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Review

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Functions and potential clinical applications of circular RNAs in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC), which accounts for 75%-80% of primary liver cancers, is recognized worldwide as a highly lethal cancer. Adjunctive diagnostic biomarkers for HCC are α-fetoprotein (AFP), induced by the absence of vitamin K or its antagonist (PIVKA-II), and AFP-L3. Using multiple biomarkers, or biomarkers in combination with other clinical tests, is recommended from the perspective of sensitivity and specificity; however, this approach does not lend itself to early detection. Therefore, novel biomarkers are needed urgently. Non-coding RNAs, mainly circular RNAs (circRNAs), are key players in the progression and suppression of diseases, including cancer. Unlike linear RNAs, circRNAs are cyclic RNAs generated by 3′ to 5′ back-splicing. Advancements in RNA sequencing technology and bioinformatic pipelines mean that numerous circRNAs have been predicted and identified. These RNA molecules play a role in the progression/suppression of cancer through direct or indirect epigenetic regulation via microRNAs and proteins in cancer cells, as well as in the surrounding tumor microenvironment. Some circRNAs are tissue- or cell line-specific and are detected in exosomes, blood, and saliva. Importantly, circRNAs are more stable than linear cognate RNAs *in vivo*. This unique characteristic positions circRNAs as versatile materials with potential for use as biomarkers, in clinical tests, and as therapeutic applications. In the context of HCC, numerous circRNAs have been identified. Here, we describe the functions of circRNAs in HCC, the experimental methods in which they can be used, and their potential clinical applications.

Keywords: Non-coding RNA, circular RNA, biomarker, experiment

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent histological type of primary liver cancer worldwide, accounting for approximately 75%-80% of cases. It is widely recognized as having a dismal prognosis, regardless of geographical location[1]. Consequently, early detection and treatment are important[2].

α-fetoprotein (AFP), a protein induced by vitamin K absence or antagonist-II (PIVKA-II), and AFP-L3 are used as supplementary diagnostic biomarkers in clinical practice. Nevertheless, neither of these biomarkers exhibits both high sensitivity and specificity, making them unsuitable as definitive diagnostic tools. Although the sensitivity of these biomarkers increases when they are used in combination with abdominal ultrasound, computed tomography, or magnetic resonance imaging[3], we still have no effective biomarker that enables definitive diagnosis. Furthermore, HCC often becomes resistant to standard molecular-targeted therapies such as sorafenib and lenvatinib, limiting their long-term efficacy[4]. Therefore, the identification of reliable HCC biomarkers, as well as the development of alternative novel therapeutic strategies, are urgent issues to be addressed.

As a possible answer to this problem, circular RNAs (circRNAs), a type of non-coding RNA (ncRNA), have attracted much attention. A recent study showed that < 3% of the human genome comprises exons, which are the regions that encode genetic information; the remaining (approximately 97%) gene sequences are transcribed into ncRNAs, including microRNAs (miRNAs), long ncRNAs (lncRNAs), and circRNAs[5]. While ncRNAs do not encode proteins, they are involved in phenomena such as DNA replication, RNA splicing, translation, and epigenetic regulation. Those shorter than 200 nucleotides, e.g., miRNAs, are categorized as short ncRNAs. By contrast, those longer than 200 nucleotides are classified as lncRNAs[6]. The length of circRNAs can range from < 100 nucleotides (nt) to > 10,000 nt[7].

CircRNAs were first discovered on electron micrographs of plant viroids in 1976[8]. In contrast to linear mRNAs, which typically splice from the 5' to 3' terminus, circRNAs are generated by back-splicing, in which a downstream splice donor (5' splice site) is joined to an upstream splice acceptor (3' splice site), leading to covalent circularization[9]. Although some circRNAs encode proteins or peptides, they are classified as ncRNAs[10]. Back-splicing is significantly less efficient than canonical splicing[11], resulting in substantially lower expression of circRNAs compared with their linear RNA counterparts[12,13].

CircRNAs were also identified in eukaryotes by electron microscopic observation[14], and human circRNAs were first found in 1991[15]. The development of RNA-seq and bioinformatic pipelines has enabled the prediction or identification of various circRNAs not only in humans but also in mice, rats, and nematodes[16].

The stability of circRNAs is superior to that of linear RNAs. CircRNAs are stable *in vivo*, with a half-life 2.5-5 times longer than that of linear mRNA and miRNA[13,17]. Lipid nanoparticle (LNP)-encapsulated circRNAs can be stocked without cryopreservation[18,19]. Some circRNAs generated in the nucleus migrate to the cytoplasm, where they act as sponges that adsorb (bind in a complementary manner) miRNAs and proteins; these circRNAs directly or indirectly regulate transcription via transcription factors and ribosomes, and serve as templates for translation. Additionally, circRNAs play roles in carcinogenesis and cancer suppression through these mechanisms.

Increasingly, circRNAs have been identified in various biological fluids, including plasma[20,21], urine[22], saliva[23], and cerebrospinal fluid (CSF)[24]. Some circRNAs are even secreted via exosomes (exosomal circRNAs) and then transmitted intercellularly[25]. These unique properties enable circRNAs to attract significant attention as biomarkers and therapeutic targets.

HCC is preceded by cirrhosis, which can be divided largely into viral and non-viral causes. The process leading to cirrhosis is thought to differ depending on the cause, and hepatic circRNAs and miRNAs are thought to be involved in these differences. In short, the elucidation of comprehensive networks, including hepatic circRNAs, is crucial for the development of clinical approaches to HCC.

This review focuses on the role of circRNAs in HCC, their diagnostic/therapeutic potential, and challenges to clinical application. Because circRNAs have a relatively short research history, we also discuss their characteristics, functions, and experimental techniques. The review provides an overview of research on circRNAs in the context of HCC and aims to offer new insights that may facilitate future clinical applications.

MAIN TEXT

Categories and biogenesis of circRNAs

CircRNAs are classified into distinct categories based on the type of RNA and the specific sequence regions from which they originate. They include exon circRNAs (EcircRNAs), circular intronic RNAs (ciRNAs), and exon-intron circRNAs (EIciRNAs). EcircRNAs are found primarily in the cytoplasm and represent the most common class. In contrast, ciRNAs and EIciRNAs are localized predominantly in the nucleus[26].

Several models of circRNA biogenesis have been proposed, including intron pairing-driven circularization, RNA-binding protein (RBP)-driven circularization, and alternative back-splicing[27] [Figure 1]. In intron pairing-driven circularization, intronic complementary sequences (ICSs) such as Alu elements, which are located in the flanking regions of the exon that forms the core of the circRNA, pair with each other to form a loop[28]. During RBP-driven circularization, RBPs bind to ICSs in the flanking regions and bring these regions into close contact, thereby facilitating loop formation and circRNA generation[29]. Known RBPs involved in back-splicing are Quaking, heterogeneous nuclear ribonucleoprotein L, Fused in Sarcoma, and muscleblind/muscleblind-like splicing regulator 1[30-33].

A mechanism that regulates circRNA expression in a context specific to HCC has also been identified. The androgen receptor (AR) upregulates the p110 isoform of adenosine deaminase, leading to broad suppression of circRNA expression[34].

The roles of circRNAs

As mentioned above, circRNAs are thought to be involved in cell proliferation, metastasis, apoptosis, aging, and drug resistance through various mechanisms. Next, we describe the functions of representative circRNAs in each of these contexts [Figure 2].

Regulation of gene transcription

Some nuclear EcircRNAs are capable of interacting with promoters to regulate transcription. For example, circACTN4 activates transcription of intrahepatic cholangiocarcinoma cells by recruiting transcriptional regulatory proteins to promoter regions[35]. Additionally, ciRNAs such as ci-ankrd52 and certain EIciRNAs bind to RNA polymerase II in the nucleus of human cells to modulate transcription[36,37]. Moreover, gene expression is functionally regulated by competition between back-splicing and linear splicing. For instance, circMBL competes with the splicing of pre-MBL mRNA, thereby inhibiting canonical splicing[33].

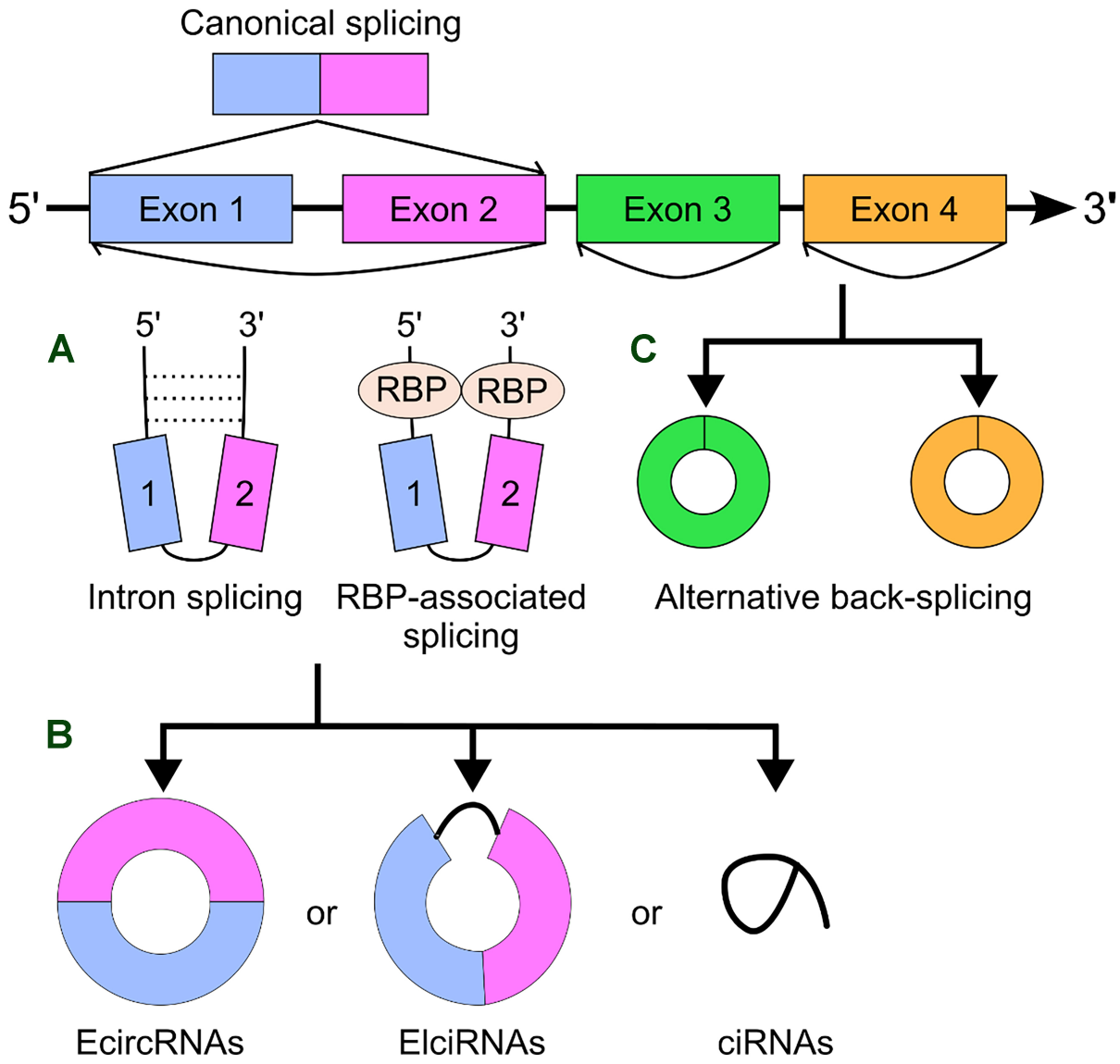

Figure 1. Categories and biogenesis of circRNAs. (A) Intron pairing-driven circularization and RBP-driven circularization; (B) After back-splicing, EcircRNAs or ElciRNAs are generated; (C) Alternative back-splicing. EcircRNAs: Exonic circRNA; ElciRNAs: exonic intronic circRNAs; RBP: RNA-binding protein; 1: exon 1; 2: exon 2.

miRNA sponges

miRNAs regulate gene expression epigenetically. However, they can also be regulated by other types of RNA that have sequences complementary to miRNAs; binding to these sequences means that miRNAs compete to regulate mRNA expression. In general, RNAs that competitively regulate the dynamics of their targets are referred to collectively as competing endogenous RNAs (ceRNAs), whereas RNAs that target miRNAs specifically are called "miRNA sponges"[38].

