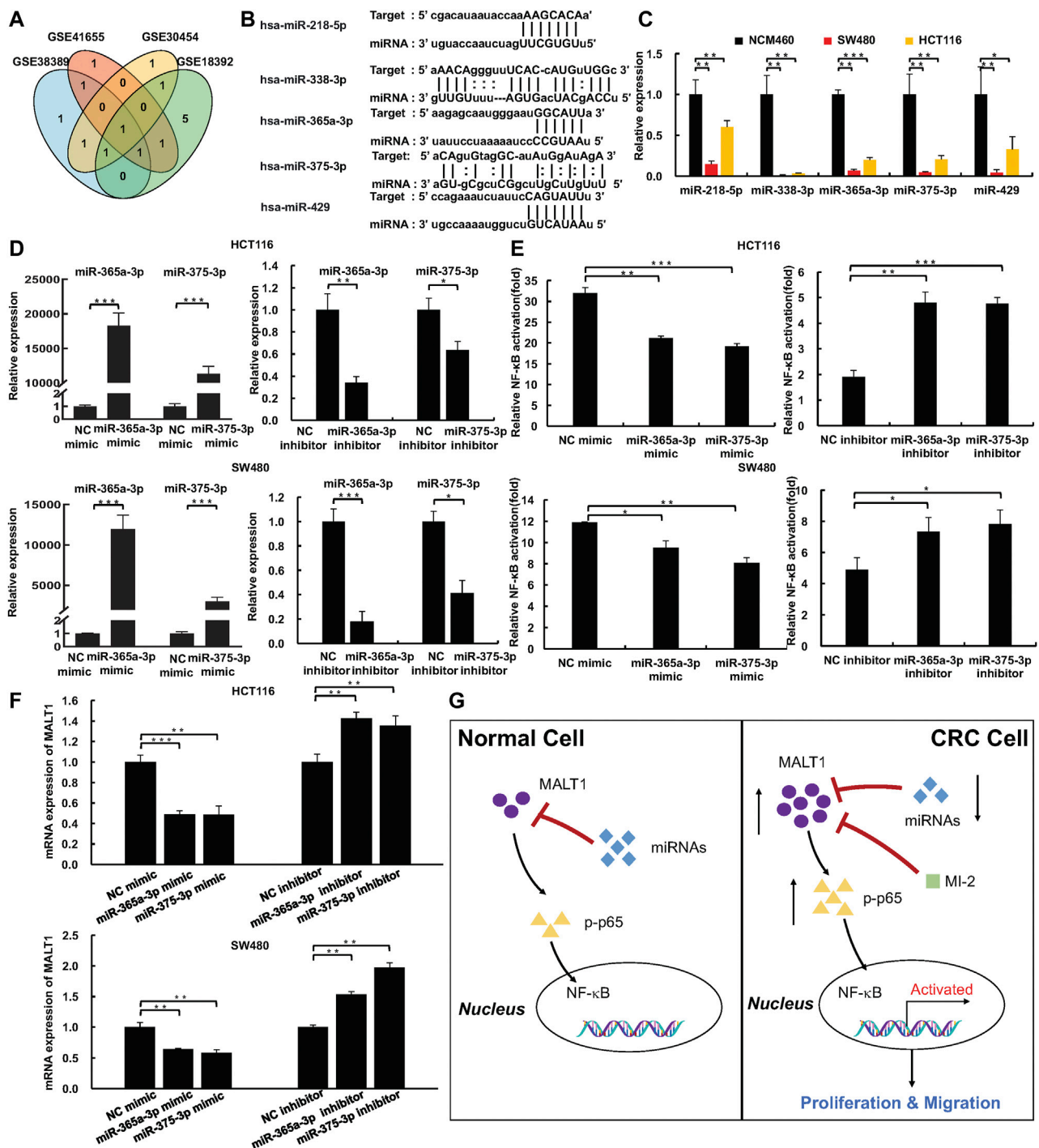
### High Expression of MALT1 Is Associated With CRC Poor Prognosis

To explore the relationship between MALT1 and the malignant progression of CRC, we first analyzed the difference of the MALT1 mRNA level between CRC and adjacent tissues based on the Gene Expression Omnibus (GEO) database (GSE21510). The data demonstrated that MALT1 expression was significantly increased in CRC tissues ( $n = 123$ ) compared with adjacent tissues ( $n = 25$ ) (**Figure 1A**). Another GEO series GSE17536 showed that patients with high MALT1 expression ( $n = 22$ ) had a worse prognosis compared with those with low MALT expression ( $n = 123$ ) (**Figure 1B**), which reminded us of the significance of MALT1 in CRC. To further confirm the expression of MALT1 in

CRC, we examined the protein level of MALT1 by immunohistochemistry (IHC) staining in colorectal tissues from 58 CRC patients compared to adjacent tissues. It was observed by microscope that MALT1 was mainly expressed in tumor tissues, and the IHC scores of tumor tissues were significantly higher than those in adjacent tissues ( $p < 0.001$ ) (**Figure 1C**). The protein and mRNA levels of MALT1 in CRC lines (SW480, HCT116) and a normal colorectal mucosa cell line (NCM) were detected by immunoblotting and qPCR. Consistent with the results from IHC, the protein and mRNA levels of MALT1 in the SW480 and HCT116 cells were both higher than those in NCM (**Figures 1D,E**). In light of these findings described, it is concluded that MALT1 was highly expressed in CRC cells and tissues, and we speculated that MALT1 may be associated with the malignancy of CRC.

### Downregulation of MALT1 Inhibits the Proliferation of CRC Cells

In order to identify the role of MALT1 in CRC malignancy, we transiently transfected HCT116 or SW480 cells by siRNA and an expression plasmid, and the protein levels of MALT1 were determined by immunoblotting (**Supplementary Figures S1A,B**). The CCK8 assays were conducted, and the results revealed a significant decrease of absorbance in HCT116 or SW480 cells following MALT1 knockdown, compared with the control group (**Figure 2A**). As shown in **Figures 2B,C**, silencing



**FIGURE 6 |** miR-375 and miR-365a-3p inhibited NF-κB pathway activation via targeting MALT1. **(A)** miR-375, miR-365a-3p, miR-429, miR-218, and miR-338-3p targeted MALT1 predicted by miRcode and were downregulated in CRC based on GEO database. **(B)** miR-375, miR-365a-3p, miR-429, miR-218, and miR-338-3p binding sites within 3'UTR of MALT1 mRNA. **(C)** The expression of miR-375, miR-365a-3p, miR-429, miR-218, and miR-338-3p in NCM and CRC cell lines detected by qPCR. **(D)** The expression of miR-375 and miR-365a-3p in CRC cells transfected with miRNA mimic or miRNA inhibitor. **(E)** Dual-luciferase reporter assays were used to analyze NF-κB activation in CRC cells. **(F)** The mRNA level of MALT1 detected by qPCR in CRC cells after transfection as described. **(G)** Simplified model of MALT1 promoting CRC development via miR-375/miR-365a-3p/NF-κB pathway. Each experiment was performed in triplicate, and data are presented as mean ± SD. One-way ANOVA and Dunnett's multiple comparison test were used to analyze the data (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).



the expression of MALT1 also decreased the number of colonies and the staining intensity of the proliferation marker Ki67. A small molecule inhibitor MI-2 was selected to inhibit MALT1 protease activity irreversibly. The cell proliferation of HCT116 and SW480 cells treated with MI-2 was significantly inhibited compared with control (Figure 2D). Then, a dose-dependent decrease of cleaved RelB or A20 was induced by MI-2, verifying its inhibitory effect (Figure 2E). When MALT1 was upregulated in CRC cells, the results were opposite with the silence of MALT1 (Supplementary Figures S1C–E). These data above suggested that MALT1 could promote the cell proliferation of CRC cells *in vitro*.

## MALT1 Inhibition Impairs Migration Ability of CRC Cells

Metastasis is a vital hallmark of carcinomas progressing to higher pathological grades of malignancy. Therefore, to further investigate the effect of MALT1 on the malignancy of CRC cells, we performed transwell assays to test the migration ability of CRC cells after transfection. The results showed that the migration abilities in both HCT116 and SW480 cells were significantly suppressed after MALT1 was silenced by siRNA transfection (Figure 3A). In addition, the expression of EMT-related proteins, which were indicators of tumor metastasis, were detected by immunoblotting. As shown in Figure 3B, the knockdown of MALT1 resulted in decreased expressions of vimentin, fibronectin, Snail, and N-cadherin and the increased expression of E-cadherin. Meanwhile, we examined the expression of EMT-associated proteins by IF, and the results were consistent with those by immunoblotting (Figure 3C). Similar to the knockdown of MALT1, MI-2 treatment inhibited CRC cell migration in a dose-dependent manner (Figures 3D,E). Inversely, overexpressed MALT1 promoted cell migration in CRC cells (Supplementary Figures S2A–C). Generally, the data mentioned above demonstrated that MALT1 strongly enhanced the migration ability of CRC cells to promote the malignant phenotype.

## Loss of MALT1 Inhibits the Growth of Tumor *In Vivo*

To evaluate the pro-oncogenic function of MALT1 *in vivo*, we established a subcutaneous tumor model. HCT116 cells were transfected with two different siRNAs targeting MALT1, and  $1 \times 10^7$  cells with MALT1 knockdown or negative control were injected subcutaneously into the two flanks of nude mice, respectively. Compared with the negative control group, the tumor volumes in the MALT1 knockdown group were significantly decreased in both two siRNAs (Figure 4A and Supplementary Figure S3A). Tumors were removed 21 days after inoculation. Then, the results showed that the MALT1 expression level, detected by IHC, was significantly decreased in the MALT1 knockdown group (Figure 4C and Supplementary Figure S3B). In accordance with our *in vitro* observations, the staining intensity of the proliferation marker Ki67 was much lower in xenografts derived from the MALT1 knockdown group than those in the control group

(Figure 4C and Supplementary Figure 3B). As for the migration indicator, we performed IHC assays to analyze the expression of EMT-associated proteins, and the results were similar to *in vitro* results (Figure 4C and Supplementary Figure S3C).

