Review

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Emerging roles of circular RNAs in liver cancer

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Summary

Hepatocellular carcinoma and cholangiocarcinoma are the most common primary liver tumours, whose incidence and associated mortality have increased over recent decades. Liver cancer is often diagnosed late when curative treatments are no longer an option. Characterising new molecular determinants of liver carcinogenesis is crucial for the development of innovative treatments and clinically relevant biomarkers. Recently, circular RNAs (circRNAs) emerged as promising regulatory molecules involved in cancer onset and progression. Mechanistically, circRNAs are mainly known for their ability to sponge and regulate the activity of microRNAs and RNA-binding proteins, although other functions are emerging (*e.g.* transcriptional and post-transcriptional regulation, protein scaffolding). In liver cancer, circRNAs have been shown to regulate tumour cell proliferation, migration, invasion and cell death resistance. Their roles in regulating angiogenesis, genome instability, immune surveillance and metabolic switching are emerging. Importantly, circRNAs are detected in body fluids. Due to their circular structure, circRNAs are often more stable than mRNAs or miRNAs and could therefore serve as promising biomarkers – quantifiable with high specificity and sensitivity through minimally invasive methods. This review focuses on the role and the clinical relevance of circRNAs in liver cancer, including the development of innovative biomarkers and therapeutic strategies.

Keywords: circRNA; hepatocellular carcinoma; cholangiocarcinoma; cancer hallmarks; biomarker

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Introduction

Circular RNAs (circRNAs) belong to a family of recently rediscovered RNA molecules. They are produced during the maturation of RNA transcripts. Structurally, circRNAs are covalently closed by a phosphodiester linkage between downstream donor and upstream acceptor RNA splice domains. Mainly considered as splicing errors during the past decade, circRNAs are now accepted as functional RNA molecules. They display tissue- and cell-specific expression patterns and are encoded from thousands of genes.[1] Emerging evidence demonstrates that circRNAs are involved in biological processes contributing to the onset and progression of cancer.[2–4] In addition, due to their circular structure, circRNAs are resistant to the action of exoribonucleases and therefore exhibit an expanded half-life compared to their parental linear counterparts, allowing detection even when expressed at a low level.[5,6] It is estimated that exonic circRNAs are very stable in cells, with most species exhibiting a half-life of over 48 hours,[7] compared to an average half-life of 10 hours for mRNAs.[6] These properties suggest that circRNAs could represent clinically relevant biomarkers for the management of patients with cancer. This review specifically highlights recent discoveries on the functions and clinical relevance of circRNAs in liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA).

CircRNA discovery, biogenesis and function

CircRNA discovery

CircRNAs were first discovered in 1976 on viroid particles.[8] Then, circular structures were identified by electron microscopy in the cytoplasm of eukaryotic cells, notably in HeLa cells.[9] Nevertheless, it was only in 1993 that circRNAs (circSry, a 1.3 kb circRNA derived from a single exon of the sex-determining region Y transcript in mice) were molecularly identified by dedicated experiments, including RNAse H digestion followed by northern blotting, reverse-transcription PCR and sequencing.[10] Afterward, several circRNAs previously described as “scrambled exon RNAs” were reported, including a circRNA from the rat cytochrome P450 2C24 gene.[11] However, no specific biological function was reported for circRNAs at that time.

Next-generation sequencing (NGS) ushered in the beginning of the genome-wide identification of circRNAs. Traditional RNA-sequencing pipelines remove non-conventional reads that do not map with the reference genome, such as reads that arise from gene fusion or those originating from back-splicing events. New bioinformatic algorithms based on back-splice junction overlapping reads recognition made it possible to efficiently detect *de novo* circRNAs and to differentiate them from their

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linear counterparts.[12–15] In addition, novel specific protocols for NGS library preparation were established to enrich the RNA fraction with circRNAs, including ribosomal and polyA+ RNA depletion and RNAse R treatment to degrade linear RNAs.[16] Accordingly, the number of circRNA libraries drastically increased and repository databases (*e.g.* circBase) were established to annotate circRNAs.[17] Recently, Xin *et al.* used rolling circle amplification followed by nanopore long read sequencing to decrypt full-length circRNA sequences, thereby increasing the roster of known circRNA isoforms.[18] It is now accepted that circRNAs are the most abundant RNA isoforms, originating from thousands of human genes,[19] and that their expression is conserved in eukaryotes.[20]

Key points

- circRNAs contribute to liver cancer by regulating cellular processes involved in cancer onset and progression.
- circRNAs are currently mainly described as sponges regulating the activity of microRNAs but more diverse functions are emerging.
- circRNAs represent promising biomarkers due to the stability linked to their intrinsic circular structure.
- circRNAs are detected in body fluids, freely or embedded into vesicles.
- circRNA-based therapeutics represent a promising approach in cancer.