The best-known miRNA sponge within circRNAs is cerebellar degeneration-related protein 1 antisense RNA (CDR1as, also known as ciRS-7), which is associated with neurogenesis and neurological diseases. CDR1as contains 73 binding sites for miR-7. The sequence of the binding sites has a certain degree of variability, which prevents dicer cleavage and allows CDR1 to absorb miRNA-7 (miR-7)[13].

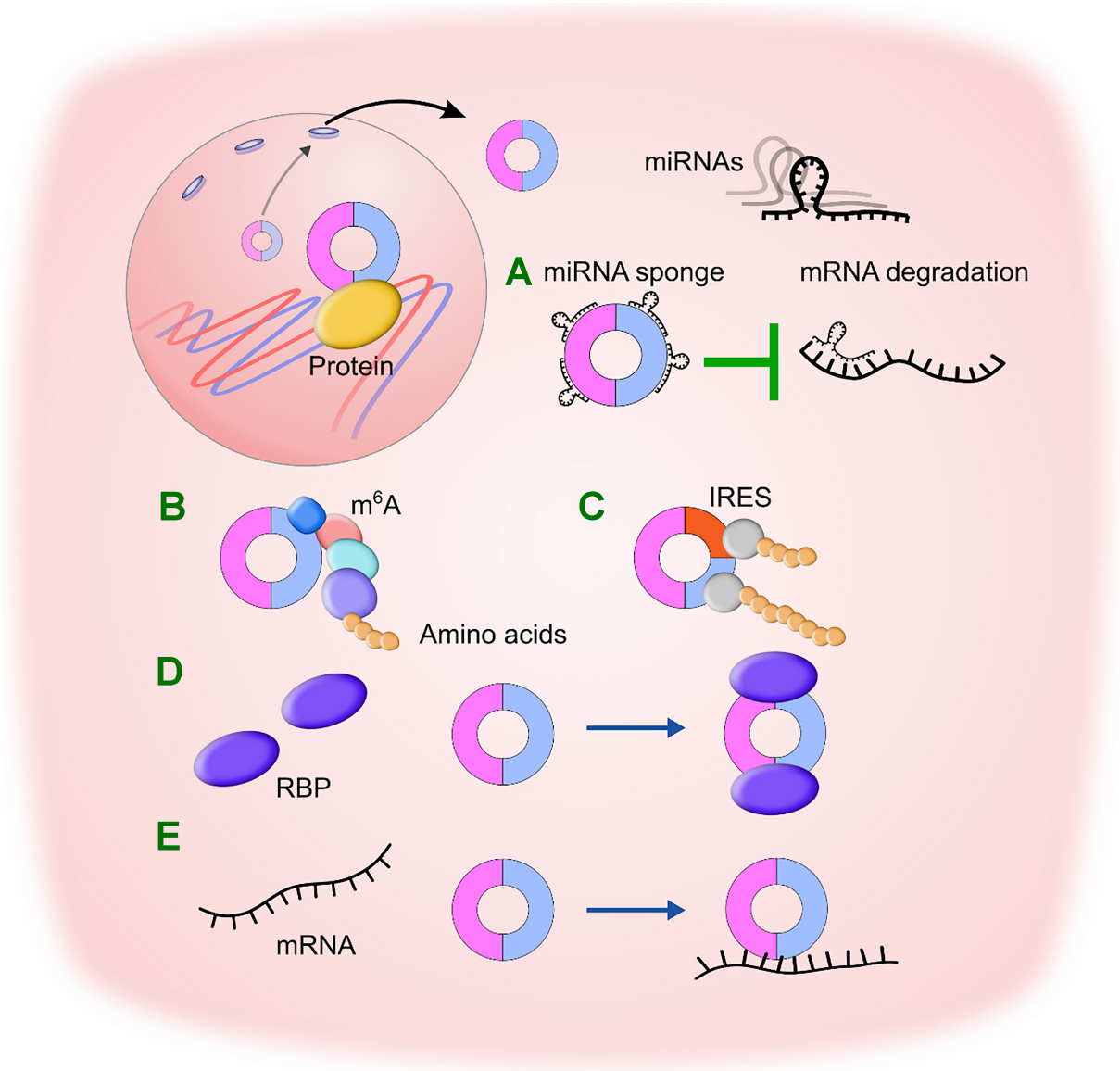

Figure 2. The functions of circRNAs. (A) Nuclear-localized EcircRNAs can interact with promoters to regulate transcription by recruiting transcriptional regulatory proteins to promoter regions; (B) miRNA sponge. CircRNAs with multiple miRNA binding sites can act as competing endogenous RNAs, thereby modulating the accessibility of miRNAs on mRNAs; (C) Translatable circRNAs. Cytoplasmic circRNAs with m^6A sites or IRESs are potentially translated through different mechanisms; (D) Protein sponge. Some circRNAs bind to proteins such as ribonucleoproteins and RBPs and are involved in epigenetic regulation; (E) Direct interaction with mRNAs. RBP: RNA-binding protein; m^6A: N^6-methyladenosine modification; IRESs: internal ribosome entry sites.

Recent reports show that circular RNA ACVR2A and circSNX6 induce the progression of HCC via miR-511-5p and miR-383-5p, respectively[39,40].

Many circRNAs have been identified as miRNA sponges in the context of HCC, including circRNA mitochondrial tRNA translation optimization 1 (circMTO1) and circSWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily A member 5 (cSMARCA5), both of which play similar roles as tumor suppressors [Table 1 and Figure 3].

Table 1. circRNAs that work as miRNA sponges

circRNAs	Up/Down regulation in HCC	Functions in HCC	Pathway	Reference
circular RNA ACVR2A	Up	Proliferation (+), invasion (+), migration (+), apoptosis (-)	miR-511-5p/PI3K-Akt signaling pathway	[39]
circSNX6	Up	Proliferation (+), invasion (+), apoptosis (-)	miR-383-5p/VEGFA signaling pathway	[40]
circ-β-catenin	Up	Proliferation (+), invasion (+)	β-catenin370aa/Wnt/β-catenin signaling pathway	[41]
circDCUN1D4	Down	Proliferation (-), migration (-), invasion (-), apoptosis (+)	miR-590-5p/TIMP3	[42]
circRNA-SORE	Up	Chemotherapy resistance	miR-103a-2-5p/miR-660-3p/Wnt/β-catenin signaling pathway	[43]
circRNA-100338	Up	Proliferation (+), metastasis (+), invasion (+), angiogenesis (+)	miR-141-3p/RHEB/PAM signaling pathway	[44,45]
cSMARCA5	Down	Proliferation (+), invasion (+), metastasis (+)	miR-17-3p and miR-181b-5p/TIMP3	[46]
circRHOT1	Up	Proliferation (+), migration (+), invasion (+), apoptosis (-)	TIP60-dependent NR2F6	[47]
circMET	Up	Invasion (+), metastasis (+), EMT (+), immunosuppression (+)	miR-30-5p/snail/DPP4	[48]
circRNA-0015004	Up	Proliferation (+)	YY1/circRNA-0015004/miR-330-3p/RCC2	[49]
circHIPK3	Up	Proliferation (+), migration (+)	miR-124/AQP3	[50]
circ-CDYL	Up	Proliferation (+)	miR-892a/HDGF/PAM signaling or Wnt/β-catenin signaling pathway miR-328-3p/HIFIAN/Notch signaling pathway	[51]
circASAP1	Up	Proliferation (+), migration (+), invasion (+)	miR-326/miR-532-5p-MAPK1/CSF-1	[52]
circ_0091579	Up	Migration (+)	miR-136-5p/TRIM27 miRNA-490-3p miR-1225-5p/PLCB1 miR-1287/PDK2 miR-1270/YAP1	
circ_0005394	Up	Proliferation (+), migration (+), EMT (+)	miR-507/E2F3 miR-515-5p/CXCL6	[53]
circZKSCAN1	Down	Proliferation (+), metastasis (+), invasion (+)	Qki5/circZKSCAN1/FMRP/CCAR1/Wnt/β-catenin signaling pathway	[54]
circTRIM33-12	Down	Proliferation (+), migration (+), invasion (-), immune evasion abilities (-)	miR-191/TET1	[55]
circMTO1	Down	Proliferation (-), migration (-), apoptosis (+)	miR-9/p21 miR-9-5p/NOX4 miR-541-5p/ZIC1/Wnt/β-catenin signaling pathway	[56-58]
circC3P1	Down	Proliferation (+), migration (+), invasion (-)	miR-4641/PCK1	[59]
circADAMTS13	Down	Proliferation (+), migration (+), invasion (-)	miR-484	[60]

(+): Gain/progress; (-): reduce/inhibit. EMT: Epithelial to mesenchymal transition; PAM: phosphatidylinositol 3-kinase/after kidney transplantation/mTOR.

Translatable circRNAs

Experiments using recombinant circRNA vectors and protein analysis techniques such as mass spectrometry, polysome fractionation assays, and dual-luciferase reporter systems show that circRNAs can be translated. Most nuclear-localized circRNAs are not translated, whereas cytoplasmic-localized circRNAs with an internal ribosome entry site (IRES) or N^6-methyladenosine (m^6A) modification sites are potentially translated through different mechanisms.