Meanwhile, HCT116 cells were injected subcutaneously to identify the effect of MI-2 *in vivo*. Three days after injection, mice were randomly divided into two groups, the DMSO and MI-2 groups. The results showed that the tumor volumes in the MI-2 group were significantly reduced compared to the DMSO group (Figure 4B). Consistently, the expression of Ki67 and EMT-associated proteins was lower in MI-2 treated mice than that in DMSO (Figure 4D). Taken together, these results showed that the inhibition of MALT1 or its protease activity could suppress tumor growth and migration *in vivo*.

## MALT1 Activates NF- $\kappa$ B Signaling Pathway in CRC Cells

To investigate the downstream molecular mechanism of MALT1 promoting CRC malignancy, we detected the activation of the NF- $\kappa$ B pathway, which has been reported as the downstream. The phosphorylation of p65 is a hallmark of NF- $\kappa$ B activation, which results in p65 nuclear translocation and further transcriptionally activates the target genes. Given that, we examined the expression of phosphorylated p65 by immunoblotting, and the results showed that the downregulation of MALT1 inhibited the phosphorylation of p65, whereas the upregulation of MALT1 increased it (Figure 5A). *In vivo* experiments also demonstrated that MALT1 knockdown or its protease activity inhibition led to a decrease of phosphorylated p65 in mice subcutaneous tumor tissues (Figure 5B and Supplementary Figure S3B). To verify the activation of the NF- $\kappa$ B pathway, luciferase reporter assays were also performed. As shown in Figures 5C,D, the luciferase activity of the NF- $\kappa$ B reporter was 4–6 times in the negative control group that of the inhibition of the MALT1 group, and overexpressed MALT1 increased the transcriptional activity compared with the control group. According to our data, MALT1 could promote CRC development by targeting the NF- $\kappa$ B signaling pathway (Figure 5E).

## miR-365a-3p and miR-375 inhibit NF- $\kappa$ B Signaling Pathway *via* Targeting MALT1

To gain a better understanding of the complete regulatory axis of MALT1-NF- $\kappa$ B, miRcode and GEO databases were carried out to identify a number of miRNAs targeting MALT1. Through miRcode database prediction, 97 miRNAs probably targeted MALT1 (Supplementary Table S1). Among these miRNAs, downregulation miRNAs in CRC tissues compared with normal tissues were sorted out based on GEO databases (GSE18392, GSE38389, GSE41655, and GSE30454, Supplementary Table S2). We chose miR-375, miR-365a-3p, miR-338-3p, miR-218, and miR-429 for further exploration, which was included in more than two GEO databases (Figures 6A,B). Then, real-time PCR experiments were exerted to detect the relative RNA expression of each miRNA in NCM and CRC cells, and the results showed that all those expressions were higher



in NCM cells as expected (**Figure 6C**). Since MALT1 was targeting the NF- $\kappa$ B pathway to increase the malignancy of CRC, miR-365a-3p and miR-375 were selected as reported in previous studies (Sun et al., 2018; Yin et al., 2019). Through the luciferase reporter assays, the results confirmed that the overexpression of miR-365a-3p or miR-375 inhibited NF- $\kappa$ B activation, which is in accordance with the results of MALT1 inhibition, and the downregulation of miR-365a-3p or miR-375 promoted NF- $\kappa$ B activation (**Figures 6D,E**). Furthermore, the mRNA expression of MALT1 regulated by miR-365a-3p or miR-375 was detected by qPCR. As expected, MALT1 expression was significantly decreased after the miR-365a-3p or miR-375 mimic treatment and increased after the miR-365a-3p or miR-375 inhibitor treatment (**Figure 6F**). In conclusion, miR-365a-3p or miR-375 might target MALT1 to inhibit NF- $\kappa$ B activation in CRC cells (**Figure 6G**).

## DISCUSSION

CRC is the third commonly diagnosed cancer and the world's second most deadly cancer in 2020 (Sung et al., 2021). Although there are many treatments including endoscopic and surgical local excision, targeted therapy, and immunotherapy, the morbidity and mortality of CRC are still rising steadily every year (Dekker et al., 2019; Siegel et al., 2020). MALT1 has been found have a vital role in lymphoma and immune cell development in the last two decades. The loss of MALT1 contributes to infantile combined immunodeficiency and immune dysregulation coupled with lymphocyte signaling impaired *via* the NF- $\kappa$ B pathway (Punwani et al., 2015). Indeed, MALT1 promotes cancer malignant progression not only in lymphoma but also in other types of cancer, such as melanoma and lung cancer (Pan et al., 2016; Rosenbaum et al., 2019). Here, in our study, we have firstly verified that MALT1 was upregulated in CRC, and the expression MALT1 was negatively correlated with the prognosis of CRC. Also, we verified that MALT1 promotes the cell proliferation and migration of CRC with the activation of the NF- $\kappa$ B pathway.

NF- $\kappa$ B has been found over 30 years as a rapidly inducible transcription factor, which has been confirmed a crucial role in tumor malignancy. NF- $\kappa$ B is directly bound to the receptors of TNF or IL-1, leading to the upregulation of anti-apoptosis genes, such as BCL-XL (Karin and Greten, 2005). Besides, the transcriptions of SLUG, TWIST1, and SNAIL were also stimulated by NF- $\kappa$ B, which initiated EMT and augmented cell migratory behavior (Taniguchi and Karin, 2018). In CRC, a number of studies have confirmed that some molecules, such as MyD88, SREBP1, and CXCL5, promoted CRC cell proliferation, migration, invasion, and tumor growth through NF- $\kappa$ B activation (Chen et al., 2019; Gao et al., 2019; Zhu et al., 2020). In addition, there were biological crosstalks between the NF- $\kappa$ B pathway and other signaling pathways, such as the EGFR pathway (Wang et al., 2019). NF- $\kappa$ B inhibitors have been exploited and studied for a long time, mainly proteasome blockers and IKK inhibitors. Bortezomib, the best studied proteasome inhibitor of NF- $\kappa$ B, reduced over 50% of patients

with resistance to the therapy in a phase 2 study (Richardson et al., 2003). NSAIDs, mainly targeting COX2, which is regulated by NF- $\kappa$ B signaling, are clinically used for preventing the recurrence of CRC (Katona and Weiss, 2020). In spite of their widespread applications, side effects are still in company with the inhibitors for CRC treatment, such as neutrophilia, fever, and abnormal IL-1 release (Zhang et al., 2017). Thus, exploring new targeted agents seems to be a long-term and beneficial strategy.

It is explicit that MALT1 activates the NF- $\kappa$ B pathway through the CBM complex in lymphoma. After the activation of TCR or BCR, CARMA1 is recruited to the plasma membrane, followed by BCL10 and MALT1; then, the CBM complex is assembled. This complex activates the IKK complex through ubiquitination, which further phosphorylates I $\kappa$ B, and its degradation activates the NF- $\kappa$ B pathway. MI-2 is a small molecule inhibitor that directly binds to MALT1 and suppresses the protease function, which is irreversible (Fontan et al., 2012). DSS-induced colitis reduced the diversity of the intestinal microbiome of mice, which was reversed by MI-2. MI-2 also reshaped the host's immune-modulating capacity by reducing inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-17 $\alpha$ , and IL-22 (Lee et al., 2018). In addition, the activation of the NLRP3 inflammasome was also inhibited by MI-2 (Liu et al., 2016). The above researches suggest a positive role of MALT1 or its protease function at least in the treatment of colorectal diseases. Previous studies proved that MI-2 inhibited ABC-DLBCL and melanoma tumor growth *in vivo* and inhibited MALT1 proteolytic activity and NF- $\kappa$ B activation *in vitro* (Fontan et al., 2012; Saba et al., 2017; Di Pilato et al., 2019). In our study, MI-2-treated CRC cells had dampened migration capability *in vitro*, which put up a promising target agent for CRC.

Nowadays, more and more miRNAs have been identified as biomarkers and potential therapeutic targets of cancers. The five miRNAs in our study have also been identified as crucial mediators of CRC, and all of them were downregulated in CRC, while only miR-365a-3p and miR-375 have been confirmed to directly suppress NF- $\kappa$ B activation (Sun et al., 2018; Yin et al., 2019). miR-365a-3p expression was associated with the overall survival of CRC patients and inhibited CRC cell invasion, migration, and chemoresistance through targeting KLF3 (Hong et al., 2020; Li et al., 2021). Plenty of literature verified that miR-375 was downregulated in CRC and suppressed the malignant progression of CRC (Wang et al., 2014; Xu et al., 2019). Moreover, miR-375 enhanced the chemosensitivity of CRC to 5-fluorouracil, which provides a hopeful therapeutic strategy (Xu et al., 2020). Some researches considered miR-429 as a biomarker for CRC, which had lower expression in CRC patients with poor prognosis, and proved that miR-429 inhibited the cell proliferation and migration of CRC (Sun et al., 2014a; Sun et al., 2014b; Guo et al., 2020). miR-218 also plays a pivotal role in CRC malignancy, including being associated with poor prognosis in patients and suppressing EMT and angiogenesis (Yu et al., 2013; Lun et al., 2018). In accordance with the other four miRNAs, miR-338-3p also inhibited CRC cell growth and migration and conferred 5-fluorouracil resistance in p53 mutant CRC (Han et al., 2017; Lu et al., 2019). Besides identifying the downregulation of five miRNAs in CRC, our study also proved that miR-375 and miR-365a-3p inhibited

the NF- $\kappa$ B pathway *via* targeting MALT1, which provided a new insight of CRC therapy.