CircRNA biogenesis

Contrary to their linear counterparts, circRNAs are generated by a non-canonical splicing mechanism, called back-splicing. A downstream 5' splice donor site from a pre-RNA transcript reacts with an upstream 3' splice acceptor site, which results in the 3' extremity of a downstream exon joining to the 5' extremity of an upstream exon. Therefore, the circularisation junction is formed of 2 mis-ordered exons (according to their genomic location). CircRNA back-splicing is an active process that can be promoted through various mechanisms (Fig. 1). For instance, many studies demonstrated that complementary inverted sequences localised into introns flanking the circularised exon junction could promote RNA circularisation.[21,22] By hybridising to each other, inverted complementary sequences form a loop bringing distant splice sites into proximity, and thus facilitating back-splicing events. Further studies reported that circRNA biogenesis is driven by RNA-binding proteins (RBPs) located upstream and downstream of the circularised exons, promoting back-splicing by direct interaction or dimerisation. For example, by binding to specific intronic motifs, the RBP Quaking (QKI) induces circRNA biogenesis during epithelial-to-mesenchymal transition (EMT).[23] It was further demonstrated that artificial insertion of QKI motifs was sufficient to induce back-splicing. Altogether,

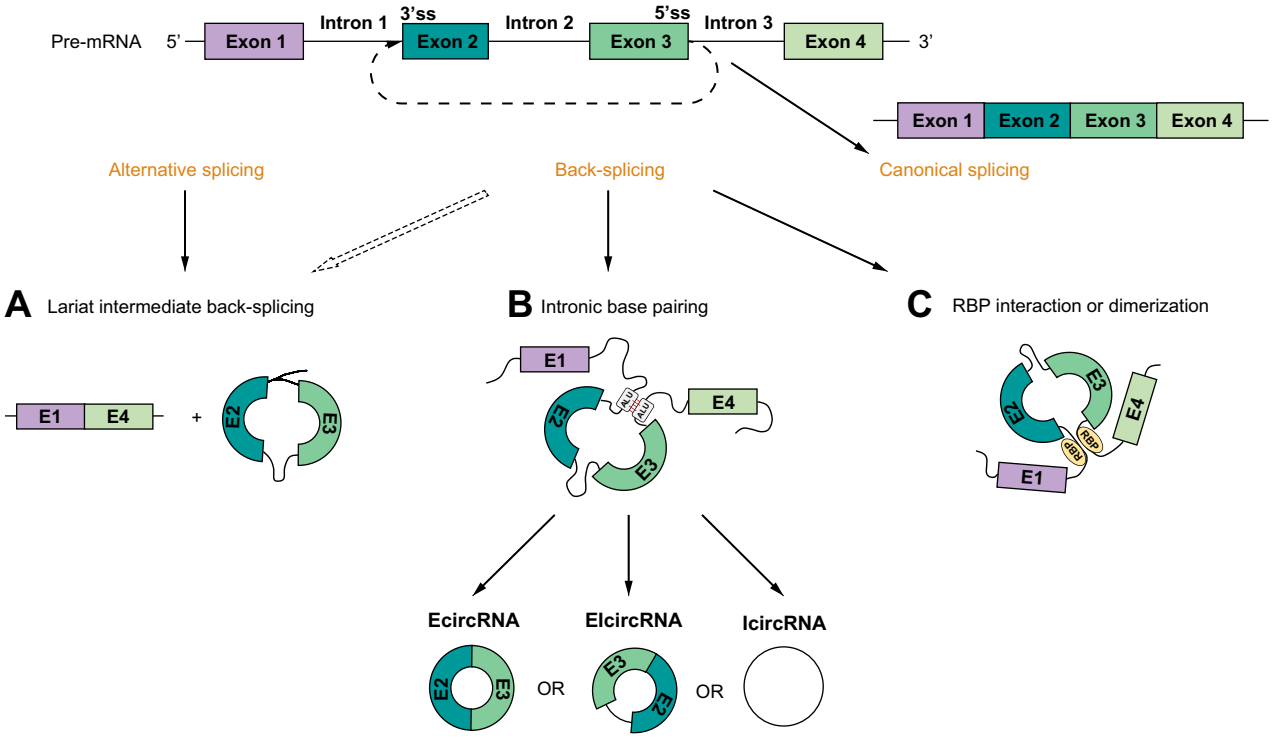

Fig. 1. The biogenesis of circRNA. CircRNAs are generated by a non-canonical back-splicing, where a 5' donor splice site (5'ss) of a downstream exon (here, exon 3, E3) will react with a 3' acceptor splice site (3'ss) of an upstream exon (here, exon 2, E2), resulting in the formation of a unique back-splice junction that does not exist in linear RNA (here, the 5' extremity of E2 with the 3' extremity of E3). So far, 3 main mechanisms spearhead the RNA circularisation. (A) Lariat intermediate back-splicing. The back-splicing process occurs after alternative splicing generates an intermediate structure called a lariat, which includes the excluded exons (here, E2 and E3) and which can be secondly spliced. (B) Intronic base pairing. A direct physical proximity between distant splice sites allows the back-splicing process. This proximity is mediated by a complementary base pairing between the introns flanking the circRNA. (C) RBP interaction or dimerisation. Here, the physical proximity between 5'ss and 3'ss is mediated by RBP interaction/dimerisation located on the exons flanking the circularisation junction. These 3 main mechanisms result in the formation of either EcircRNA, EIcircRNA or IcircRNA. CircRNA, circular RNA; EcircRNA, exonic circRNA; EIcircRNA, exonic-intronic circRNA; IcircRNA, intronic circRNA; RBP, RNA-binding protein.