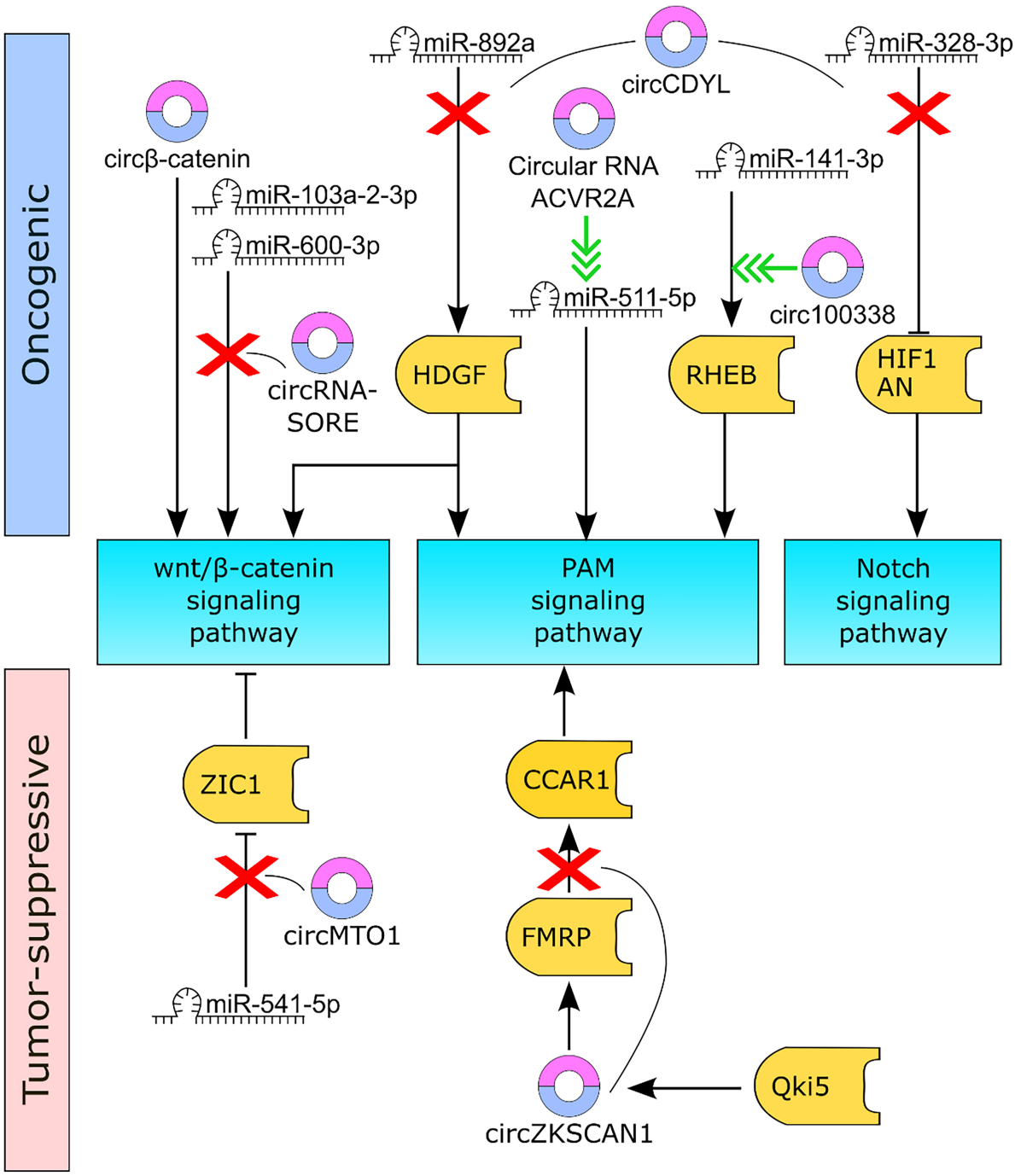

Figure 3. The relationship between circRNAs and signaling pathways. The shapes at the top represent oncogenic elements, while those at the bottom indicate tumor-suppressive elements. Pink and blue circles represent circRNAs, while orange shapes denote proteins. Linear shapes indicate miRNAs, and blue squares represent major pathways. X indicates pathway inhibition. A triple arrowhead signifies pathway activation. An arrow with a T-shaped tip represents downregulation, whereas a normal arrow represents upregulation.

Translation of circRNA is cap-independent and inefficient compared with cap-dependent translation under steady conditions; however, it becomes more efficient under stress conditions such as starvation or heat shock.

Li *et al.* constructed a plasmid containing a m^{6}A-modified start codon and a Flag tag coding sequence immediately upstream of the stop codon, in addition to exon 3 to exon 2 of Rho GTPase-activating protein 35 (p-circARHGAP35-Flag), which conferred high expression efficiency. The plasmid was transfected into HEK-293T cells, and Flag-tagged proteins were detected by western blotting, suggesting that circARHGAP35 encodes a protein in a m^{6}A-dependent manner. Using p-circARHGAP35-Flag-transfected cancer cells, they conducted a colony formation assay, a CCK-8 proliferation assay, and Transwell migration and invasion assays. Overexpression of circARHGAP35 protein increased the migratory and invasive capabilities of cancer cell lines. Unlike normal ARHGAP35, which is localized in the cytoplasm, the circARHGAP35 protein lacks the Rho GTPase-activating protein-binding domain. Immunofluorescence assays showed that the circARHGAP35 protein was found mainly in the nucleus, suggesting that p-circARHGAP35 migrates from the cytoplasm to the nucleus. They then performed a RhoA activation assay, and revealed that the circARHGAP35 protein had no significant effect on RhoA activity, whereas ectopic expression of the ARHGAP35 protein suppressed RhoA activation. They confirmed the interaction between transcription factor II-I (TFII-I) and the circARHGAP35 protein by performing co-immunoprecipitation assays using anti-Flag or anti-TFII-I antibodies. Furthermore, they conducted a screening using small interfering RNAs (siRNAs) to individually knock down 63 RBPs known to participate in RNA splicing, and confirmed that silencing of HNRNPL resulted in downregulation of circARHGAP35 but not linear ARHGAP35. They also observed that HNRNPL was upregulated significantly in HCC compared with matched non-tumor liver tissues. These results suggest that in HCC, circARHGAP35, derived from the locus of the tumor suppressor gene *ARHGAP35*, is regulated by HNRNPL, and that the circARHGAP35 protein exerts its oncogenic role by interacting with TFII-I while ARHGAP35 suppresses cancer cell motility by decreasing RhoA activity[61].

Other studies show that circZKSaa, a translation product of circ zinc fingers harboring KRAB and SCAN domains 1 (circZKSCAN1), promotes interaction between mechanistic target of rapamycin kinase (mTOR) and F-box and WD repeat domain-containing 7 (FBXW7), and promotes ubiquitylation of mTOR, thereby exerting tumor-suppressive functions via the phosphatidylinositol 3-kinase/after kidney transplantation/mTOR (PAM) pathway[62].

Circ β-catenin is also translated. Circ β-catenin shares a start codon with linear *β-catenin* mRNA, and translation terminates at a new stop codon created by cyclization. Translation yields a novel β-catenin isoform of 370 amino acids, which antagonizes phosphorylation and degradation of β-catenin induced by glycogen synthase kinase 3 β (GSK3β), stabilizes full-length β-catenin, activates the Wingless (Wnt) pathway, and promotes HCC cell growth, invasion, and metastasis[41].

Protein sponges

In addition to miRNA sponges, circRNAs function as protein sponges that adsorb proteins. In HCC, several circRNAs bind to proteins such as RNPs and RBPs, and are involved in epigenetic regulation.

Zhu *et al.* conducted RNA immunoprecipitation (RIP)-seq and siRNA experiments on circZKSCAN1, and demonstrated that circZKSCAN1 acts as a decoy for FMRP interacting protein 2 (FMRP), an RBP. They found that circZKSCAN1 deficiency had no effect on the expression of FMRP, but significantly increased the expression of cell division cycle and apoptosis regulator 1 (CCAR1), which interacts with FMRP. Additionally, they performed fluorescence *in situ* hybridization (FISH) at the subcellular level, and confirmed the competitive binding of circZKSCAN1 or *CCAR1* mRNA to the FMRP protein. Moreover, they used a luciferase reporter gene assay to assess the transcriptional activity of β-catenin downstream targets in CCAR1-knockdown cells, and found that the transcriptional activity of Wnt signaling was

restored. These results suggest that circZKSCAN1 suppresses HCC progression via the Wnt signaling pathway[54].

Circ vesicle-associated membrane protein 3 (VAMP3), back-spliced from exon 3 to exon 4, interacts with cell cycle-associated protein 1 and Ras GTPase-activating protein-binding protein 1 in stress granules, thereby promoting their formation. It also inhibits the translation of the proto-oncogene protein c-Myc, thereby suppressing HCC progression[63].

Circular RNA upregulated in sorafenib-resistant HCC cells (circRNA-SORE) binds competitively to tumorigenic protein Y-box binding protein 1 (YBX1) in the cytoplasm, and prevents YBX1 from translocating to the nucleus[43] [Figure 4].

Circ Ras homolog family member T1 (circRHOT1) recruits the 60 kDa Tat-interactive protein to nuclear receptor subfamily 2 group F member 6 (NR2F6), an oncogenic RNP, to the promoter region and initiates transcription of NR2F6. These reactions promote HCC growth and metastasis[47].

Direct interactions with mRNAs

CircZNF609, which drives the progression of HCC, is also the first molecule suggested to interact directly with mRNA in rhabdomyosarcoma[64]. The back-splicing junctions (BSJ) of circZNF609 bind directly to mRNA encoding cytoskeleton-associated protein 5 (CKAP5), and facilitate binding of Hu-antigen R (HuR, also known as ELAV-like protein 1), thereby stabilizing *CKAP5* mRNA and increasing its translation. CKAP5 is a microtubule polymerization factor essential for mitosis; therefore, these findings suggest that the circZNF609/CKAP5 axis plays a vital role in cell cycle progression[65].

Roles in HCC development and prognosis

Classical HCC progresses from chronic hepatitis and hepatic sclerosis. Progression to HCC is often dependent on the tumor microenvironment (TME), which consists of tumor cells, immune cells, stromal cells, endothelial cells, cancer-related fibroblasts, and extracellular matrix (ECM)[66]. CircRNAs regulate signaling pathways via the epigenetic mechanisms described above. In other words, circRNAs are involved in the various developmental stages of HCC: liver sclerosis, cell proliferation, migration/invasion, and angiogenesis. Here, we explain how circRNAs are involved at each step.