Taken together, our study demonstrated that MALT1 promotes CRC progression *via* NF- $\kappa$ B activation. The inhibition of MALT1 proteolytic function or silencing its expression suppressed the proliferation and metastasis of CRC cells through NF- $\kappa$ B activation. Moreover, miR-375 and miR-365a-3p inhibited NF- $\kappa$ B activation by targeting MALT1 in CRC. Our research unveiled that MALT1 acted as an oncogene in the progression of CRC and provided a novel therapeutic target for CRC clinical treatment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Nanfang Hospital affiliated to Southern Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Southern Medical University Animal Care and Use Committee.

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## AUTHOR CONTRIBUTIONS

HD, MZ, YW, and RQ contributed to the conception and design of the study. RQ completed animal experiments and cell experiments. XN designed experimental schedule and was responsible for WB and qPCR analysis. ZG carried out the immunohistochemistry assay. XD performed immunofluorescence and statistical analysis. RQ and XN wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.845048/full#supplementary-material>

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# circDNMT1 Promotes Malignant Progression of Gastric Cancer Through Targeting miR-576-3p/Hypoxia Inducible Factor-1 Alpha Axis

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**Background:** Circular RNAs (circRNAs) regulate multiple malignant behaviors of various types of cancer. The role of circDNMT1, a newly identified circRNA, remains unknown in gastric cancer (GC). This study aimed to elucidate the underlying mechanisms of circDNMT1 in regulating GC progression.

**Methods:** microRNA (miRNA) and circRNA expression was detected by quantitative real-time PCR. Western blotting was performed to measure hypoxia inducible factor-1 alpha (HIF-1 $\alpha$ ) protein expression. Sanger sequencing, gel electrophoresis and fluorescence *in situ* hybridization were performed to identify the presence of circDNMT1. The clinicopathological features and overall survival of patients were analyzed based on circDNMT1 expression. The proliferation, migration and invasion of GC cells were determined by cell counting kit-8, 5-ethynyl-2'-deoxyuridine, wound healing and transwell assays. Glycolysis of GC cells was detected based on the levels of glucose uptake, the lactate acid, ATP and pyruvic acid production and the extracellular acidification and oxygen consumption rates. The binding sites between miR-576-3p and circDNMT1 or HIF-1 $\alpha$  were predicted by online bioinformatic tools and were validated using RNA pull-down and luciferase reporter assays. Xenograft models were established to determine the effects of the circDNMT1/miR-576-3p/HIF-1 $\alpha$  axis on GC growth and metastasis *in vivo*.

**Results:** circDNMT1 was successfully identified and shown to be overexpressed in GC tissues and cell lines. The expression levels of circDNMT1 were correlated with pathological T stage, pathological TNM stage and shorter survival time of GC patients. circDNMT1 knockdown inhibited the proliferation, migration, invasion and glycolysis of GC cells. circDNMT1 functioned as an oncogenic factor by sponging miR-576-3p. HIF-1 $\alpha$  was negatively regulated by miR-576-3p via binding its mRNA 3' untranslated region. circDNMT1 promoted malignant behaviors and metabolic reprogramming of GC by targeting the miR-576-3p/HIF-1 $\alpha$  axis both *in vitro* and *in vivo*.



**Conclusion:** These findings demonstrated that circDNMT1 knockdown inhibited GC proliferation, migration, invasion and glycolysis through sponging miR-576-3p/HIF-1 $\alpha$  axis. circDNMT1 may be a novel target for GC treatment.

**Keywords:** gastric cancer, circDNMT1, miR-576-3p, HIF-1 $\alpha$ , cancer progression, glycolysis

## INTRODUCTION

According to the latest epidemiological investigation, gastric cancer (GC) ranks fifth in incidence and forth in mortality worldwide (1). Due to its atypical symptoms, patients are usually diagnosed with GC at advanced or late stages, which limits survival benefits from current therapeutic methods. Gastrectomy and chemotherapy serve as classical regimens, whereas the long-term survival of patients is unsatisfactory. The side effects induced by these interventions also reduce their quality of life. In recent years, targeted drugs have been widely applied as alternative GC treatment regimens (2, 3). Nevertheless, a significant proportion of patients cannot harvest survival benefits from these treatment due mutations in specific gene sites. GC heterogeneity in a single tumor is also an important reason for therapy failure. Cancer cells with drug resistance will be naturally selected out and become the dominant portion of tumors, leading to the insensitivity to targeted drugs. It is urgent to develop regimens that interfere with more common characteristics based on the achievements of basic research.

Circular RNAs (circRNAs) are a type of non-coding RNAs, which are covalently closed and single-stranded (4). circRNAs have a notable stability serves as a feature of circRNAs. Their half-life periods are usually more than 48 h (5). They were first identified in 1976 by Kolakofsky (6) and regarded as the product of error splicing for a long time. With the development of research, circRNAs have been reported to regulate various physiological and pathological processes (7, 8). The biological functions of circRNAs can be primarily classified into four aspects: microRNA (miRNA) sponges, modulation of protein activity, regulation of gene expression and direct translation of peptides (8–11). The most attractive role of circRNA is miRNA sponging, which can abolish the biological functions of downstream miRNAs based on complementary sequences. Mounting studies have revealed close associations between the dysregulation of circRNA-miRNA networks and GC progression (12, 13). For instance, Zhang et al. indicated that circNRIP1 acts as a sponge of miR-149-5p and potentiates the malignant behaviors of GC cells (12). Xie et al. showed that exosomal circSHKBP1 promotes GC malignancy through suppressing the miR-582-3p/HUR/VEGF axis and inhibiting HSP90 degradation (13). Collectively, circRNAs are promising targets for cancer treatment.

Glucose metabolism is the main energy source for cancer growth and development, and is also regarded as a hallmark of cancer. Cancer cells are more dependent on glycolysis, rather than oxidative phosphorylation, to provide sufficient energy (12). Inspired by this phenomenon, researchers have indicated that suppression of cancer glycolysis might be a novel direction for overcoming drug resistance

and cancer heterogeneity. Some glycolytic inhibitors, such as genistein, lonidamine and 2-deoxy-D-glucose, have been developed and validated by clinical trials (14–16). However, the existing inhibitors are commonly used as combined drugs due to their limited efficacies and severe adverse reactions in recipients. Some circRNAs, such as circMAT2B, circATP2B1 and circBFAR, have shown capabilities of reprogramming cell glycolysis (9, 17, 18). These evidences suggested that inhibition of important circRNAs may attenuate GC glycolysis and progression. Despite the rapid progress of related basic research, remarkable translational advances in GC therapy are lacking. More circRNA targets should be explored and proved by rigorous validation.

Hsa\_circ\_0049224 (circDNMT1) is a newly identified circRNA in cancer treatment. It has been reported to be an autophagy modifier and miRNA sponge that potentiates breast cancer development (19, 20). Since the biological functions of circDNMT1 in GC remain unknown, in this study, we aimed to explore the role and underlying mechanisms of circDNMT1 in GC.

## MATERIALS AND METHODS

### Clinical Specimens

50 pairs of GC and adjacent normal tissues were harvested from patients who were diagnosed with advanced gastric carcinoma at Chinese PLA General Hospital from August 2016 to September 2017. The specimens were stored in liquid nitrogen. These patients did not receive any preoperative chemotherapy or radiotherapy. Informed consent was obtained from the included patients, who were followed-up to determine the 3-year overall survival (OS). This study was approved by the Ethics Committee of Chinese PLA General Hospital.

### Cell Culture

Cell lines were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). HGC-27 cells labeled with firefly luciferase (luc-HGC-27) were previously constructed and stored in our laboratory. Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher, MA, USA) with supplementary of 10% fetal bovine serum (FBS, Kangyuan, Tianjin, China) and 1% streptomycin/penicillin (Corning, NY, USA). Cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### Plasmid Transfection, Lentivirus Packaging and Cell Infection

The small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), miRNA mimics and overexpression plasmids used in this study were designed and synthesized by JTSBIO Scientific

(Wuhan, China, **Table S1**). Lipofectamine 2000 (Thermo Fisher) was used to conduct cell transfection according to the protocol of the manufacture. A Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia, USA) was used to perform the lentivirus packaging. Cells were chemically selected by puromycin (Yuan, Shanghai, China) at the working solution of 1 µg/mL for 10 days.

## RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

To detect RNA expression in cells and tissues, RNA extraction and qRT-PCR analysis were performed according to methods described in our previous study (18). The expression of relative genes was analyzed by the  $2^{-\Delta\Delta Ct}$  method. Vinculin and U6 served as the internal controls. The oligo dT and qRT-PCR primers are listed in **Table S1**.

## Western Blot (WB) Analysis

Protein was extracted by RIPA buffer (Solarbio, Beijing, China). A BCA Protein Assay Kit (Thermo Fisher) was used to conduct protein quantification of lysed samples. Total protein underwent high-temperature denaturation at 100°C for 15 min. 25 µg of protein was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, MA, USA). The membranes were then incubated with the primary antibodies and the anti-rabbit immunoglobulin G secondary antibody (Abcam, Cambridge, UK). The Super ECL Plus (Biorigin, Beijing, China) was used to make the bands visualized.