these data suggest that a combination of *cis*-acting elements and *trans*-acting factors bring into proximity a downstream 5' donor splice site with an upstream 3' splicing acceptor site, facilitating back-splicing events. Another so-called "lariat splicing" mechanism has also been reported to promote circRNA formation. During alternative splicing events, excluded exon(s) are incorporated into an intermediate circular lariat containing intron-exon structures, which could be spliced again to generate circRNAs[7,11](Fig. 1). While the majority of circRNAs are only comprised of exonic sequences (EcircRNA), a substantial portion of circRNAs also include retained intronic sequences. The so-called exonic-intronic circRNAs (EIcircRNAs) have been reported to interact with U1 small nuclear ribonucleoproteins (snRNP), thereby regulating the transcription of their parental gene.[24] Likewise, some circRNAs consisting of only intronic sequences (IcircRNAs; lariat-derived circRNAs) have been reported.[25]

CircRNA functions

Although circRNAs were discovered decades ago, defining their role remains a major challenge because of their low abundance and their sequence similarity with parental linear RNAs. To date, the most frequently reported mechanism of action of circRNAs is their capacity to sponge microRNAs (miRNAs) although other functions are emerging (*e.g.* transcriptional and post-transcriptional regulation, protein scaffolding) (Fig. 2).[26,27] MiRNAs are small non-coding RNAs regulating gene expression at the post-transcriptional level. By binding the 3' untranslated regions of mRNAs, miRNAs repress mRNA translation and/or induce their degradation.[28] Accordingly, miRNA abundance and activity are tightly regulated. Notably, miRNAs can be sequestered by RNA sponges, including circRNAs.[29] Thus, CDR1 antisense circular RNA (circCDR1as) contains more than 70 miR-7 responsive elements. Through its sponging capability, circCDR1as acts as a competitive inhibitor of the tumour suppressor