Cell proliferation

Uncontrolled cell growth is one of the characteristics of cancer. Numerous circRNAs are involved in cell proliferation, and some regulate typical cell growth signaling pathways.

miR-9 is an oncogenic miRNA that downregulates tumor suppressor genes *p21* and *NADPH oxidase 4*[56-58], thereby promoting proliferation, migration, and invasion. By using a biotin-labeled circMTO1 probe to perform RNA *in vivo* precipitation in HCC cells, Han *et al.* identified miR-9 as a circMTO1-associated miRNA. Furthermore, they showed that knocking down circMTO1 in HCC, which downregulates p21 (the target of oncogenic miR-9), promoted the proliferation and invasion of HCC cells. This suggests that circMTO1 suppresses HCC progression by acting as a sponge for miR-9 to eliminate the oncogenic effects of miR-9 through the circMTO1/miR-9/p21 axis. Furthermore, intratumoral administration of cholesterol-conjugated siRNA targeting circMTO1 promoted tumor growth in HCC-bearing mice[56].

Another example is cSMARCA5, the back-splicing of which is inhibited by DExH-box Helicase 9 (DHX9). cSMARCA5 downregulates the tumor suppressor TIMP metallopeptidase inhibitor 3 (TIMP3) via miR-17-3p and miR-181b-5p, thereby driving cell proliferation, invasion, and metastasis[46].

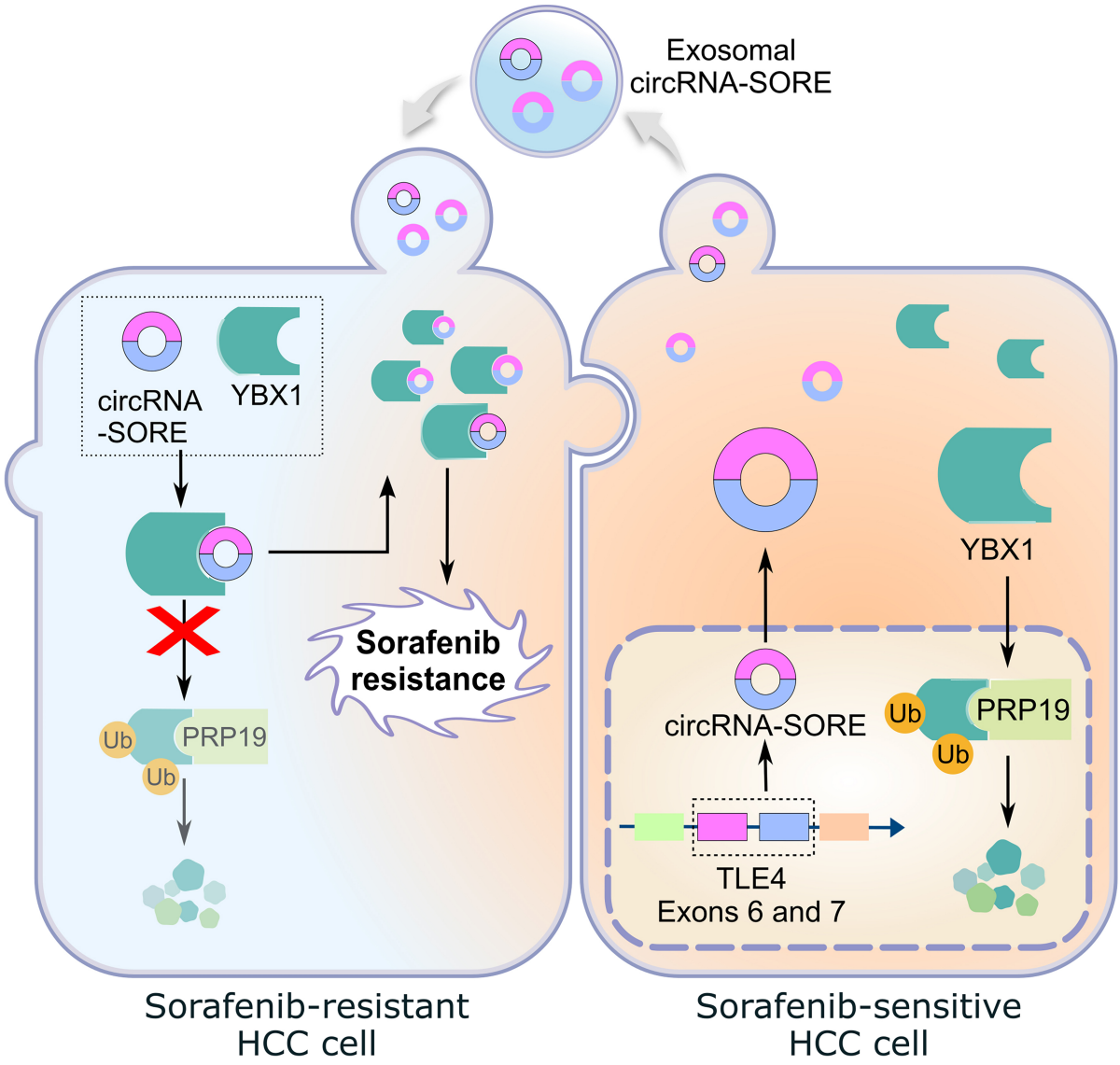

Figure 4. CircRNA-SORE, one of the well-studied circRNAs, is transmitted via exosomes. Dotted squares indicate nuclei. Green shapes represent YBX1, while the light green shape denotes PRP19. Solid squares indicate exons. In sorafenib-sensitive HCC cells (left), YBX1 translocates from the cytoplasm to the nucleus, where it is ubiquitinated and degraded. CircRNA-SORE is generated from Exons 6 and 7 of TLE4 and is secreted via exosomes. In sorafenib-resistant HCC cells (right), transmitted circRNA-SORE binds to free YBX1 in the cytoplasm. This complex then activates various signaling pathways and confers sorafenib resistance. HCC: Hepatocellular carcinoma.

Finally, circ zinc finger protein 609 (circZNF609) increases cell proliferation, metastasis, and stemness by adsorbing miR-15a-5p/15b-5p, increasing expression of GLI family zinc finger 2, and activating the hedgehog pathway[67].

Epithelial-mesenchymal transition/migration/invasion

Epithelial-mesenchymal transition (EMT), the morphological-functional cellular change from epithelial to mesenchymal cells, promotes migration and invasion. Thus, EMT is a crucial component of the mechanism that drives HCC progression and metastasis.

Huang *et al.* reported that circMET upregulates EMT-related genes and promotes migration and invasion of HCC. They created mutant HepG2 cells that overexpressed circMET. Unlike normal epidermal cells, these mutant cells had a spindle shape and gained the ability to migrate and invade. Moreover, they analyzed RNA-seq datasets from the mutant cells and proved that they had an EMT-specific gene profile, i.e., reduced expression of E-cadherin and increased expression of N-cadherin and Vimentin[48].

Angiogenesis

HCC is a highly vascularized tumor because it receives blood exclusively from the hepatic artery[68]. Angiogenesis increases metastasis, leading to a fatal prognosis[69]; therefore, elucidating the mechanism underlying angiogenesis in HCC is crucial for developing new treatments and clinical tests.

CircRNAs-100338 is an exosomal circRNA. Huang *et al.* reported higher expression of circRNAs-100338 in exosomes from aggressive metastatic HCC cell lines than in those from HCC cell lines with lower metastatic potential. They also used exosomes extracted from HCC cells with either overexpression or knockdown of circRNA-100338 to show that circRNA-100338 triggered the proliferation of human umbilical vein endothelial cells, as well as angiogenesis, increased vascular permeability, and vasculogenic mimicry. These findings suggest that circRNA-100338 is transmitted via exosomes, and that it promotes angiogenesis in HCC tumors[45].

Apoptosis

Li *et al.* showed that circMTO1, which acts as a tumor suppressor by sponging miR-541-5p, is downregulated in HCC. miR-541-5p, in turn, suppresses a repressor Zic member family 1 (ZIC1), a component of the Wnt/β-catenin pathway, thereby driving HCC progression. Through rescue experiments and flowcytometric analysis in HepG2 cells with circMTO1 knockdown, the authors showed that circMTO1 promotes apoptosis. Therefore, the downregulation of circMTO1 inhibits apoptosis[58].

CircRHOT1 also appears to regulate apoptosis. Flowcytometric analysis revealed that knockdown of either circRHOT1 or NR2F6 promoted apoptosis. Since circRHOT1 knockdown reduced the expression of NR2F6 and genes involved in Notch2 signaling, it is suggested that circRHOT1 promotes apoptosis by upregulating NR2F6 and activating the Notch2 signaling pathway[47].

TME

The TME plays a central role in forming tumor bulk, which mainly comprises desmoplastic stroma composed of cancer-associated fibroblasts (CAFs), collagens, and hyaluronic acid. Desmoplastic stroma is a key factor driving malignancy (i.e., recurrence, metastasis, chemotherapy resistance, and immune escape). Recent studies identified crosstalk between circRNAs and the TME (immune cells and the ECM in particular), which regulates tumorigenesis and the development of HCC. Understanding this crosstalk is essential to prevent tumor progression and immune evasion[70].

Repressive immune cells

CircRNAs regulate both tumor-antagonizing immune cells and tumor-promoting immune cells. Cytotoxic T lymphocytes ($CD8^+$ T cells) play a critical role in immune defense against tumors[71]. Exosomal hsa_circ_0000240 (also called circCCAR1) derived from HCC cells is transmitted and taken into $CD8^+$ T cells, after which circCCAR1 binds to the PD-1 protein directly to stabilize it, resulting in "T cell exhaustion", a major characteristic of impaired $CD8^+$ T cell function[72].

Similarly, the cytotoxicity of natural killer (NK) cells in the TME of HCC patients is impaired. Exosomal circUHRF1 secreted from HCC cells is delivered to NK cells, where it induces NK cell exhaustion, upregulating expression of T cell immunoglobulin and mucin domain 3 by sponging miR-449c-5p[73].

Regulatory T cells (Tregs) secrete immunosuppressive factors and impair the function of $CD8^+$ T cells, leading to tumor immune escape[74]. Exosomal circGSE1 derived from HCC cells is transmitted to $CD4^+$ T cells, where it works as a sponge for miR-324-5p, thereby activating the TGF-β pathway and inducing expansion of Tregs[75].

Progressive immune cells

CAFs, the primary source of inflammatory factors and chemokines in the TME, secrete more of the chemotactic cytokine CXCL11 than normal fibroblasts to promote metastasis of HCC[76]. CXCL11 upregulates circUBAP2 in HCC cells and promotes expression of the antiviral proteins interferon-induced protein with tetratricopeptide repeats (IFIT) 1/3. Moreover, upregulation of IFIT1/3 upregulates IL-17 and IL-1β, which drives metastasis and invasion of HCC cells[77].

Tumor-associated macrophages are the most common immune cells in the TME of various cancers that secrete inflammatory cytokines to induce tumor migration and invasion[78,79]. Depending on environmental cues, macrophages are polarized to a classically activated (M1) phenotype that has cancer-suppressive functions; alternatively, they can polarize to an activated (M2) phenotype that has anti-inflammatory and cancer-promoting functions[80]. Wang *et al.* revealed that HCC-derived exosomal hsa_circ_0074854 is transported into macrophages, and promotes polarization to an M2 phenotype, which promotes migration and invasion of HCC cells[81].