## Identification of circDNMT1 Existence

To confirm the authenticity of circDNMT1 detection by qRT-PCR, the amplified product was harvested. Agarose (2%) was prepared with Tris-acetate-EDTA buffer and heated until the agarose was sufficiently dissolved. GelGreen (Biomed, Beijing, China) nucleic acid dye was added into the 2% agarose which was poured into a mold. The mixture solidified into a gel after 20 minutes. Then, the amplified product underwent gel electrophoresis to verify circDNMT1 existence and observed under ultraviolet irradiation. A 50 bp Ladder DNA Marker (Biomed) was used to show the locations of DNA sizes in the gels. Sanger sequencing was also conducted to compare the nucleotide sequences of the amplified product and circDNMT1 junction site.

## RNA Pull-Down Assay

An RNA Pull-down Kit (Genesee, Guangzhou, China) was used to determine the binding efficiencies between circDNMT1 and miRNAs. The biotin-labeled circDNMT1 probe was mixed with magnetic beads (Thermo Fisher, USA) at room temperature for 2 h. The lysates of HGC-27 and AGS cells were prepared using lysis buffer and sonication, followed by incubation with probes at 4°C for 8 h. The magnetic beads were washed by washing buffer three times. The miRNAs were extracted by Trizol and were analyzed by qRT-PCR. The probe sequences can be viewed in **Table S2**.

## Luciferase Reporter Assay

To examine the binding efficiencies of miR-576-3p, the corresponding wildtype and mutant reporter plasmids as predicted by bioinformatic analysis were constructed (JTSBIO

Scientific, Wuhan, China). The miRNA mimics and plasmids were co-transfected into cells. The cells were harvested after 48 h, and the Dual-Luciferase Reporter Assay System (Promega, USA) was used to determine the luciferase activities.

## Fluorescence *In Situ* Hybridization (FISH)

Seeded cells were washed by phosphate buffered saline (PBS) two times and 4% paraformaldehyde was used to fix cells. A Situ Hybridization Kit (BersinBio, Guangzhou, China) and Cy3-labeled circDNMT1 probe (HonyiBio, Guangzhou, China) were used to label circDNMT1 location. The images were recorded using a laser scanning confocal microscope. The circDNMT1 probe sequence can be viewed in **Table S2**.

## Cell Proliferation Assay

For the CCK-8 assay,  $3 \times 10^3$  cells were seeded into 96-well plates. After 10 h, the medium was replaced with CCK-8 working solution. The plates were placed at 37° for 1 h protected from light. Then, the absorbance at 450 nm was measured by a microplate reader (Biotech, USA). The experiments were repeated at the indicated times. For the EdU assay,  $2 \times 10^4$  cells were cultivated in 96-well plates. The proliferative cells were stained with EdU and 4,6-diamidino-2-phenylindole (DAPI) according to the manufacturers' protocols. An inverted fluorescence microscope was used to observe the stained cells.

## Cell Migration Assay

To evaluate the capability of GC cells migration, the cells were seeded and the density could reach 100% after 10 h. Cells were vertically scratched using pipette tips and gently washed by PBS for three times. The medium was replaced with DMEM without FBS. The wounds at the specific sites were observed under an inverted microscope at 0 h and 24 h.

## Cell Invasion Assay

The 10% Matrigel (Corning, NY, USA) was prepared in DMEM. Then, 50 µL of 10% Matrigel was added to the bottom of the Transwell chamber.  $1 \times 10^4$  cells were suspended in 200 µL serum-free DMEM and seeded on a concretionary Matrigel. The lower chambers were immersed in 600 µL DMEM containing 20% FBS. After 24 h, the cells were stained with 0.1% crystal violet and counted under an inverted microscope.

## Glycolytic Experiments

A Glucose Uptake Assay Kit (Biovision, CA, USA) was used to measure the uptake of glucose. The production of lactate acid, ATP and pyruvic acid was examined by D-Lactate Assay Kit, ATP Colorimetric/Fluorometric Assay Kit and Pyruvate Colorimetric/Fluorometric Assay Kit (Biovision), respectively. The corresponding absorbances were detected using a microplate reader.

## Extracellular Acidification Rate (ECAR) and Oxygen Consumption Rate (OCR) Assays

To further evaluate the metabolism levels of GC cells, ECAR and OCR were determined with a Glycolysis Stress Test Kit and Cell

Mito Stress Test Kit (Agilent, USA). Briefly, cells were seeded in Seahorse XF96 plates (Agilent). After 10 h of incubation, the ECAR and OCR were detected according to the manufacture's protocols. In the detection of ECAR, glucose (10 mM), oligomycin (1  $\mu$ M) and 2-deoxy-d-glucose (100 mM) were injected into medium of cells sequentially. For the measurement of OCR, cells were treated with oligomycin (1  $\mu$ M), protonophore trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, 0.5  $\mu$ M) and antimycin A (0.5  $\mu$ M). The signaling data were read and recorded by a Seahorse XFe96 Analyzer (Agilent).

## Animal Experiments

To establish xenograft models of subcutaneous tumors and lung metastasis, BALB/c nude mice (4 weeks, male, Charles River, Beijing, China) were fed and housed under specific pathogen-free conditions. After 2 weeks of acclimatization, the mice were randomly divided into experimental groups, with 5 mice in each group. For generation of the model with subcutaneous tumors, mice were subcutaneously injected with  $1 \times 10^7$  luc-HGC-27 cells suspended in PBS. The tumor volumes were measured by a vernier caliper every 5 days. The evaluation method of tumor volumes as follows: Volume = (length  $\times$  width<sup>2</sup>)  $\times$  0.5.  $5 \times 10^6$  luc-HGC-27 cells were injected into the tail vein to simulate lung metastasis. After 30 days, 1.5 mg D-Luciferin (Solarbio) was intraperitoneally injected into each mouse. The tumor locations and loads were displayed by bioluminescence. Mice were sacrificed by cervical dislocation after anesthesia.

## Statistical Analysis

The SPSS 25.0 and GraphPad Prism 8.0 software were used to conduct all statistical analysis. The normal distribution was conducted using Q-Q plot and Shapiro-Wilk test. The significance of variables was evaluated by Student's t-test after distribution examinations. The Chi-square test was used to compare the differences in clinicopathological features. The survival time of included patients was analyzed using Kaplan-Meier method. Data were presented as means  $\pm$  standard deviation (SD). A p value < 0.05 was considered as the statistical significance.

## RESULTS

### Identification of circDNMT1 Characteristics in GC

To confirm the existence of circDNMT1 in GC, we first investigated its origins from genomic DNA. circDNMT1 is derived from exon 6 and exon 7 of DNMT1. qRT-PCR primers for sequence amplification across the junction sites were designed. The concrete base sequences of the amplified product were confirmed using Sanger sequencing (Figure 1A). The images of DNA gel electrophoresis showed that the circDNMT1 amplification product could only be obtained using divergent primers derived from cDNA. It cannot be amplified with convergent primers or gDNA as templates (Figure 1B). FISH

images indicated that circDNMT1 was mainly located in the cytoplasm of GC cells (Figure 1C). The data identified the existence of circDNMT1 in GC and proved the feasibility of these primers for circDNMT1 amplification.

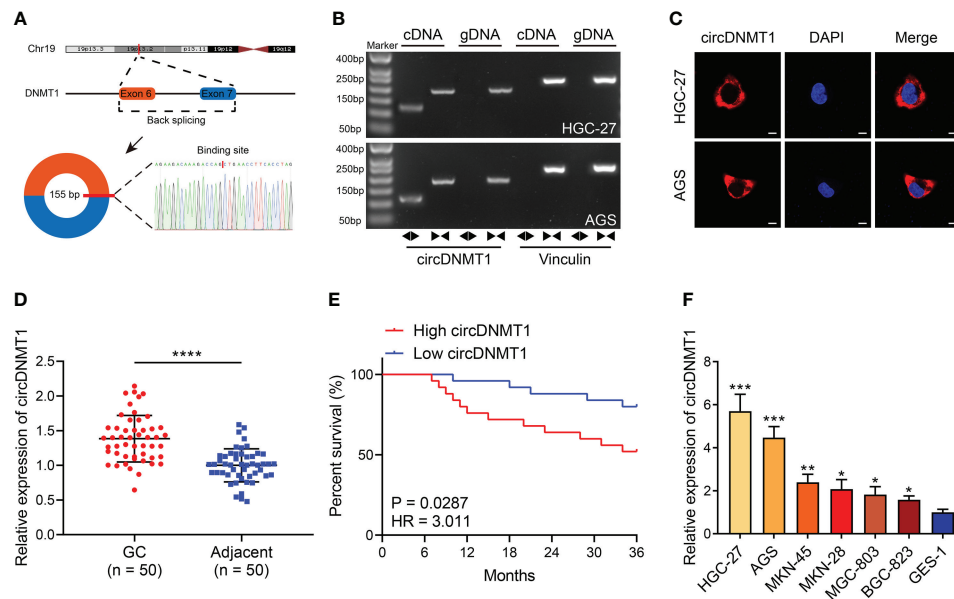
Next, we measured circDNMT1 expression in clinical specimens from 50 GC patients. circDNMT1 was significantly overexpressed in GC tissues (Figure 1D). The median value of circDNMT1 was set as the dividing line between the high-circDNMT1 and low-circDNMT1 groups. The clinicopathological information of these patients was further analyzed. circDNMT1 levels were correlated with pathological T (pT) stage and pathological TNM (pTNM) stage (Table 1). High expression of circDNMT1 indicated poorer patient survival time (Figure 1E). The circDNMT1 expression in GC and gastric epithelial cell lines was also determined using qRT-PCR analysis. All GC cell lines had higher expression levels than human gastric epithelial cells, and HGC-27 and AGS cells had the highest levels (Figure 1F).