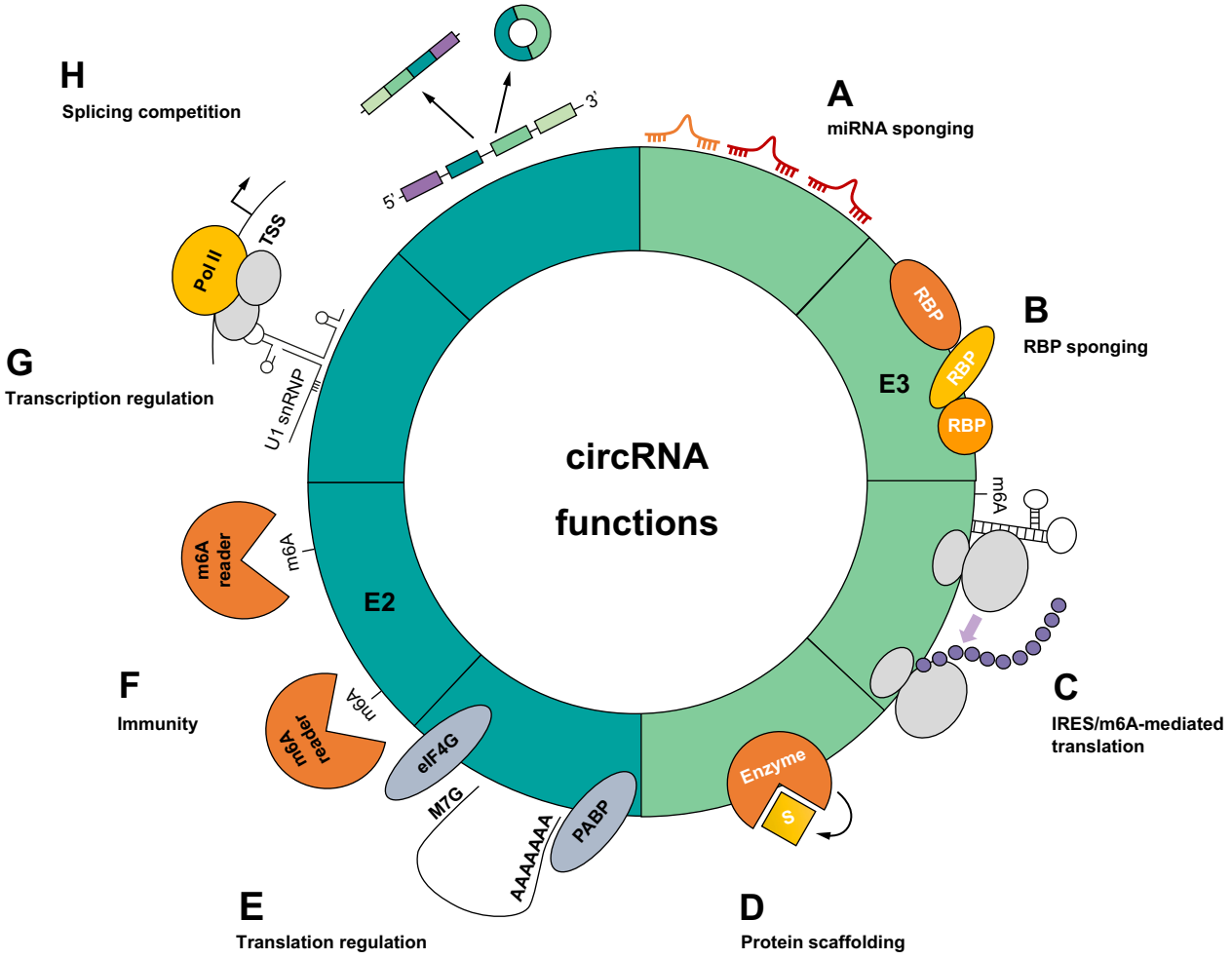

Fig. 2. CircRNA functions. (A) miRNA sponging: Numerous circRNA harbour miRNA response elements. Therefore, by sponging miRNA, circRNA act as competitive endogenous RNA, preventing miRNA post-transcriptionally binding to and repressing their natural targets. (B) RBP sponging: CircRNA display specific protein binding motifs offering them the capability to sequester RBP, regulate their activity and influence their localisation. (C) IRES/m6A-mediated translation: CircRNA containing IRES or with m6A epitranscriptomic modifications can be translated in a cap-independent manner. (D) Protein scaffolding: Proteins can be recruited by circRNA, facilitating enzymatic reactions. (E) Translational regulation: The translation initiation complex factors PABP and eIF4G are able to bind to circRNA. In this case, the interaction negatively regulates the translation initiation process. (F) Immunity: The endogenous m6A modification is necessary to distinguish self and non-self circRNA like those coming from viruses. (G) Transcription regulation: EIcircRNA regulate transcription by interacting with U1 snRNP and promoting the transcription of their parental genes. (H) Splicing competition. Spliceosome machinery can foster circRNA biogenesis under specific conditions leading to the reduction of linear mRNA production. CircRNA, circular RNA; EIcircRNA, exonic-intronic circRNA; IRES, internal ribosome entry sites; miRNA, microRNA; RBP, RNA-binding protein.

miR-7, preventing it from binding to and repressing its natural targets involved in tumour promotion.[27,30] However, this feature is uncommon. Indeed, an expanded identification and characterisation of circRNAs in mammals reported that circCDR1as is only 1 of 2 circRNAs with an over-representation of binding sites for a single miRNA.[31] In addition, it should be noted that circCDR1as is detected in circular but not in linear form, complicating interpretations of its role as a sponge for miR-7.[27] circRNA databases (*e.g.* CircInteractome, starBase) also predict the presence of circRNA/miRNA networks based on bioinformatics and/or experimental cross-linking immunoprecipitation (CLIP)-sequencing data.[32,33] These databases also include information on RBP-circRNA interactions. Indeed, emerging evidence demonstrated that circRNAs can sponge RBPs, with functional consequences for their subcellular localisation and activity.[34] For example, it was recently demonstrated that circFOXK2 interacts with YBX1 and hnRNPK to promote the expression of NUF2 and PDXK oncogenes in pancreatic cancer.[35] In some cases, back-splicing also competes with canonical splicing. A typical example concerns muscleblind (*MBL* or *MBNL1* in humans) RNA which contains several intronic motifs which the MBL protein can bind to. When overexpressed, MBL binds to these specific intronic RNA motifs, thus promoting an exon 2 back-splicing event and reducing its own protein expression as a feedback mechanism.[36] Another study described a regulatory network involving YAP circRNA (circYAP) and its associated linear RNA. In breast cancer cell lines, it was demonstrated that circYAP interacts with *YAP* mRNA, PABP and the translation initiation protein eIF4G. This interaction inhibits the assembly of the translation initiation complex on *YAP* mRNA, resulting in the inhibition of translation.[37] Interestingly, circRNAs can promote transcription of their parental gene by interacting with U1 snRNP and the RNA polymerase II complex.[24] It is also important to emphasise that circRNAs may be coding, as demonstrated in *Drosophila* by ribosome footprinting sequencing specifically focused on reads that overlap with specific circRNA circularisation junctions.[38] Likewise, translation of circRNAs can be mediated by internal ribosome entry sites (IRES).[39–41] In addition, emerging data suggest that circRNA translation could be driven by epitranscriptomic modifications, including N6-methyladenosine (m6A), in a cap-independent manner.[42] Recent studies also demonstrated that the m6A modifications could distinguish self-circRNAs from immunogene foreign circRNAs (*i.e.* those coming from viruses).[43] Although the functions of circRNAs have not been fully elucidated, it is clear that circRNAs contribute to the regulation of gene expression. Thus, one can assume that the deregulation of circRNAs under pathological conditions will greatly affect fundamental processes required to maintain cellular and tissue homeostasis.

Overview of circRNAs in cancer: mechanisms and clinical relevance

As reviewed in 2020, circRNAs are involved in physiological processes by regulating gene expression and protein activity.[44] Growing evidence demonstrates that circRNAs are deregulated in cancer.[2,4,44] Indeed, up- or downregulation of specific circRNAs correlates with clinical features like TNM stage, differentiation or survival.[45] In addition, the ratio between the expression of circRNAs and the corresponding linear RNAs can be modified, suggesting that back-splicing is an actively regulated process involved in cancer. In 2019, a loss-of-function screen using small-hairpin RNAs (shRNAs) directed against highly expressed circRNAs indicated that 11.3% of circRNAs are necessary to promote cell growth in V16A prostate cancer cells. In contrast, among the related linear counterpart transcripts, 91.8% were not essential to fulfil this function.[46] Thus, by exhibiting tumour suppressive or oncogenic activities, circRNAs may contribute to cancer onset and progression. As mentioned above, a well-described function of circRNAs is their capability to sponge miRNAs, thereby promoting or suppressing tumour progression, depending on the nature of the miRNA targets.[47,48]