ECM

As another example, circRNAs modulate cell-cell communication by regulating the release of signals from the ECM. Exosomal circTMEM181 works as a sponge for miR-488-3p and induces the expression of CD39 by macrophages. CD39 binds to extracellular ATP (eATP) and hydrolyzes it to AMP. AMP is hydrolyzed to adenosine by CD73 expressed on HCC cells. Adenosine then impairs $CD8^+$ T cell function and causes resistance to anti-PD1 therapy[69].

These findings suggest that exosomal circRNAs play a crucial role in the progression of HCC by facilitating immune escape. In other words, oncogenic exosomal circRNAs may be a potential therapeutic target.

Therapy resistance

Systemic chemotherapy is the recommended treatment for advanced HCC. Sorafenib, Lenvatinib, and Atezolizumab plus Bevacizumab are used as first-line agents and demonstrate excellent efficacy[82,83]. However, HCC usually acquires resistance to these molecularly targeted drugs within 6 months[84]. Accordingly, elucidating the factors that enable HCC to become drug-resistant is crucial to improving patient survival rates and prognoses.

It is thought that exosomes are responsible for spreading characteristics associated with resistance to therapeutic agents throughout the TME[45]. In addition, various exosomal circRNAs facilitate immune escape and resistance to immune checkpoint inhibitors (ICI) by stabilizing or upregulating PD-1 or PD-L1. In other cases, intracellular circRNAs play a role in the development of resistance to molecularly targeted drugs.

CircRNA-SORE (hsa-circ-0000437) traps YBX1 and inhibits its transit to the nucleus, where it is degraded via ubiquitination by pre-mRNA processing factor 19 (PRP19). Failure to inactivate YBX1 leads to resistance to sorafenib. Moreover, circRNA-SORE is transported by exosomes to other sorafenib-sensitive HCC cells, resulting in the dissemination of sorafenib resistance[43] [Figure 4].

Oncogenic circRNAs represent promising therapeutic targets; however, exosomal circRNAs are taken up by tumor cells and immune cells, in which they regulate the expression of PD-1 and PD-L1. Exosomal circRNAs such as circCCAR1 and circRNA-SORE could be effective targets in cases of HCC that are resistant to ICI and molecularly targeted drugs.

cSMARCA5 is thought to be associated with resistance to radiation therapy because it is expressed at significantly higher levels in the tumor tissues of HCC patients who are resistant to radiation therapy than in normal adjacent tissues[85], but the underlying mechanism is unknown.

Multiple studies consistently show that circRNAs such as circMTO1, circZKSCAN1, circSMARCA5, and circRNA-SORE exert similar functions in HCC, underscoring their high degree of robustness.

In contrast, these circRNAs also exhibit similar or distinct functions in other cancers and diseases. For example, circMTO1 acts as a suppressor in viral liver fibrosis[58], polycystic ovary syndrome (PCOS)[86], glioblastoma[87], and osteosarcoma[88], functioning through different miRNA pathways. However, circMTO1 also shows oncogenic effects in gallbladder cancer and cervical cancer by sequestering miR-219a-5p[89] and miR-6893[90], respectively. Similarly, circZKSCAN1, in contrast to its tumor-suppressive role in HCC, promotes the progression of lung adenocarcinoma via the miR-185-5p/transgelin 2 axis[91].

Although the mechanisms by which circMTO1 and circZKSCAN1 switch functions remain unclear, the interaction between these circRNAs and different disease-specific miRNAs is thought to determine their properties. CircRNAs that act as miRNA sponges can bind to multiple types of miRNAs because they tolerate a certain degree of mismatching of their complementary strands[92]. MiRNAs and transcription factors exhibit distinct expression profiles across different tissues, which may result in the binding of different miRNAs and, consequently, differences in function.

Given that the expression of some circRNAs differs in HCC cells with differing malignant potential[93], it is suggested that circRNA profiles vary at the cellar level during tumor development. Unfortunately, current knowledge remains insufficient at each developmental stage, meaning that further research is required.

Abnormal regulation of circRNAs can trigger tumorigenesis and tumor progression, but it is not yet clear how this abnormal regulation occurs. Some circRNAs can be upregulated by IL-1β, TNF-α, and IFN-γ[94]. Since chronic inflammation is a background factor in HCC, it is possible that this abnormality is caused by exogenous factors such as exposure to cytokines and growth factors (either long-term or at high levels). Since a single circRNA is involved in regulating multiple signaling pathways, and the circRNA profile changes over time, the reported signaling pathways are likely to form more complex networks. It is also possible that circRNAs mediate crosstalk between pathways. We look forward to new data that may shed light on these issues.

Clinical applications of circRNAs in HCC

As previously discussed, circRNAs exhibit notable tissue specificity and have been detected in various body fluids, suggesting their potential as promising biomarkers and therapeutic agents. In HCC, preliminary

efforts toward clinical application have been initiated. Representative examples of these applications are introduced below. A summary of key clinical highlights is presented in Table 2.

Diagnostic potential of circRNAs

The exploration of circRNAs as diagnostic biomarkers for HCC has attracted significant attention in recent years. Although the field remains in its early stages, several meta-analyses have emphasized the diagnostic value of circRNAs.

Yuan *et al.* conducted a comprehensive meta-analysis of exosomal circRNAs across six types of cancer (i.e., HCC, lung cancer, colorectal cancer, gastric cancer, multiple myeloma, and osteosarcoma). The meta-analysis included 1,609 cases and 1,498 controls, resulting in pooled sensitivity and specificity values of 0.72 [95% confidence interval (CI): 0.62-0.81] and 0.83 (95%CI: 0.78-0.88), respectively. The area under the curve (AUC) for the summative receiver operating characteristic (ROC) was 0.86 (95%CI: 0.83-0.89), suggesting that exosomal circRNAs have favorable diagnostic accuracy across these malignancies[95].

In 2018, Wang *et al.* conducted a meta-analysis that included 1,752 patients with circRNA expression data from both the tumor and adjacent non-tumor tissues. The pooled sensitivity, specificity, negative likelihood ratio, diagnostic odds ratio (DOR), and AUC were 0.72 (95%CI: 0.67-0.76), 0.74 (95%CI: 0.69-0.78), 2.80 (95%CI: 2.40-3.10), 0.38 (95%CI: 0.33-0.44), and 7.00 (95%CI: 6.00-9.00), respectively. These findings suggest that circRNAs have moderate diagnostic utility as cancer biomarkers. Furthermore, subgroup analysis demonstrated that circRNAs in HCC tissues exhibit higher specificity, DOR, and AUC values than those in other tumor types[96].

Additionally, Nie *et al.* performed a meta-analysis focusing on the diagnostic accuracy of serum/plasma circRNAs, either alone or in combination with AFP, for HCC detection. CircRNAs showed a sensitivity of 0.82 (95%CI: 0.78-0.85) and a specificity of 0.85 (95%CI: 0.78-0.86). In comparison, AFP had a sensitivity of 0.65 (95%CI: 0.61-0.68) and a specificity of 0.90 (95%CI: 0.85-0.93). The AUC for circRNAs was 0.89 (95%CI: 0.86-0.91). The combination of circRNA plus AFP yielded a higher sensitivity of 0.88 (95%CI: 0.84-0.92), a specificity of 0.86 (95%CI: 0.80-0.91), and an AUC of 0.94 (95%CI: 0.91-0.96), indicating that combining circRNAs with AFP improves diagnostic accuracy[97]. Additionally, subgroup analysis comparing healthy control versus HCC patients showed that the diagnostic accuracy was higher than all other comparisons.

These meta-analyses highlight the potential of circRNAs to enhance diagnostic performance when combined with existing biomarkers such as AFP, even though they are not yet established for use in definitive diagnosis. Their integration may prove particularly valuable in the context of screening tests. For such applications, the use of easily accessible specimens such as serum and urine is ideal; however, clinical data on circRNA levels in bodily fluids remain limited. Continued research, especially subgroup analyses involving various liver diseases and HCC of different etiologies (e.g., viral versus alcoholic), is highly anticipated to further validate and expand the clinical utility of circRNAs.

Screening and staging biomarkers

In addition to their diagnostic utility, circRNAs have garnered attention as potential biomarkers for screening and staging HCC. Timely detection and precise staging are imperative for expanding therapeutic strategies and improving patient prognosis.

Table 2. Key findings related to potential applications

Potential application	Outline
Screening biomarkers	The combination of circRNA and AFP yielded a high sensitivity of 0.88 (95%CI: 0.84-0.92), specificity of 0.86 (95%CI: 0.80-0.91), and an AUC of 0.94 (95%CI: 0.91-0.96) A comprehensive meta-analysis of exosomal circRNAs across six types of cancer, including HCC, reported pooled sensitivity and specificity values of 0.72 (95%CI: 0.62-0.81) and 0.83 (95%CI: 0.78-0.88), respectively, with an AUC of 0.86 (95%CI: 0.83-0.89)
Prognostic biomarkers/treatment selection indicators	In HCC, survival analysis revealed that patients with high circTMEM181 expression had a shorter OS than those with low expression. Similarly, recurrence analysis indicated that patients with high circTMEM181 expression tended to experience early recurrence after surgery Lower circRNA-SORE expression was associated with better recurrence-free survival, suggesting its potential as a predictive indicator of sorafenib efficacy in HCC patients
Vaccines for infectious diseases	By acting as a decoy for miRNA-122, the artificial circRNA prevents the replication of HCV
Cancer vaccine	In the context of melanoma, LNP-artificial circRNAs demonstrated non-inferiority in inducing an immune response, suppressing tumor growth, and extending survival compared with their LNP-mRNA counterparts Wang *et al.* reported the development of a circRNA-based vaccine that expresses HCC-specific tumor neoantigens in mice[109] Direct injection of transfected circular siRNA into glioblastoma tumor tissues downregulated the target gene

AFP: α-fetoprotein; AUC: area under the curve; CI: confidence interval; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; LNP: lipid nanoparticle; OS: overall survival.

Wei *et al.* showed that circ-CDYL interacts with mRNAs encoding hepatoma-derived growth factor (HDGF) and hypoxia-inducible factor asparagine hydroxylase (HIF1AN) by trapping miR-892a and miR-328-3p, respectively. They also showed that the combination of circ-CDYL (derived from peritumoral tissues), HDGF, and HIF1AN may be potential diagnostic biomarkers for early-stage HCC (odds ratio: 124.58; 95%CI: 13.26-1170.56)[51].

In another study, ROC analysis showed that circZKSaa, the peptide translated from circZKSCAN1, had a diagnostic specificity of 100%. Moreover, because circZKSaa is secreted extracellularly, it can be detected by blood tests[62].