### circDNMT1 Knockdown Inhibits the Proliferation, Migration and Invasion of GC Cells

Regarding the clinical value of circDNMT1, it has been speculated that circDNMT1 might participate in the progression of GC. The two cell lines with the highest circDNMT1 expression, HGC-27 and AGS, were chosen for the subsequent experiments. We generated the GC cells with stable knockdown of circDNMT1. The interference efficiencies of shRNA and overexpression plasmids were examined using qRT-PCR analysis (Figures 2A, S1). Downregulation of circDNMT1 expression attenuated the proliferation rates of GC cells, while circDNMT1 overexpression rescued the inhibitory effects on cell proliferation (Figures 2B–D). circDNMT1 knockdown inhibited GC migration and additional transfection with its overexpression plasmids restored the suppression caused by circDNMT1 knockdown (Figure 2E). Transwell assays also verified the effects of circDNMT1 on GC cells invasion (Figure 2F). The data of *in-vitro* experiments prove that knockdown of circDNMT1 suppresses the malignant progression of GC cells and there is no off-target effect that interfered with the results.

### circDNMT1 Promotes Glycolysis of GC Cells

Glycolysis is an important initiator of cancer malignancy. To detect glycolysis of GC cells, we determined the metabolite levels using colorimetric methods, which included glucose uptake and lactate acid, ATP, pyruvic acid production. The ratio of glucose uptake to lactate acid production is also an indicator of measuring cell glycolysis. The increased ratio demonstrates the inhibition of glycolysis. Downregulation of circDNMT1 expression suppressed ATP and pyruvic acid production. The decreases of glucose uptake, lactate acid production and a relative increase of their ratio were observed. Restoration of circDNMT1 expression brought them back as the control groups (Figures 3A–E). The ECAR and OCR represent glycolysis and oxidative phosphorylation, respectively. circDNMT1 knockdown inhibited the ECAR, while the OCR was apparently elevated. This imbalance



**FIGURE 1** | Identification of circDNMT1 characteristics in GC. **(A)** The schematic illustration of circDNMT1 origination and the result of Sanger sequencing. **(B)** The gel electrophoresis to examine amplified product of qRT-PCR using convergent and divergent primers. **(C)** FISH to display the distributions of circDNMT1 in HGC-27 and AGS cells. Scale bar: 10  $\mu$ m. **(D)** The qRT-PCR analysis to show circDNMT1 expression in 50 pairs of GC and adjacent normal tissues. **(E)** The Kaplan-Meier plot to show survival time of 50 GC patients who were divided into high-circDNMT1 and low-circDNMT1 groups. **(F)** The qRT-PCR analysis to show circDNMT1 expression in HGC-27, AGS, MKN-45, MKN-28, MGC-803, BGC-823 and GES-1 cell lines. Data were presented as means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

could be mitigated by additional circDNMT1 overexpression (Figures 3F, G). These findings suggest that circDNMT1 may be an important contributor of glycolysis during GC progression.

**TABLE 1** | Correlation between circDNMT1 expression and clinicopathological characteristics of 50 GC patients.

Characteristics	Case number	High (n=25)	Low (n=25)	P value
Age at surgery (years)				0.390
<60	29	13	16	
$\geq 60$	21	12	9	
Gender				0.208
Male	36	16	20	
Female	14	9	5	
pT stage				<b>0.037</b>
pT1+pT2	17	5	12	
pT3+pT4	33	20	13	
Tumor size (cm)				0.157
<5	25	10	15	
$\geq 5$	25	15	10	
Location				0.777
Cardiac	23	12	11	
Non-cardiac	27	13	14	
pTNM stage				<b>0.045</b>
I+II	21	7	14	
III+IV	29	18	11	
Differentiation				0.157
Poorly	45	24	21	
Well	5	1	4	

The bold values mean the data with significant difference ( $P < 0.05$ ).

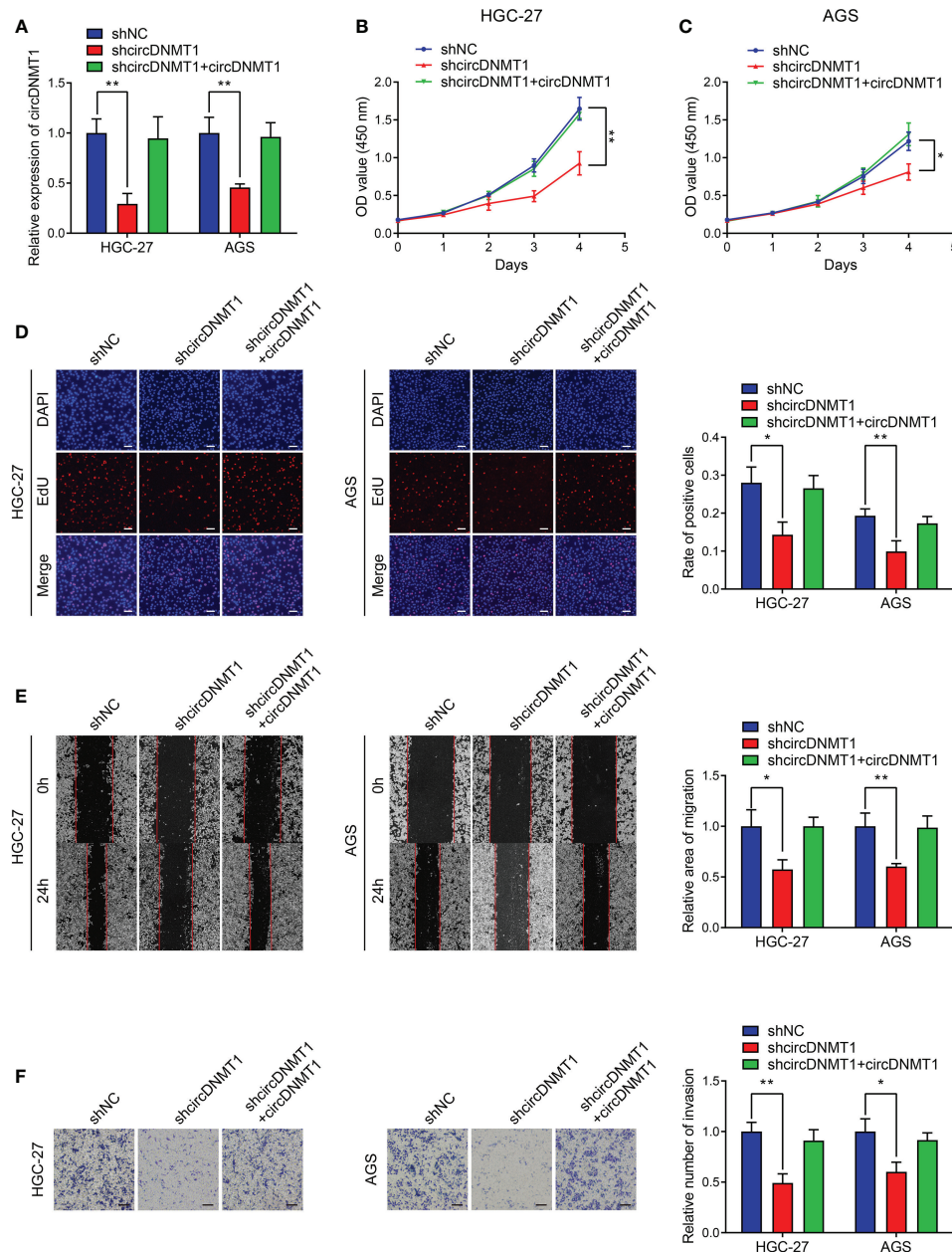
## miR-576-3p Is Directly Sponged by circDNMT1 in GC

To further reveal the mechanisms of circDNMT1 as a miRNA sponge, we employed a bioinformatic tool, Circinteractome, to predict candidate downstream miRNAs. A total of five miRNAs with over 90 context+ score percentiles were selected out, including miR-876-3p, miR-1200, miR-576-3p, miR-661 and miR-1236 (Figure 4A). The probe targeting circDNMT1 was designed and used for baiting miRNAs that directly bind to circDNMT1 in GC cells. Among the five miRNAs, miR-576-3p had the highest binding efficiencies both in HGC-27 and AGS cell lines (Figures 4B, C). Next, we constructed the luciferase reporter plasmids that were inserted with wildtype or mutant circDNMT1 sequences (Figure 4D). miR-576-3p significantly reduced the luciferase activities of wildtype plasmids in HGC-27 and AGS cells. Whereas, the luciferase activities of mutant plasmids were free from the suppression of miR-576-3p (Figures 4E, F). These results suggest that circDNMT1 inhibits miR-576-3p functions by direct complementary binding.