Given that the field is still in its infancy, there is currently little overlap between studies,[3] similar to what we observed years ago for miRNAs. Although the current literature has not yet enabled the identification of master oncogenic or tumour suppressive circRNAs, such as *RAS* or *TP53* for coding genes, several circRNAs have been detected in multiple cancers (*e.g.* circHIPK3, ciRS-7, circFOXO3, circMTO1). One archetype of a possible oncogenic circRNA is circHIPK3, which harbours 18 binding sites for sponging 9 tumour suppressor miRNAs involved in cell growth in several cancers, including breast cancer, colorectal cancer and HCC.[49] This observation has revealed new therapeutic opportunities, such as using target site blocker oligonucleotides to inhibit the sponging activity of circHIPK3. However, circHIPK3 was also reported to be downregulated in bladder cancer and involved in cell growth and metastasis inhibition, suggesting that circRNA function could be highly context-dependent, making it difficult to define a particular circRNA as oncogenic or tumour suppressive.[3,50] Interestingly, circRNAs have also been shown to derive from master oncogenic or tumour suppressive genes, including circTP53, which was reported to promote colorectal cancer by sponging miR-876-3p and subsequently increasing cyclin-dependent kinase-like 3 expression.[51] An exome capture RNA-sequencing project was recently conducted in more than 2,000 cancer samples, including cell lines, tumours from diverse organs and non-pathological tissues. Data were compiled into MiOncoCirc, the first international cancer-specific circRNA database.[5]

CircRNAs and the hallmarks of liver cancer

Liver cancer ranks sixth among the most prevalent cancers worldwide. Dramatically, both its incidence and associated mortality have steadily increased over the last decade. HCC and intrahepatic cholangiocarcinoma (iCCA) account for ~80% and ~15% of primary liver tumours, respectively.[52–54] Both are associated with limited therapeutic options as they are frequently diagnosed late.[53,54] Although our understanding of liver carcinogenesis has improved, notably through functional genomic studies, effective long-term targeted therapies are still lacking. By integrative genomics based on gene expression, the core transcriptional hallmarks of human HCC were previously highlighted, identifying potentially targetable signalling pathways that were commonly altered.[55] In 2011, Hanahan and Weinberg updated the hallmarks of cancer and described 10 hallmarks that govern the evolution of normal cells to a neoplastic state in most cancers.[56] Defining circRNAs that are involved in the hallmarks of liver cancer could provide new insights into liver carcinogenesis and open avenues for the development of new therapeutic options. However, to what extent circRNAs contribute to liver carcinogenesis still requires experimental investigations. In order to determine how circRNAs could contribute to liver cancer, we reviewed the current

literature on cancer hallmarks impacted by circRNAs (Fig. 3 and Table 1).

Hepatocellular carcinoma

Data from the recent literature suggest that circRNAs are relevant molecules involved in regulatory networks governing HCC onset and progression. While the role of circRNAs in cell proliferation, apoptosis, migration and invasion is rather well described, their role in angiogenesis, immune surveillance and metabolic switching is still under investigated. The involvement of circRNAs in enabling replicative immortality, another hallmark of cancer cells, has not been investigated in HCC.

CircRNAs in cell proliferation and resistance to cell death

Sustained proliferation is one of the distinguishing features of cancer cells. Some circRNAs exhibit tumour suppressor activities and display low expression in HCC (Table 1). For instance, circMTO1 was downregulated in HCC and was reported to sponge miR-9, a well-known oncogenic miRNA. Reduced expression of circMTO1 in HCC results in increased miR-9 activity, leading to reduced expression of its natural targets, such as the cell cycle inhibitor p21. Accordingly, circMTO1 suppresses HCC progression and low expression of circMTO1 predicts poor overall survival.[47] Deregulation of cell proliferation pathways in HCC is also mediated by pro-oncogenic circRNAs. Among them, SCD-circRNA 2 was upregulated in HCC tissues. A gene regulatory network was proposed, in which the RBP RBM3 promotes HCC cell proliferation in a SCD-circRNA 2-dependent manner.[57] Oncogenic circRHOT1 was also related to sustained HCC cell proliferation by recruiting TIP60 (also known as KAT5), a member of the MYST sub-family of histone acetyltransferases, to the promoter of NR2F6 and subsequently enhancing its transcription.[58] Interestingly, NR2F6 has also been reported as a central intracellular immune checkpoint for cancer immune surveillance that suppresses adaptive anti-cancer immune responses.[59] An