In addition, some researchers suggest that circRNAs may be useful for staging and predicting invasion. The potential of circular RNA SMARCA5 as a screening biomarker for virus-related HCC has been investigated. Expression of circular RNA SMARCA5 (circSMARCA5) and *SMARCA5* mRNA in whole blood was measured using qRT-PCR across three groups: healthy controls, patients with chronic hepatitis C, and patients with HCC. Although no significant correlation was observed with the Barcelona Clinic Liver Cancer, both circSMARCA5 and *SMARCA5* mRNA levels were significantly lower in the HCC group than in the other two groups. ROC analysis for early HCC diagnosis demonstrated that circSMARCA5 had an AUC of 0.917 at a cut-off value of 4.55, with a specificity of 83.8% and a sensitivity of 91.7%. By comparison, AFP at a cut-off value of 515 ng/mL had an AUC of 0.913, with a specificity of 89.2% and a sensitivity of 91.3%. These findings suggest that circSMARCA5 may be a more sensitive biomarker for HCC than AFP[98].

Li *et al.* reported that circDCUN1D4 suppresses HCC, and demonstrated that its expression was associated significantly with TNM staging, particularly N staging ($P = 0.011$; stages I and II *vs.* III and IV, $P = 0.002$; $N = 0$ *vs.* $N \geq 1$), based on analysis of 90 paired HCC primary tumor and adjacent non-tumor tissues[42].

Zhao *et al.* demonstrated that hsa-circ-0015004 upregulates the expression of the regulator of chromatin condensation 2 via the antitumor miRNA miR-330-3p, thereby promoting HCC. They also found that in a cohort of 80 HCC patients, expression levels of has-circ-0015004 in HCC tissues varied according to tumor size ($P = 0.0441$; < 5 cm *vs.* ≥ 5 cm) and TNM stage ($P = 0.0132$; stages I and II *vs.* III and IV)[49].

Some circRNAs are associated with characteristics beyond TNM staging and tumor size. For example, Ji *et al.* revealed that circCRIM1 is highly upregulated in HCC cells and tissues, and that this correlated significantly with a poor prognosis and clinicopathological features such as tumor size ($P = 0.0116$; < 5 cm *vs.* ≥ 5 cm), TNM stage ($P = 0.0216$; stages I and II *vs.* III and IV), and Edmondson grade ($P = 0.0216$; stages I and II *vs.* III and IV)[99].

Another example is hsa-circ-0007386. Feng *et al.* conducted a cohort study using HCC tissue samples from 80 patients, and suggested that expression of circ_0007386 correlated positively with tumor volume ($P = 0.0014$; < 5 cm *vs.* ≥ 5 cm), TNM stage ($P = 0.0032$; stage I *vs.* II and III), and vascular invasion ($P = 0.0007$; yes *vs.* no), as determined through clinicopathological examination[100].

These circRNA markers are likely to become increasingly valuable for cases in which staging and assessment of vascular invasion are challenging through imaging diagnosis based on computed tompgraphy (CT) and magnetic resonance imaging (MRI).

Prognostic biomarkers and treatment stratification

Beyond their role in screening and staging, circRNAs are being investigated as prognostic biomarkers that may guide treatment decisions.

Lu *et al.* conducted survival and recurrence analyses using tissue microarray data from 204 HCC patients, stratified into high and low cricTMEM181 expression groups. Survival analysis revealed that patients with high cricTMEM181 expression had shorter overall survival (OS) than those with low expression. Additionally, recurrence analysis revealed that patients with high cricTMEM181 expression tended to experience early recurrence after surgery. Thus, circTMEM181 may be useful as a prognostic biomarker[69].

Chemoresistance remains an inevitable challenge when treating HCC. Sorafenib, a multi-target tyrosine kinase inhibitor, is the first-line therapy for advanced HCC. Nevertheless, drug resistance is common following prolonged administration. Several mechanisms that contribute to sorafenib resistance have been identified, including activation of the Wnt/β-catenin pathway, EMT, alterations in the TME, and epigenetic modifications. In addition, growing evidence suggests that circRNAs play a critical role in regulating the chemosensitivity of HCC[101].

Xu *et al.* demonstrated that patients with lower expression of circRNA-SORE have better recurrence-free survival, suggesting that circRNA-SORE may predict the efficacy of sorafenib in HCC patients[43].

Lenvatinib is non-inferior to sorafenib with respect to OS, while exhibiting significant advantages across multiple secondary endpoints; however, the emergence of acquired resistance, largely attributable to drug resistance mutations under selective therapeutic pressure, remains a significant clinical obstacle. Although there is a correlation between elevated tumor stemness and lenvatinib resistance in HCC, the detailed molecular mechanisms and resistance targets have yet to be fully characterized. Tang *et al.* highlighted this critical knowledge gap, reporting that expression of circRNA-mTOR (hsa_circ_0009792) is upregulated markedly in HCC tissues and correlates strongly with poor prognosis. Mechanistic studies such as RNA pull-down assays, FISH, and co-immunoprecipitation reveal that circRNA-mTOR interacts with PC4 and SRSF1 interacting protein 1 (PSIP1), one of the RBPs, and translocates to the nucleus, with both *in vitro* and *in vivo* models demonstrating its pivotal role in promoting lenvatinib resistance in HCC via the PSIP1/c-Myc axis[102].

These studies offer valuable insight into therapeutic stratification. Nevertheless, given the small sample sizes used in existing research, validation in larger, multicenter cohorts remains essential.

Drug development applications

The therapeutic potential of circRNAs extends beyond biomarker discovery. Pharmaceutical companies have recognized their value, as exemplified by the partnership between Orna Therapeutics and Merck to develop circRNA-based therapies at a cost of $3.5 billion[103].

CircRNAs are resistant to exonucleases and harbor miRNA binding sites; thus, a miRNA sponge-like therapeutic approach may lead to good outcomes. CircRNAs are also being considered for use as a preventative medicine. One notable example is the development of circRNA-based therapies that target HCV through their function as miRNA sponges. miRNA-122 is essential for the HCV life cycle because it binds to the 5' end of HCV RNA, thereby stabilizing the viral RNA to promote its translation and replication. Synthetic circRNAs designed to act as sponges for miRNA-122 inhibit viral protein synthesis in hepatocytes, thereby suppressing HCV replication[104]. Although this method is still experimental, it can potentially prevent future HCV-related HCC outbreaks by curbing viral proliferation.

Production of circRNA drugs encompasses the design, synthesis, purification, and encapsulation of circRNA, followed by efficacy and safety evaluations, manufacturing processes, and clinical trials[105].

Another promising avenue is the application of circRNAs to vaccine development[106]. Owing to their covalently closed-loop configuration, circRNAs demonstrate remarkable stability and resistance to degradation by exonucleases, and are capable of inducing robust humoral immunity along with a substantial titer of neutralizing antibodies[107]. Unlike linear RNAs, circRNAs with m^6A modifications or IRES elements can be translated in a cap-independent manner, enabling efficient translation even under pathological conditions. In addition, the absence of recognition motifs for innate immune receptors such as RIG-I, TLR3, TLR7, and TLR8 means that circRNAs show low immunogenicity[108]. These characteristics highlight the potential of circRNAs as platforms for antibody-based immunotherapy and cancer vaccine development, offering novel avenues for therapeutic intervention.

Interestingly, Wang *et al.* reported the development of a circRNA-based vaccine that expresses HCC-specific tumor neoantigens in mice. This vaccine activated dendritic cells, leading to T cell stimulation and effective tumor suppression. When the circRNA vaccine was encapsulated in lipid nanoparticles, it elicited robust immune responses. These findings suggest potential for clinical application in humans[109].

Moreover, regarding SARS-CoV-2 circRNA vaccines, vaccinated nonhuman primates did not exhibit clinical signs of illness or exacerbated pathology, which is encouraging evidence of its safety[106].

LNP-encapsulated circRNA vaccines encoding chicken ovalbumin have been developed as a treatment for intractable melanoma. Compared with its LNP-mRNA counterpart, the LNP-circRNA vaccine exhibited significantly greater intracellular stability and induced a comparable cytotoxic T cell response along with greater secretion of certain cytokines. In a melanoma model, the LNP-circRNA vaccine suppressed tumor growth to a level similar to that of the LNP-mRNA vaccine. All untreated mice showed tumor progression and succumbed within 25 days, whereas all vaccinated mice had undetectable tumors and survived for at least 25 days. Additionally, the LNP-circRNA vaccine effectively suppressed pulmonary metastasis, with untreated mice succumbing within 41 days and all vaccinated mice surviving for at least 60 days. Furthermore, a subcutaneous colorectal cancer model showed that vaccination with LNP-circRNA led to

tumors of significantly smaller volumes than those in the untreated group, suggesting potent tumor suppression[110]. The biostability of LNP-encapsulated circRNAs is higher than that of linear RNAs; indeed, they remain stable for at least 4 weeks at 4°C and for about 2 weeks at room temperature[18,19].

The first step is to design a linear RNA precursor. Similar to conventional mRNA vaccines, circRNA vaccines depend on the host translation machinery to produce antigens, thereby inducing an immune response. Therefore, the incorporation of an open reading frame (ORF) encoding the antigen, along with translation-facilitating elements such as m^{6}A or IRES, is essential for the design of the linear precursor. Considering antigen reactivity, these fundamental elements should be optimized to increase translation efficiency. Furthermore, it is crucial to incorporate design features that promote circularization and reduce immunogenicity. By synchronizing these multiple design strategies, it is possible to establish circRNA as a superior and practical platform for therapeutic applications[105].

CircRNAs are covalently closed RNA molecules that lack a 5' end; therefore, they rely on cap-independent mechanisms for translation. Translation of circRNAs depends on specific sequence elements. As noted previously, translation can be initiated through elements such as m^{6}A modifications and IRESs. IRES-mediated translation requires a seamless, scar-free connection between the IRES and the ORF, as intervening sequences can reduce translation efficiency. Therefore, designing sequence elements that closely mimic a structurally-optimized IRES and an appropriate ORF is critical. Representative IRESs used in circRNA translation are derived from poliovirus type 1, human rhinovirus A1, encephalomyocarditis virus, HCV, cricket paralysis virus, or coxsackievirus B3 (CVB3). Among these, the IRES derived from CVB3 exhibit superior performance with respect to initiating circRNA translation; however, direct comparisons with other human-derived IRESs have not been reported. Furthermore, since the efficiency of IRES-mediated translation can vary depending on cell type and species, further reevaluation is necessary for both research and practical applications[105].

In general, the synthesis of linear RNA precursors is performed using the *in vitro* transcription (IVT) method. This approach employs bacteriophage-derived RNA polymerases such as T7 RNA polymerase or SP6 RNA polymerase to amplify the target sequence within a DNA template, thereby producing large quantities of linear precursors. To synthesize nucleotide-modified linear RNA precursors such as those containing m^{6}A, the amplification buffer can be supplemented with N6-methyl-ATP (m^{6}ATP).