## circDNMT1 Potentiates GC Progression and Glycolysis by Targeting miR-576-3p

The oncological functions of miR-576-3p in GC were not reported. Thus, we examined the effects of miR-576-3p on the malignant phenotypes of GC cells. We designed miR-576-

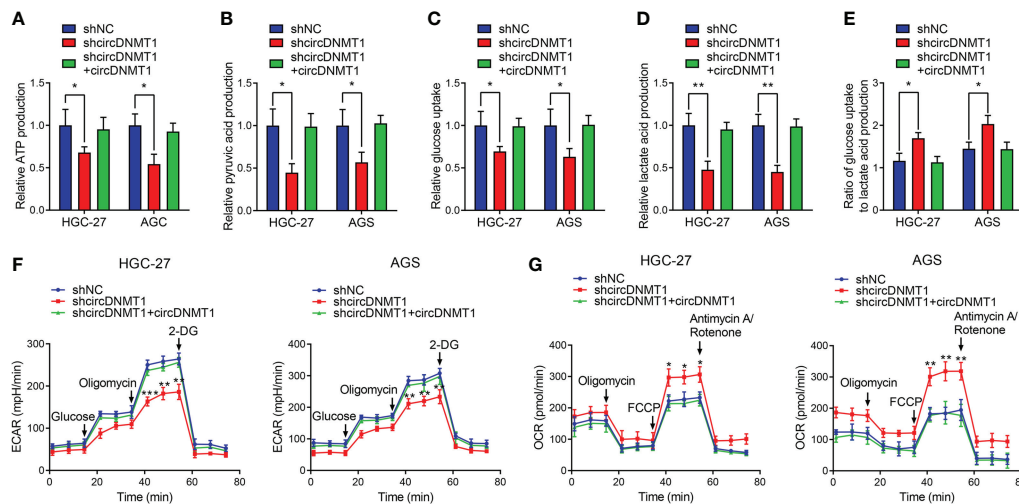




**FIGURE 2 |** circDNMT1 knockdown inhibits proliferation, migration and invasion of GC cells. **(A)** The qRT-PCR analysis to show circDNMT1 expression in HGC-27 and AGS cells stably carrying lentivirus with NC shRNA or circDNMT1 shRNA and additionally transfected with vector or circDNMT1 overexpression plasmids. **(B, C)** The CCK-8 assay to show proliferation of cells as in **(A)**. **(D)** The EdU assay to show proliferation of cells as in **(A)**. Scale bar: 100  $\mu$ m. The histogram is displayed on the right. **(E)** The wound healing assay to show migration of cells as in **(A)**. The histogram is displayed on the right. **(F)** The transwell assay to show invasion of cells as in **(A)**. The histogram is displayed on the right. Scale bar: 100  $\mu$ m. Data were presented as means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ .

3p mimics, and their overexpression efficiencies were examined by qRT-PCR (**Figure 5A**). The *in-vitro* experiments showed that upregulation of miR-576-3p suppressed GC proliferation, migration and invasion. circDNMT1 overexpression counteracted the inhibition induced by miR-576-3p (**Figures 5B–F**). For cell glycolysis, miR-576-3p had

inhibitory effects on glucose uptake, lactate acid, ATP, pyruvic acid production and promotive effects on the ratio of glucose uptake to lactate acid production (**Figures S2A–E**). The ECAR was attenuated while the OCR was inversely enhanced. The data show that circDNMT1 could mitigate the effects of miR-576-3p (**Figures S2F, G**).

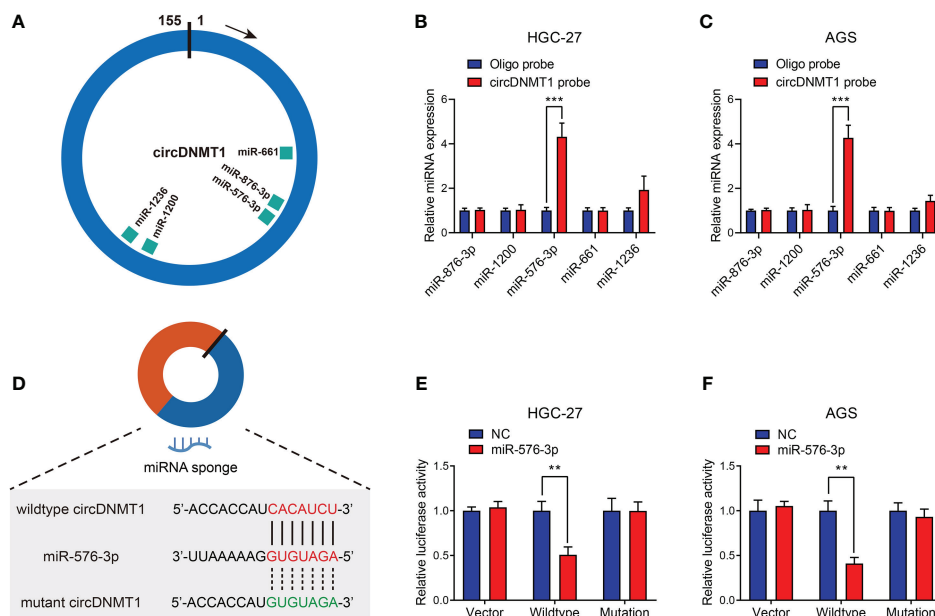


**FIGURE 3 |** circDNMT1 promotes glycolysis of GC cells. (A–E) ATP production (A), pyruvic acid production (B), glucose uptake (C), lactate acid production (D) and ratio of glucose uptake to lactate acid production (E) were determined in HGC-27 and AGS cells stably carrying lentivirus with NC shRNA or circDNMT1 shRNA and additionally transfected with vector or circDNMT1 overexpression plasmids. (F, G) ECAR (F) and OCR (G) assays of cells as in (A–E). A series of compounds were added at the indicated time. Data were presented as means  $\pm$  SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

## miR-576-3p Suppresses Hypoxia Inducible Factor-1 Alpha (HIF-1 $\alpha$ ) Expression by Directly Binding to Its mRNA 3'UTR

miRNAs can inhibit downstream gene expression by binding its mRNA 3'UTR and accelerating mRNA degradation. miR-576-3p

has been reported to regulate HIF-1 $\alpha$  expression in cancer (21, 22). Therefore, it was speculated that circDNMT1 could promote GC progression by sponging miR-576-3p/HIF-1 $\alpha$  axis. HIF-1 $\alpha$  was inhibited by miR-576-3p while circDNMT1 overexpression elevated its expression. circDNMT1 could also mitigate the



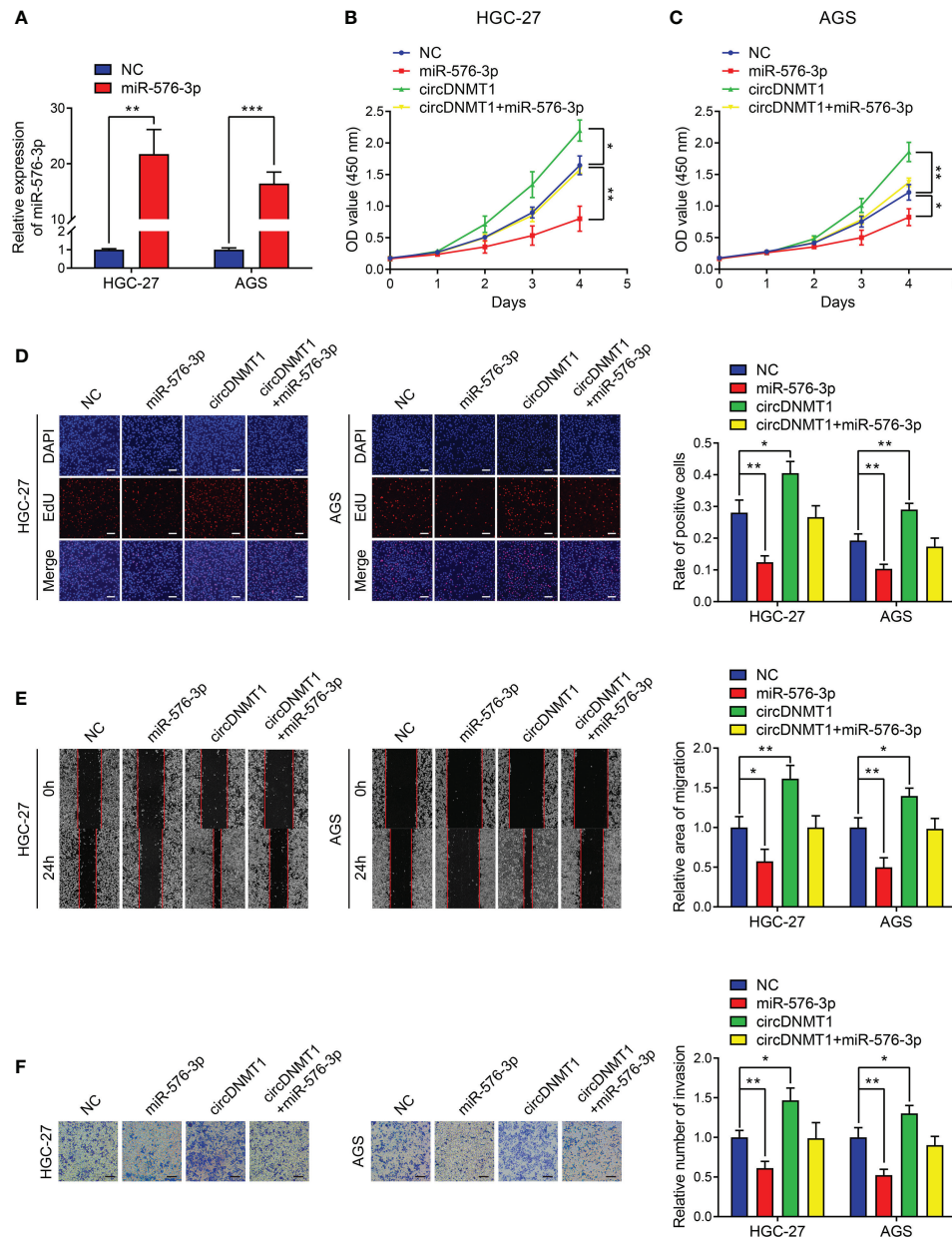
**FIGURE 4 |** miR-576-3p is directly sponged by circDNMT1 in GC. (A) The schematic illustration of miRNAs that can be sponged by circDNMT1 predicted by Circinteractome. (B, C) The qRT-PCR analysis to show the miRNA levels that were pulled down by NC probe or circDNMT1 probe in HGC-27 (B) and AGS (C) cells. (D) The schematic illustration of wildtype (red) and mutant (green) sequences of binding sites of circDNMT1 and miR-576-3p. (E, F) Luciferase reporter assay to show the relative luciferase activities in HGC-27 (E) and AGS (F) cells that were cotransfected with empty luciferase reporter plasmids (vector) or plasmids inserted with wildtype or mutant sequences and NC and miR-576-3p mimics. \*\*P < 0.01, \*\*\*P < 0.001.

inhibitory effects of miR-576-3p (**Figure S3A**). We then aimed to confirm the mechanistic associations between miR-576-3p and HIF-1 $\alpha$ . According to the sites predicted by Targetscan, we designed luciferase reporter plasmids that were inserted with wildtype or mutant sequences of the mRNA 3'UTR of HIF-1 $\alpha$  (**Figure S3B**). miR-576-3p could suppress the activities of wildtype luciferase reporter plasmids instead of mutant ones

(**Figures S3C, D**). These data prove that miR-576-3p directly suppresses HIF-1 $\alpha$  expression in GC cells.