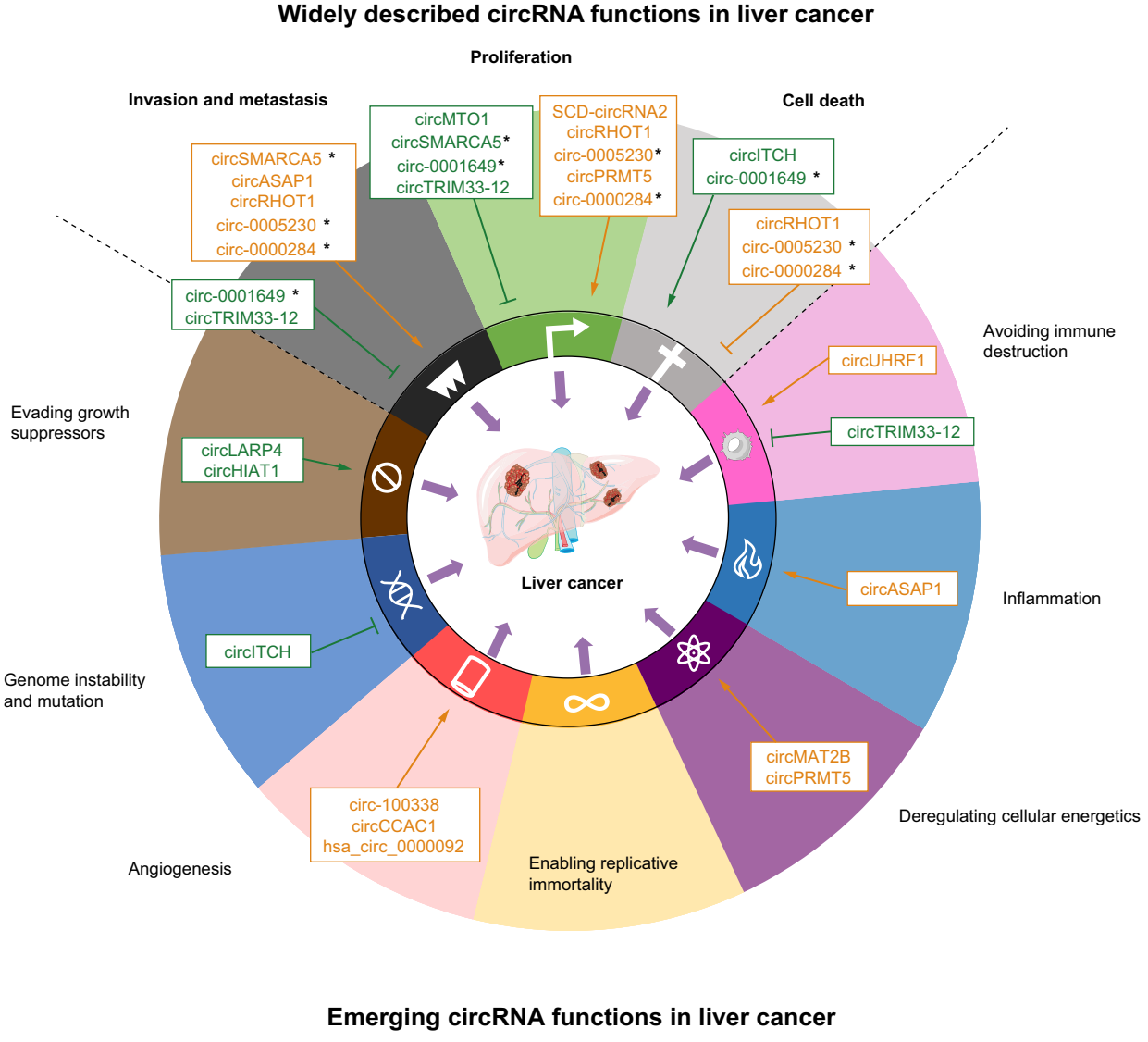

Fig. 3. CircRNA functions in liver cancer onset and progression. The upper part gathers the most described functions driven by circRNAs in liver cancer, while the lower part pinpoints potential emerging regulatory functions of circRNAs. Pro-oncogenic circRNAs are indicated in red and tumour suppressor circRNAs are indicated in green. CircRNAs deregulated in iCCA are marked by an asterisk. CircRNA, circular RNA; iCCA, intrahepatic cholangiocarcinoma.

Table 1. Reported functions of circRNAs in liver carcinogenesis.

Liver cancer	circRNA ID	circRNA function	Sponged molecules	Regulated targets	Reported functions	Ref.
circRNA induced in HCC						
HCC	SCD-circRNA2*	RBP interaction	N/A	p-ERK ↑	Sustaining proliferative signalling, invasion and metastasis	57
HCC	circRHOT1*	transcription regulation	NRF26 TIP60	NRF26 ↑	Sustaining proliferative signalling, resisting cell death, invasion and metastasis	58
HCC	circ-100338*	RBP interaction	N/A	MMP9 ↑	Sustaining proliferative signalling, invasion and metastasis, inducing angiogenesis	110
HCC	circ-0000092*	miRNA sponging	miR-338-3p ↓	HN1 ↑	Sustaining proliferative signalling, invasion and metastasis, inducing angiogenesis	73
HCC	circPRMT5*	miRNA sponging	miR-188-5p ↓	HK2 ↑	Sustaining proliferative signalling, invasion and metastasis, deregulating cellular energetics	111
HCC	circMAT2B*	miRNA sponging	miR-338-3p ↓	PKM2 ↑	Sustaining proliferative signalling, invasion and metastasis, deregulating cellular energetics	75
HCC	circASAP1*	miRNA sponging	miR-326 ↓ miR-532-5p ↓	MAPK1 ↑ CSF-1 ↑	Sustaining proliferative signalling, invasion and metastasis, tumour-promoting inflammation	80
HCC	circβ-catenin*	translated, decoy	N/A	β-catenin ↑	Sustaining proliferative signalling, invasion and metastasis	60
HCC	circUHRF1*	miRNA sponging	miR-449c-5p ↓	TIM3 ↑	Sustaining proliferative signalling, immune surveillance escape	112
circRNA repressed in HCC						
HCC	circTRIM33-12*	miRNA sponging	miR-191 ↑	5hmc ↓	Sustaining proliferative signalling, immune surveillance escape, invasion and metastasis	77
HCC	circHIAT1*	miRNA sponging	miR-3171 ↑	PTEN ↓	Sustaining proliferative signalling, evading growth suppressors, resisting cell death	61
HCC	circLARP4*	miRNA sponging	miR-761 ↑	RUNX3 ↓ p53 ↓ p21 ↓	Sustaining proliferative signalling, evading growth suppressors	62
HCC	circMTO1*	miRNA sponging	miR-9 ↑	p21 ↓	Sustaining proliferative signalling, invasion and metastasis	47
HCC	circITCH	miRNA sponging	miR-224-5p ↑	mafF ↓	Sustaining proliferative signalling, resisting cell death, genome instability and mutation	65,113
circRNA induced in CCA						
iCCA	circ-0000284*	miRNA sponging	miR-637 ↓	LY6E ↑	Sustaining proliferative signalling, resisting cell death, invasion and metastasis	64
iCCA	circ-0005230*	miRNA sponging	miR-1238 ↓ miR-1299 ↓	N/A	Sustaining proliferative signalling, resisting cell death, invasion and metastasis	63
iCCA	circACTN4*	miRNA sponging transcription regulation	miR-424-5p ↓	YAP ↑ FZD7 ↑	Sustaining proliferative signalling, invasion and metastasis	82
circRNA repressed in HCC and CCA						
HCC iCCA	circ_0001649*	miRNA sponging	miR-127-5p ↑ miR-612 ↑ miR-4688 ↑	SHPRH ↓	Sustaining proliferative signalling, resisting cell death, invasion and metastasis	66,67
HCC iCCA	circSMARCA5*	miRNA sponging	miR-17-3p ↑ miR-181b-5p ↑	TIMP3 ↓	Sustaining proliferative signalling, invasion and metastasis	48,81