Following IVT synthesis, various methods can be applied to circularize the linear precursors. These approaches can be broadly categorized into intron-catalyzed splicing, which requires intronic sequences, and chemical or enzymatic ligation, which enables circularization using only exonic sequences[105]. In particular, T4 RNA ligases that produce circRNA without extraneous fragments exhibit minimized innate immunogenicity[111].

Maintaining the purity of circRNA is critical for preserving low immunogenicity and achieving sustained and high protein expression. Extra products generated during synthesis, such as linear precursor RNAs, nicked circRNAs, and excised intron fragments, can be recognized by pattern recognition receptors (PRRs), potentially inducing innate immune responses. Therefore, rigorous purification is required[105,106].

Electrophoretic techniques such as denaturing agarose gel electrophoresis (DAGE), capillary electrophoresis (CE), and polyacrylamide gel electrophoresis (PAGE) can be utilized for this purpose. Notably, DAGE and PAGE enable the discrimination of circRNAs from linear RNAs and byproducts. In addition, specific detection of intact circRNAs can be achieved by combining these methods with oligonucleotide-directed

RNase H cleavage. However, electrophoretic methods are generally unsuitable for large-scale purification because they may increase RNA instability due to heat generation within gels and running buffers. Consequently, their use is limited mainly to quality control applications such as quantification, purity assessment, and identity verification[105].

A purification method combining high-performance liquid chromatography (HPLC) and RNase R treatment has also been developed. HPLC is a chromatographic technique that enables rapid and high-resolution separation of RNA mixtures; however, complete separation from byproducts such as nicked circRNAs and linear RNA precursors, which have similar structures and molecular weights, remains challenging. Issues such as peak overlap and incomplete digestion persist. Therefore, to further improve the purity of circRNAs, it is necessary to develop more selective separation techniques, as well as supplementary processing methods[105].

Drug delivery systems (e.g., LNPs)

CircRNA vaccines function by inducing specific antigens in the cytoplasm of host cells; however, due to their large size, circRNAs cannot easily cross the cell membrane. In addition, an appropriate delivery system enables circRNAs to evade immune surveillance and reach target sites for effective expression, which is crucial for ensuring successful immunization[105,106].

Lipid-based delivery is the mainstream delivery system. Because of their exceptional versatility as nanocarriers, liposomes have become pioneers in the field of nanomedicine delivery platforms. More recent studies recognized that smaller liposomes are more likely to evade phagocytic uptake and reach target cells more effectively. In particular, stabilized LNPs less than 100 nm in diameter are considered the next generation of liposomes: such liposomes encompass solid LNPs, nanostructured lipid carriers, and cationic lipid amphiphiles. These systems offer enhanced stability *in vivo* and are able to control both the destination and timing of delivery. As a result, next-generation LNPs are used widely for the delivery of nucleic acid-based therapeutics[105].

At present, LNPs are the most commonly used carriers in the context of circRNA vaccines. Compared with their linear RNA counterparts, circRNAs have a relatively compact conformation, which increases their loading capacity onto delivery vehicles[112]. Cationic ionizable LNPs comprise neutral phospholipids, cholesterol, polyethylene glycol (PEG)-lipids, and cationic ionizable lipids. During delivery, the cationic ionizable lipids exhibit a neutral surface charge at physiological pH, but regain a positive charge under acidic conditions during cellular uptake, thereby facilitating membrane fusion and endosome formation. The PEG-lipids provide a hydrophilic outer surface to the LNPs, preventing aggregation during storage and functioning as a stabilizing barrier. Cholesterol and neutral phospholipids contribute to the stabilization of both the LNP structure and "*in vivo*" behavior. *In vitro* studies report that LNPs achieve high RNA vaccine encapsulation efficiency. Moreover, LNPs protect RNA from degradation by endosome-derived enzymes *in vivo*[113]. Optimization of lipid components, LNP adjuvants, and administration routes determines the distribution and expression kinetics of RNA vaccines. Modifying the head and tail groups of the lipids can improve delivery efficiency. Combining adjuvants with LNP delivery is a potential strategy to further promote endosomal escape. For instance, using manganese (Mn) as a delivery adjuvant activates the direct synthesis of 2'3'-cyclic GMP-AMP (2'3'-cGAMP), stimulates the STING pathway, and promotes both maturation of antigen-presenting cells (APCs) and endosomal escape[114]. Common administration routes include subcutaneous, intramuscular (IM), and intradermal injection. As exemplified by SARS-CoV-2 mRNA vaccines, appropriate administration induces a potent and sustained local humoral immune response[106].

Recently, several novel cationic ionizable LNPs and strategies have been reported for the delivery of circRNAs. In mice, charge-altering releasable transporters (CARTs) are molecules that temporarily display cationic properties to mediate mRNA delivery. CARTs have been used for intravenous (IV) delivery of circRNAs, demonstrating consistent circRNA coding capacity over 96 h[115]. Furthermore, cases using multi-armed ionizable lipids such as AX4-based lipid nanoparticles (AX4-LNPs; FDA-approved) as carriers have been reported. In these cases, RNA vaccines packaged in LNPs were administered IM, and exhibited antigen coding and induced immune responses. Additionally, AX4-LNPs are thought to be degraded rapidly within splenic cells, thereby accelerating the release of circRNA vaccines. Multi-armed ionizable lipids, exemplified by AX4-LNPs, induce cytokine production, and this LNP system is considered suitable for activating cytotoxic T cells via the formation of an inflammatory TME without eliciting distinct adverse effects[110]. Overall, while LNP delivery systems are a promising platform for circRNA delivery, research remains at an early stage, and preclinical trials have not yet been conducted. Although various LNPs have been developed and tested as nucleic acid formulation delivery platforms, further studies are required to determine whether these novel strategies can be applied to circRNA-based vaccines.

In addition, carrier-free administration has been proposed. For example, Zhang *et al.* reported that they synthesized circular siRNAs and injected them directly (dissolved in transfection reagent) into green fluorescent protein (GFP)-expressing glioblastoma tumor tissue transplanted into mice. They observed downregulation of GFP, suggesting that direct injection of circular siRNA suppresses tumor growth[116].

Studies of subcutaneous xenograft models of lung cancer, melanoma, and colorectal cancer report that direct intratumoral injection of a carrier-free circRNA vaccine in combination with Ringer's solution induces localized cytokine expression, modulates the inflammatory TME, and increases the efficacy of tumor immunotherapy[117]. This strategy relies on the presence of APCs present at the injection site, and serves as an adjuvant approach to the treatment of solid tumors[105]. In recent years, increasing attention has focused on exosome-mediated transmission. Exosomes are nanoscale extracellular vesicles derived from endogenous cells, characterized by high affinity for recipient cells and the ability to transport circRNAs between cells. These properties make them promising nanocarriers for overcoming the challenges associated with circRNA delivery[118].

Clinical trials

In the field of gastrointestinal cancers, ongoing clinical investigations are assessing the potential of circRNAs as diagnostic and prognostic biomarkers [Table 3] (https://clinicaltrials.gov/). However, the early stage of circRNA research remains a limiting factor, and the number of studies registered in ClinicalTrials.gov is currently very limited. To enable future clinical application of circRNAs, it is essential to conduct clinical trials under diverse clinical conditions and across different geographical regions[112].

Functional analysis

Annotation

circRNAs are typically expressed at low abundance, which limits the sensitivity of RNA-seq and RT-PCR analyses when using untreated total RNA extracted from tissues. To concentrate circRNAs, it is recommended to treat total RNA with RNase R, 3′-5′ exonuclease that selectively digests linear RNAs[28,119].

For RNA-seq annotation, BSJs are the annotation targets. The number of predicted circRNAs, as well as mispredictions, varies among pipelines. Therefore, it is highly recommended that multiple pipelines are used[120]. Well-known pipelines such as CIRI, CIRCexplorer, and KNIFE are considered superior to other algorithms because they provide a better balance between accuracy and sensitivity, ensuring a thorough

Table 3. Clinical trials on circRNAs related to gastrointestinal cancers

NCT ID	Study title	Disease	Target circRNA(s)	Samples	Enrollment	Status
NCT06042842	The value of circRNAs (hsa_circ_0004001) in early diagnosis of HCC	HCC	Hsa_circ_0004001	Plasma	102	Not yet recruiting
NCT04584996	Circular and non-coding RNAs as clinically useful biomarkers in pancreaticobiliary cancers	Pancreaticobiliary cancer	Multiple circRNAs	Plasma	186	Recruiting
NCT03334708	Development of biomarkers for the early detection, surveillance and monitoring of PDAC	Pancreatic cancer	Multiple circRNAs	Plasma, tumor tissue, cyst fluid	700	Recruiting

HCC: Hepatocellular carcinoma.

process[121]. Regardless of advances in RNA-seq technology and pipeline development, it is essential to identify predicted circRNAs experimentally by combining qRT-PCR with Sanger sequencing or Northern blot analysis[122]. Combined application of qRT-PCR and Sanger sequencing means that divergent and convergent primers can be used to identify the full-length sequences of target circRNAs[123,124]; however, in cases in which target circRNAs are highly expressed, Northern blotting with a probe specifically targeting the BSJ is more appropriate than RT-PCR followed by Sanger sequencing[125]. Regarding circRNA profiling, Northern blotting is considered more suitable than RNA-seq, as the latter typically requires hundreds of reads for reproducible expression analysis, but often lacks sufficient BSJ alignments[126].

Characterization

Given their low abundance *in vivo*, circRNAs implicated in epigenetic regulation are likely not distributed uniformly throughout the cell. Instead, their subcellular localization is predicted to be closely associated with their functional roles. Consequently, after determining the subcellular localization of specific circRNAs, it is critical to select an appropriate analytical approach that considers both copy number and coding potential[127].

Subcellular localization can be investigated using Northern blotting/qRT-PCR following subcellular fractionation and FISH; however, bulk analyses with cell fractionation carry a risk that nuclear fractions may inadvertently include the endoplasmic reticulum (ER) (attached to the outer nuclear membrane); this may lead to misclassification of ER-derived RNA as nuclear RNA[128]. Additionally, many nuclear RNAs are tightly associated with proteins, chromatin, the nuclear matrix, and dense condensates[129], making them difficult to extract, leading to potential underestimation of their nuclear localization[130].

While FISH is advantageous for single-cell analysis[131], detecting circRNAs remains challenging due to their low expression levels, as well as inherent difficulties associated with the design of probes specific for BSJs[127].

Gain of function analysis

Two primary methods for gain-of-function (GoF) analysis are used to assess durable overexpression of circRNAs: the vector-based approach and the Twister-optimized RNA overexpression system called "Tornado". In the vector-based method, vectors containing the exons and flanking ICSs that comprise the main body of the target circRNA must be constructed and introduced into cells via transfection[132]. Upon transcription, these vectors produce circRNAs in an ICS-dependent manner, while simultaneously generating linear cognate RNAs[133-135]. Consequently, it is necessary to analyze the expression kinetics of these cognate linear RNAs at the same time; these serve as background controls to ensure accurate interpretation of the results[136].