### circDNMT1 Promotes GC Malignancy by Targeting the miR-576-3p/HIF-1 $\alpha$ Axis

We then knocked down HIF-1 $\alpha$  using a specific siRNA, and the results were verified by WB analysis (**Figure 6A**). HIF-1 $\alpha$



**FIGURE 5 |** circDNMT1 potentiates GC progression by targeting miR-576-3p. **(A)** qRT-PCR analysis to show the miR-576-3p expression in HGC-27 and AGS cells that were transfected with NC or miR-576-3p mimics. **(B, C)** The CCK-8 assay to show proliferation of HGC-27 **(B)** and AGS **(C)** cells stably carrying lentivirus with vectors or circDNMT1 overexpression plasmids and additionally transfected with NC or miR-576-3p mimics. **(D)** The EdU assay to show proliferation of cells as in **(B-C)**. Scale bar: 100  $\mu$ m. The histogram is displayed on the right. **(E)** The wound healing assay to show migration of cells as in **(B, C)**. The histogram is displayed on the right. **(F)** The transwell assay to show invasion of cells as in **(B, C)**. The histogram is displayed on the right. Scale bar: 100  $\mu$ m. Data were presented as means  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

overexpression potentiated the proliferation of GC cells, and miR-576-3p could rescue the effects of HIF-1 $\alpha$  overexpression. HIF-1 $\alpha$  knockdown significantly reduced the proliferation capability of GC cells. However, this proliferative inhibition could not be effectively rescued by circDNMT1 overexpression (Figures 6B–D). Cell migration and invasion had the similar tendencies as proliferation detection. miR-576-3p and HIF-1 $\alpha$  had the counteracting effects. Elimination of HIF-1 $\alpha$  functions abrogated promotion induced by circDNMT1 (Figures 6E, F). These findings show that circDNMT1 potentiates GC malignant behaviors by targeting the miR-576-3p/HIF-1 $\alpha$  axis. HIF-1 $\alpha$  may serve as a mediator of circDNMT1 to contribute to GC progression.

### The circDNMT1/miR-576-3p/HIF-1 $\alpha$ Axis Regulates GC Growth and Metastasis *In Vivo*

To further verify the participation of the circDNMT1/miR-576-3p/HIF-1 $\alpha$  axis in GC development, generation of mouse models with subcutaneous tumor and lung metastasis was conducted. circDNMT1 overexpression facilitated the *in-vivo* growth of GC. Transfection of miR-576-3p mimics into tumors mitigated the promotive effects. Consistent with the *in-vitro* experiments, HIF-1 $\alpha$  knockdown induced decreases in cancer growth and abrogated the functions of circDNMT1 (Figures 7A–C). The lactate acid concentrations in cell-derived tumors were determined, which were in line with the tendencies of tumor growth (Figure 7D). Moreover, upregulation of circDNMT1 expression potentiated GC metastasis, which was counteracted by miR-576-3p. Downregulation of HIF-1 $\alpha$  expression impaired the metastasis, and circDNMT1 could not rescue these inhibitory effects (Figures 7E, F). Collectively, our work identified the oncogenic role of circDNMT1 in GC and found that the miR-576-3p/HIF-1 $\alpha$  axis might serve as the main downstream pathway of it as shown by both *in-vitro* and *in-vivo* experiments (Figure 7G).

## DISCUSSION

The research concerning biological functions of circRNA is a hot topic in recent years. circRNA participate in the regulation of many diseases, such as cardiovascular diseases, neural degradation, and endocrine disorders (23–25). Numerous studies have elucidated the mechanisms of circRNA regulatory network during the processes of carcinogenesis and metastasis. circRNA-5692 attenuates the malignant behaviors of hepatocellular carcinoma by suppressing the miR-328-5p/DAB2IP axis (26). circNDUFB2 reduces the stabilization of IGF2BPs and enhances anti-tumor immunity in lung cancer (27). The mechanistic associations between circRNAs and GC have also been studied. Dysregulation of circRNA expression serves as a hallmark of GC development and has potential value of diagnosis and prognostic prediction (28). Interference with expression of some circRNAs could reverse GC progression and

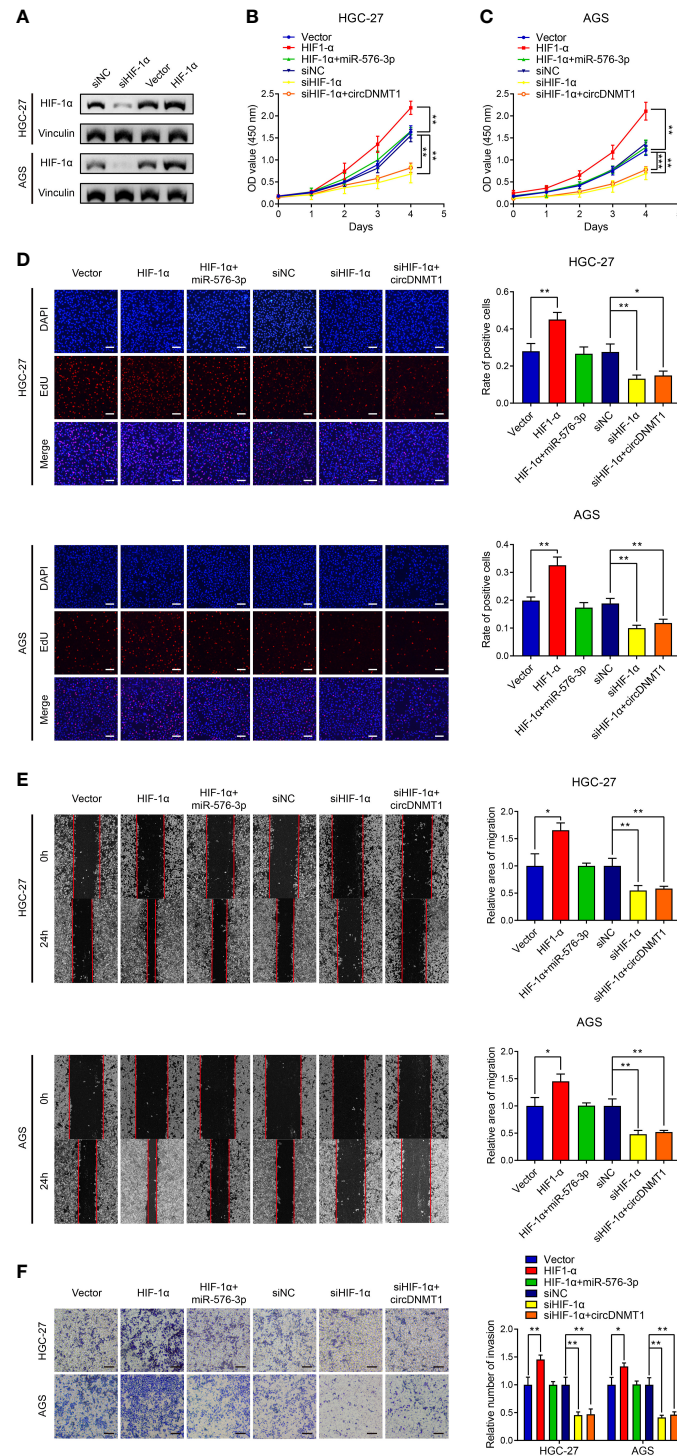
mitigate tumor burdens both *in vitro* and *in vivo* (12, 13). However, few clinical trials about circRNAs in GC treatment have been conducted. It may be attributable to the limited findings of existing reports. Therefore, it is eagerly required to explore more effective targets and to broaden horizons of the circRNA landscape in cancer development.

circDNMT1 is a novel circRNA that has been reported to promote breast cancer progression. circDNMT1 was overexpressed in breast cancer tissues. As an miRNA sponge, circDNMT1 maintains the highly proliferative state of breast cancer by regulating the miR-485-3p/ZEB1 axis (20). Another study showed that circDNMT1 promotes nuclear translocation of p53 and AUF1 and activates autophagy to increase survival capabilities of breast cancer (19). Our findings showed that circDNMT1 exhibited overexpression in GC. The clinicopathological features and 3-year OS of GC patients were collected and circDNMT1 expression indicated clinical severity and poor survival. circDNMT1 indicated shorter survival time of GC patients. Further experiments demonstrated that circDNMT1 knockdown led to inhibition of GC progression. Inversely, its upregulation could promote these malignant behaviors. circDNMT1 may have potentials as a valuable target in clinical practice of GC.