CircRNA, circular RNA; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; miR/miRNA, microRNA.
* *In vivo* functional confirmation in preclinical mouse models.

original mode of action of circβ-catenin, a circRNA derived from β-catenin, a well-characterised oncogene in liver cancer, has recently been reported.[60] Indeed, circβ-catenin is translated into a 370-amino acid β-catenin isoform and acts as a decoy. This shorter isoform can stabilise full-length β-catenin by antagonising GSK3β-induced β-catenin phosphorylation and degradation, leading to activation of the Wnt pathway, and promoting tumour cell growth.[60]

Another well-described feature of tumour cells is their ability to evade anti-proliferative signals governed by tumour suppressor genes (*i.e.* quiescence, cell cycle arrest, senescence, cell death).[56] Such properties are essential to maintain a pool of active transformed cells, notably by repressing or inactivating cell cycle regulators. For instance, a network involving circHIAT1, miR-3171 and the *PTEN* tumour suppressor gene was shown to contribute to HCC progression (Table 1). Thus, circHIAT1 downregulation in HCC was responsible for miR-3171 release, thereby promoting *PTEN* repression.[61] Similarly, it was demonstrated that miR-671 activation, as a consequence of circLARP4 sponge downregulation, drives the silencing of master tumour suppressor genes RUNX3, CDKN1A and TP53 in HCC.[62]

Programmed cell death by apoptosis is responsible for the removal of damaged cells. Notably, apoptosis serves as a bulwark against the propagation of transformed cells. Investigations into pro- and anti-apoptotic programmes will be critical to elucidate the paths whereby tumours succeed in progressing to states of high-grade malignancy and resistance to therapy.[56] Lately, circRNAs were reported for their entailment in signalling tracks governing the apoptotic machinery in liver cancer. Several studies identified circRNAs that can regulate apoptosis (Table 1). Pro-oncogenic circRNAs such as circRHOT1, circ-0005230, or circ0000284 have all been shown to exert anti-apoptotic actions on HCC and iCCA cell lines. Through intrinsic miRNA sponging properties, these circRNAs promote tumourigenesis by preserving anti-apoptotic factors, such as *NRF26* and *LY6E,* from degradation.[58,63,64] Meanwhile, a decreased expression of circITCH and circ-0001649 contributes to cell death resistance by loosening the sponging of specific anti-apoptotic miRNAs. Thus, pro-apoptotic natural targets (*e.g.* MAFF and SHRPH) lack circRNA protection against miRNA degradation and are unable to counteract tumour cell spreading.[65–67] Remarkably, most of these circRNAs are also involved in sustained abnormal cell proliferation, resulting in unbalanced cell homeostasis and cancer progression.