CircRNAs can be expressed intracellularly using the "Tornado" system. This system incorporates the target RNA flanked by Twister ribozymes, which undergo autocatalytic cleavage to generate RNA ends that are subsequently ligated by the ubiquitous RNA ligase, RNA 3'-terminal phosphate cyclase B. This approach enables the production of circRNA aptamers with increased stability inside cells[137].

Loss of function analysis

Two commonly employed techniques to study loss-of-function (LoF) of circRNAs are RNA interference (RNAi) and the clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas2 protein system. RNAi-based approaches include short hairpin RNAs[138] and siRNAs[139] targeting BSJ sites, and antisense oligonucleotides that target the fusion sites of fusion circRNAs[140]. However, RNAi methods may influence the expression of parental genes due to partial complementarity between the RNAi molecules and linear mRNAs. To reduce such off-target effects, locked nucleic acid gapmers are often employed[136].

In contrast, RNA-targeting approaches utilizing type VI CRISPR-Cas13 (RfxCas13d) are highly specific. Guide RNAs designed to span BSJ sites can effectively distinguish between linear cognate RNAs and circRNAs, thereby minimizing off-target effects[141]. Additionally, the CRISPR-Cas9-based method has been developed to knock out circRNAs by excising entire back-splice target exons[11]. Although this approach effectively disrupts the expression of circRNA, it inevitably affects the expression of linear cognate RNAs[142]. Furthermore, strategies aimed at inhibiting the synthesis of circRNAs by removing the ICS have been explored[142], but these are often ineffective at loci harboring multiple ICS pairs[125]. Thus, applying LoF strategies to circRNA research requires careful consideration of the specific biogenesis mechanisms involved.

A related technique involves using the circRNA-specific base editor system based on Cas9. This method has a lower impact on the expression of linear cognate RNAs than conventional CRISPR-Cas9 approaches because it enables more precise editing of the back-splice region[143]; however, this method is not entirely free from the effects on linear cognate RNAs, and challenges such as target restrictions due to protospacer adjacent motif (PAM) constraints remain[144].

Limitations and future expectations

Although circRNAs show great potential as biomarkers and therapeutic agents, several limitations must be addressed.

Limitations of experimental models and functional ambiguity

Numerous methodologies have been devised for the identification, functional characterization, and localization analysis of circRNAs, encompassing not only established GoF and LoF techniques and qRT-PCR, but also advanced approaches such as dual-luciferase reporter assays, FISH, and RIP. Nevertheless, the inherently low *in vivo* expression levels of circRNAs present challenges to detection, despite their existence. To increase detection sensitivity, RNase R treatment is employed during sample preparation, although extended treatment times may inadvertently lead to circRNA degradation. Consequently, the development of more efficient circRNA detection strategies is an urgent priority.

CircRNAs interact with miRNAs, mRNAs, and RBPs, leading to dynamic changes in expression levels. While considerable progress has been made in elucidating the targets of circRNAs and their impact on downstream signaling, the upstream regulatory mechanisms governing the expression of circRNA remain largely unexplored[108]. Variations in miRNA and mRNA expression profiles across tissues and under differing environmental conditions can result in functional shifts of identical circRNAs[56,62,90,91]. Moreover, a

single circRNA can affect a variety of tissues and diseases. For example, CDR1 is associated with Parkinson's disease, myocardial infarction, diabetes, and femoral head necrosis[145]. Therefore, the possibility that circRNAs could induce adverse events associated with off-target effects, including cancer progression, cannot be ruled out. Given the critical nature of off-target effects during clinical application, a thorough understanding of the mechanism(s) regulating the expression of circRNA is of paramount importance, and it is imperative to expedite pathway analyses focusing on circRNAs.

Currently, research into circRNA is at an early stage, and only a limited number of cases have advanced to clinical trials [Table 3], underscoring the need for further clinical investigations.

Obstacles to the clinical translation of circRNAs

Prior to the clinical application of circRNAs, several critical challenges must be addressed. Chief among these is the absence of established methodologies that enable large-scale synthesis, a lack of clarity regarding adverse events such as off-target effects, unavailability of safe and efficient delivery systems, and incomplete elucidation of the mechanisms controlling circRNA expression. A detailed discussion of each issue will follow.

Challenges with respect to large-scale production of circRNAs

CircRNAs possess a covalently closed circular structure, rendering them resistant to RNase-mediated degradation and conferring high biological stability; however, this unique structure poses substantial challenges for large-scale synthesis.

Synthesis of circRNA is more complex and expensive than that of linear RNA vaccines, although in general, nucleic acid drugs can be mass-produced through a simple and easily controlled process. First, stable cyclization of linear RNAs is inefficient; longer RNA sequences have lower circularization efficiency, leading to a high proportion of extra polymerization side products such as free-style excised intron fragments, remnants of linear precursors, and nicked circRNAs. These byproducts can not only hinder circularization reactions but also serve as potent immunogens that are recognized by PRRs, thereby triggering innate immune responses. Even with RNase R treatment and HPLC, it remains difficult to completely eliminate all byproducts. When intact circRNAs and byproducts have similar molecular weights, they cannot be easily distinguished by HPLC due to overlapping elution profiles. Furthermore, precursor linear RNAs with complex secondary structures often exhibit strong resistance to RNase R digestion, thereby complicating the purification process[105].

Challenges to developing delivery systems

Although LNP-mediated delivery of circRNAs shows considerable promise in preclinical studies, it is associated with several critical limitations such as low biocompatibility, use of organic solvents, intricate manufacturing processes, restrictions in administration routes, and difficulties in scaling up production. Of particular concern is the ability of LNPs to elicit innate immune responses. Exogenous cationic charges near the cell surface are recognized as danger signals, leading to the activation of PRR pathways. These challenges suggest that, despite encouraging preclinical data, many circRNA vaccines may face significant hurdles with respect to clinical translation[105].

Additionally, it is well known that delivery efficiency to hepatocytes is maximized when nanoparticles do not possess a positively charged surface in plasma; this leads to excessive accumulation in these cells. Furthermore, studies show that IV or IM administration of LNPs results in pronounced vaccine accumulation in the liver, which can induce severe post-administration hepatic injury. Therefore, a key

challenge is to develop delivery systems that either specifically target or effectively bypass the liver, while ensuring rapid metabolic clearance[146].

A novel LNP, named YK009-LNP, demonstrates a more favorable biodistribution pattern than the commercial MC3-LNP, particularly in the spleen and at the skeletal muscle injection site, which is rich in macrophages and other immune cells. Additionally, YK009-LNP is eliminated more rapidly, resulting in reduced liver toxicity[147]. However, YK009-LNP has not yet been put to practical use.

To fully realize the therapeutic potential of circRNA vaccines, minimizing off-target expression in non-target cells and tissues is of utmost importance[105]. Accordingly, a major challenge is developing a delivery system capable of achieving precise endosomal escape (both location and timing) to facilitate antigen expression.

Engineering chimeric antigen receptor (CAR)-T cell therapy with circRNAs is expected to improve antitumor specificity and reduce off-target effects; however, further research is needed to clarify whether circRNA-modified CAR-T cells have functional superiority over conventional CAR-T approaches in terms of persistence, proliferation capacity, and tumor infiltration[148].

Challenges to the development of personalized medicine, and ethical implications

Because of their close interactions with other RNAs, proteins, and DNA, circRNA-based vaccines are being actively developed, particularly for personalized immunotherapy targeting tumor-specific antigens; however, given that research into circRNAs is still in its early stages, their efficacy, immunogenicity, and safety have yet to be thoroughly validated, especially compared with those of mRNA-based vaccines[148]. Studies involving ethnically diverse patient cohorts demonstrate that circRNA polymorphisms contribute to interindividual differences in drug responses, suggesting that circRNA-based therapeutics hold strong potential for personalized medicine[149]. However, further research is required to standardize experimental methodologies and validate findings across different cancer types and ethnic backgrounds prior to the pharmacogenomic application of circRNAs. In the near future, integrated multi-omics approaches leveraging AI-driven analyses, as well as longitudinal cohort studies, will be essential to establish circRNAs as robust clinical biomarkers and therapeutic targets[148].

A critical challenge shared across all forms of personalized medicine is that if both healthcare providers (e.g., doctors) and patients lack sufficient understanding of the advantages and limitations of circRNA-based medicines, the therapeutic effects cannot be fully realized[4]. This, in turn, risks eroding patient trust and poses serious obstacles to the advancement of clinical research and trials. Currently, circRNA research remains in its early stages, and the accumulation of knowledge regarding its benefits and drawbacks is limited. Therefore, increasing the understanding of healthcare providers and patients alike will require the generation of extensive biological and clinical evidence. In particular, the clinical application of circRNA therapeutics demands a systematic collection of comprehensive evidence, including efficacy, safety, pharmacokinetics, immunogenicity, and drug-drug interactions, through large-scale, multicenter collaborative clinical trials. Looking ahead, it will be essential to formulate detailed guidelines and legal frameworks for the development and clinical application of circRNA-based drugs, grounded in these accumulated findings and with a view toward international standardization[124].

Moreover, the manufacture of circRNA therapeutics requires advanced technologies and specialized facilities, which drive up production costs and may exacerbate regional and economic disparities regarding access to treatment[4,150]. To expand the clinical applicability of circRNA drugs, the development of novel

chemical and enzymatic approaches, along with the optimization of manufacturing processes, will represent key challenges requiring urgent resolution to enable high-purity and high-yield production.

CONCLUSIONS

Advances in RNA-seq technologies have led to the discovery of circRNAs across various cell types, tissues, and body fluids, attracting considerable attention because of their biological stability and potential as biomarkers and therapeutic agents. CircRNAs engage in epigenetic regulation in coordination with miRNAs, mRNAs, and proteins, exerting both tumor-promoting and tumor-suppressive functions. In the context of HCC, an increasing number of reports highlight the involvement of circRNAs in tumor suppression, progression, and drug resistance, raising expectations for their application as diagnostic and stratification biomarkers, as well as in cancer therapeutics. However, the expression levels of circRNAs are generally lower than those of mRNAs, necessitating the development of methods for their specific detection. Furthermore, while efforts are underway to develop circRNA-based drugs that minimize off-target effects and maximize therapeutic efficacy, optimization of their design and synthesis remains insufficient, underscoring the need for further improvements.

For the clinical application of circRNAs, it is crucial to accumulate comprehensive network analyses of interactions between circRNAs and other RNAs and proteins, along with clinical insight, to avoid adverse events and enhance understanding among both healthcare providers and patients regarding treatment. As research advances and clinical trials commence, the establishment of evidence-based guidelines will become essential. Although circRNA research is still in its infancy due to the relatively recent discovery of these molecules, they hold promising potential as biomarkers and therapeutic agents, and the accumulation of further knowledge is highly anticipated.

DECLARATIONS

Authors' contributions

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