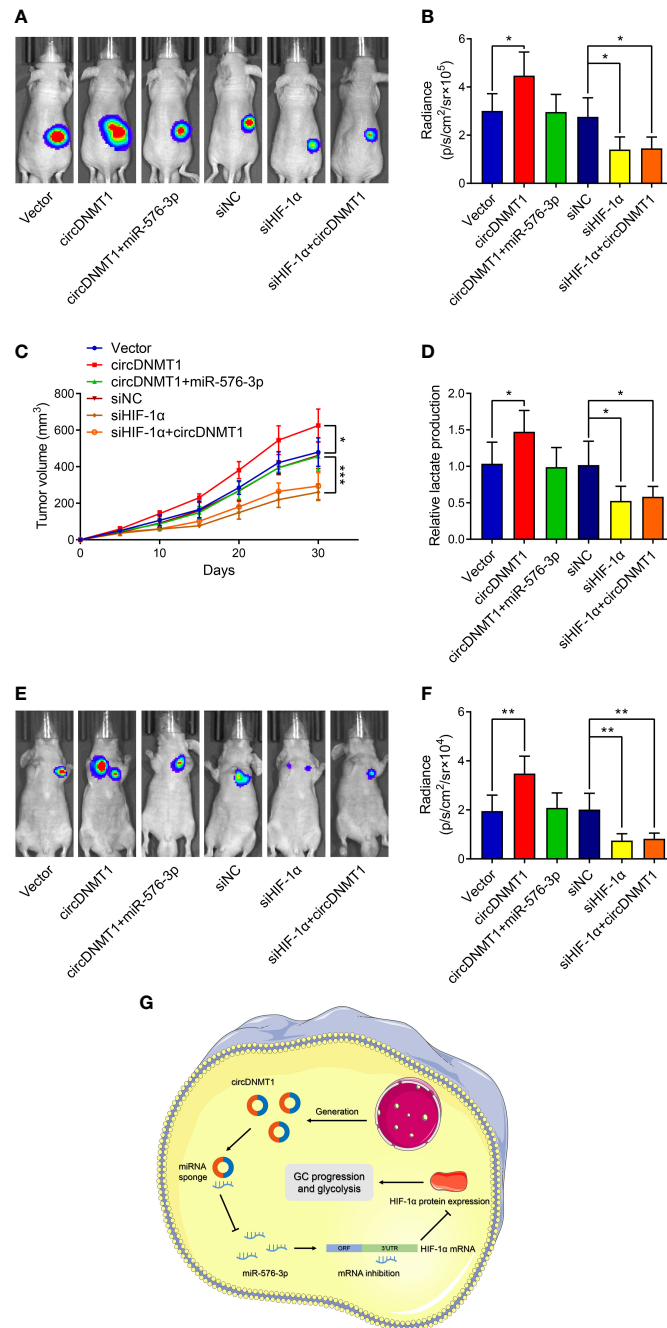
Aerobic glycolysis was firstly identified by Otto Warburg in the last century. Glycolysis can provide adequate energy and substance supply to fulfill the active state of many types of cancer cells. This metabolic reprogramming links to various malignant phenotypes, including rapid proliferation, chemoresistance formation and immune evasion (17, 29, 30). Enlightened by this evidence, researchers began to investigate the value of glycolysis inhibition in cancer therapy. As important regulators of cancer progression, some circRNAs have also been proved to affect glycolysis to modulate GC malignancy (9, 17, 18). We next speculated that circDNMT1 might facilitate cancer development by reprogramming glucose metabolisms. Downregulation of circDNMT1 expression attenuated glycolytic indicators. The ECAR reflects general glycolysis levels, while the OCR serves as an index of oxidative phosphorylation. Knockdown of circDNMT1 reduced the ECAR whereas enhanced the OCR. It suggested that GC cells rely more on oxidative phosphorylation to provide energy after the suppression of aerobic glycolysis. The data proved that circDNMT1 is a critical regulator of GC glycolysis both *in vitro* and *in vivo*, which might be a contributor of cancer development.

The role of circRNAs as miRNA sponges has received more attention from researchers compared to other circRNA mechanisms. The circRNA-miRNA regulatory network has been depicted by many studies (12, 13). Regarding the oncogenic role of circDNMT1, we aimed to investigate the downstream mechanisms of its functions. Combinational use of bioinformatic analysis and further experiments indicated high binding efficiencies between circDNMT1 and miR-576-3p based on the complementary sequences. There are significantly counteractive effects of circDNMT1 and miR-576-3p on GC malignant behaviors. Zuo et al. reported that miR-576-3p overexpression sensitizes ovarian cancer to cisplatin by reducing PD-L1 and cyclin D1 expression (31). It also





**FIGURE 6** | circDNMT1 promotes GC malignancy by targeting miR-576-3p/HIF-1 $\alpha$  axis. **(A)** The WB analysis to show HIF-1 $\alpha$  expression in HGC-27 and AGS cells transfected with NC shRNA or HIF-1 $\alpha$  siRNA, or carrying lentivirus with vector or HIF-1 $\alpha$  overexpression plasmids. **(B, C)** The CCK-8 assay to show proliferation of HGC-27 **(B)** and AGS **(C)** cells. They could be divided into two groups: i) The cells stably carrying lentivirus with vector or HIF-1 $\alpha$  overexpression plasmids and transfected with NC or miR-576-3p mimics; ii) The cells transfected with NC shRNA or HIF-1 $\alpha$  siRNA and carrying lentivirus with vector or circDNMT1 overexpression plasmids. **(D)** The EdU assay to show proliferation of cells as in **(B, C)**. Scale bar: 100  $\mu$ m. The histogram is displayed on the right. **(E)** The wound healing assay to show migration of cells as in **(B, C)**. The histogram is displayed on the right. **(F)** The transwell assay to show invasion of cells as in **(B, C)**. The histogram is displayed on the right. Scale bar: 100  $\mu$ m. Data were presented as means  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.



**FIGURE 7 |** circDNMT1/miR-576-3p/HIF-1 $\alpha$  axis regulated GC growth and metastasis *in vivo*. **(A)** Representative bioluminescence images at 30 days after subcutaneous injection of luc-HGC-27 cells. They could be divided into two groups: i) The cells stably carrying lentivirus with vector or HIF-1 $\alpha$  overexpression plasmids and transfected with NC or miR-576-3p mimics; ii) The cells transfected with NC shRNA or HIF-1 $\alpha$  siRNA and carrying lentivirus with vector or circDNMT1 overexpression plasmids. **(B)** Luminescence signals in **(A)** represented by overlaid false-color images with the signal intensity. **(C)** Curves of tumor volumes as in **(A)** at the indicated time. **(D)** Lactate acid production was determined in the tumor tissues from **(A)**. **(E)** Representative bioluminescence images at 30 days after tail vein injection of luc-HGC-27 cells as in **(A)**. **(F)** Luminescence signals in **(E)** represented by overlaid false-color images with the signal intensity. **(G)** Schematic illustration of the role and mechanisms of circDNMT1 in regulating GC progression. Data were presented as means  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

mitigates the migration and invasion capabilities of lung adenocarcinoma (32). The researches about the functions of miR-576-3p in GC were absent. Our findings reported the tumor suppressive role of miR-576-3p in GC, which is under rigorous regulation of circRNA networks.

HIF-1 $\alpha$  is a classical transcription factor that regulates the expression of many oncogenic factors and tumor suppressors. HIF-1 $\alpha$  activity is also under the control of complicated regulatory networks in cancer cells (33, 34). HIF-1 $\alpha$  promotes GC growth and metastasis by regulating miR-17-5p/PDCD4 axis (35). It also facilitate angiogenesis by upregulating VEGF-A expression and macrophage polarization (36). As a cell sensor of microenvironment, HIF-1 $\alpha$  modulates glycolytic activity to sustain cancer viability and progression (37, 38). To further clarify the downstream mechanisms of circDNMT1, we consulted the existing studies concerning miR-576-3p functions in cancer. Two studies reported that HIF-1 $\alpha$  expression is suppressed by miR-576-3p at the post-transcriptional level in glioma and hepatocellular carcinoma (21, 22). In addition, HIF-1 $\alpha$  overexpression contributes to glycolysis promotion of cancer (39). Therefore, we speculated that circDNMT1 potentiated GC progression by sponging miR-576-3p/HIF-1 $\alpha$  axis. A library of interaction experiments confirmed high binding efficiencies between the HIF-1 $\alpha$  mRNA 3'UTR and miR-576-3p. Overexpression of HIF-1 $\alpha$  mitigated its inhibitory effects on GC malignant behaviors. Moreover, the loss of circDNMT1/miR-576-3p function was observed after HIF-1 $\alpha$  knockdown. These findings demonstrated that HIF-1 $\alpha$  is a main target of the circDNMT1/miR-576-3p axis.

There are some limitations of our work. First, glycolysis is an important mediator of cancer development. In the present study, we only verified that circDNMT1 simultaneously enhanced GC malignancy and glycolysis. Its functions in glycolysis-mediated progression should be confirmed by further experiments. Second, HIF-1 $\alpha$  might serve as a critical downstream of circDNMT1, which does not mean HIF-1 $\alpha$  is the only target. More research should be performed to reveal the circRNA-miRNA networks in GC. Third, whether this axis regulates malignancy in other types of cancer remains to be explored.

Collectively, this study identified the oncogenic role of circDNMT1 in GC. circDNMT1 was overexpressed in GC and was correlated with the clinicopathological characteristics and poor prognosis of GC patients. It promoted GC proliferation,

migration, invasion and glycolysis by targeting the 576-3p/HIF-1 $\alpha$  axis both *in vitro* and *in vivo*. circDNMT1 might become a prognostic factor and therapeutic target in clinical practice of GC treatment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethical Committee of Animal Center of Chinese PLA General Hospital.

## AUTHOR CONTRIBUTIONS

BW designed the study and supervised the process of research. HL, BC, and RZ performed the experiments, collected data, and wrote the original draft. TL, XX, HC, HD, and JG analyzed and visualized the data. All authors contributed to this research and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2022.817192/full#supplementary-material>

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