CircRNAs in invasion, metastasis and angiogenesis

Invasion and metastasis are tightly associated with EMT, a critical cellular process governing the plasticity of epithelial cells which lose adherent junctions, apico-basal polarity and acquire the abilities to resist apoptosis, to invade, and to disseminate.[56,68] However, to what extent circRNAs are involved in acquiring mesenchymal features and a metastatic phenotype has not been extensively explored in liver cancer (Table 1). RNA-sequencing identified a signature of circRNAs that were significantly deregulated in HCC tissues, including circSMARCA5. Low expression of circSMARCA5 in HCC was attributed to the regulation of DExH-box helicase 9 (DHX9), an abundant nuclear RNA helicase, and was shown to promote HCC cell growth and metastasis. One mechanism of action resulting from the reduced expression of circSMARCA5 involved a decreased expression of the metallopeptidase inhibitor TIMP3 due to the increased activity of miR-17-3p and miR-181b-5p, 2 miRNAs naturally sponged by circSMARCA5. Further highlighting its importance in cancer, it was recently reported that circSMARCA5 can bind to its parent gene locus, forming an R-loop, which results in the downregulation of tumour-promoting SMARCA5 in breast cancer.[69] In glioblastoma multiform, circSMARCA5 is also a tumour suppressor and acts as a decoy for SRSF1 (serine and arginine rich splicing factor 1) through a GAUGAA binding motif, thus controlling cell migration and angiogenesis, notably by regulating *VEGFA* mRNA splicing.[70] In HCC, circSMARCA5 downregulation was associated with poor outcomes, including shorter overall and recurrence-free survival.[48] The role of circRNAs in HCC metastasis was further evaluated by circRNA expression profiling in 15 patients who displayed pulmonary metastasis and 15 patients that did not show any metastasis or recurrence. Hence, circASAP1 was specifically upregulated in HCC with a high metastatic potential. Gain and loss of function experiments demonstrated that circASAP1 promotes HCC cell metastasis by acting as a competitive endogenous RNA for miR-326 and miR-532-5p, leading to the induction of their common targets, the kinase MAPK1/ERK2 and CSF1, also known as macrophage colony-stimulating factor (M-CSF). By regulating these 2 targets, circASAP1 modulates HCC cell proliferation, invasion and tumour-associated macrophage (TAM) infiltration.[48] More recently, an original mechanism of action has been reported for circPABPC1, a tumour suppressive circRNA downregulated in HCC.[71] Although circPABPC1 is predominantly localised in the cytoplasm, its small size (91 bases) makes it unlikely to be a miRNA sponge. Indeed, circPABPC1 functions as a protein scaffold for ITGB1, a key integrin involved in HCC metastasis. Frequent loss of circPABPC1 correlates with increased ITGB1 level in patients with advanced HCC. Mechanistically, circPABPC1 physically links ITGB1 to the proteasome 26S and promotes its degradation in a ubiquitin-independent manner, thereby reducing tumour cell adhesion, migration, and metastatic spreading.[71]

Tumour cell spreading requires a tailored vascular system to ensure its operational needs. Hence, during tumour progression, a new tumour-associated vasculature is generated by uncontrolled angiogenesis.[56,72] *In silico* analysis suggested that circRNAs, including hsa_circ_0000092, could contribute to the tumour-associated angiogenic switch occurring during liver carcinogenesis (Table 1). Hsa_circ_0000092 is upregulated in HCC and significantly associated with shortened overall survival. A comprehensive analysis of both coding and non-coding RNAs suggested that hsa_circ_0000092 interferes with the negative regulation of haematopoietic- and neurologic-expressed sequence 1 (HN1) by miR-338-3p. Increased expression of oncogenic HN1 not only promotes HCC cell invasion and migration, but also angiogenesis.[73]

CircRNAs in metabolic switching

An important hallmark of cancer is the adjustment of energy metabolism to support cancer cell proliferation. During carcinogenesis, cells classically switch towards aerobic glycolysis to meet their energy demands. This typical glycolytic reprogramming is associated with well-known oncogenes.[56,74] However, there is still little evidence regarding the role of circRNAs in this process (Table 1). Meta-analysis of gene expression datasets demonstrated that circMAT2B was upregulated in HCC (compared to paired adjacent normal tissues) and strongly associated with glycolysis. More specifically, circMAT2B promotes glycolysis-dependent cell proliferation and migration

under hypoxic conditions both *in vitro* and *in vivo*. A regulatory axis was proposed in which circMAT2B positively regulates the miR-338-3p target gene pyruvate kinase M2, a key enzyme involved in glycolysis.[75] CircMAT2B is also clinically relevant since its elevated expression is associated with shortened overall survival.[75]

CircRNAs in immune system surveillance escape and inflammation

It has long been recognised that tumour onset and progression are continuously monitored by the immune system. Thus, proliferating tumour cells have somehow developed the capability to evade immune surveillance.[56,76] A recent study pinpointed that circTRIM33-12 downregulation in HCC was correlated with poor clinical features, suggesting its potential utility as an independent HCC risk factor (Table 1). Mechanistically, circTRIM33-12 expression was negatively associated with cell proliferation, migration and invasion through the miR-191-dependent positive regulation of TET1 demethylase. In addition, circTRIM33-12 was shown to participate in immune evasion via the regulation of activating receptor NKG2D (natural-killer group 2 member D) ligands. Thus, circTRIM33-12 regulates natural killer cell-, γδ+T cell- and CD8+ T cell-mediated immune responses to cancers.[77]

Inflammation is an essential mechanism to protect the body against injuries and pathogenic microorganisms. Nevertheless, in the context of carcinogenesis, substantial evidence indicates that the immune system could exert tumourigenic properties.[78] For instance, by releasing cytokines, infiltrated immune cells indirectly promote tumour growth and survival.[79] One study reported the involvement of circRNAs in tumour-promoting inflammation in HCC (Table 1). Hence, it was highlighted that an enhanced expression of circASAP1 indirectly induces CSF1 expression, a macrophage mitogenic factor, by sponging miR-532 and miR-326, and therefore promotes TAM recruitment.[80] There is growing evidence that TAM infiltration is strongly associated with tumour onset, progression and aggressiveness.[79] Thus, circASAP1 could promote HCC development by participating in TAM infiltration.[80]

Intrahepatic cholangiocarcinoma

Only few studies have reported on the role of circRNAs in iCCA so far (Table 1). One study focused on circSMARCA5, a tumour suppressor circRNA already largely described for its role in the progression of several cancers, including HCC. The expression of circSMARCA5 was reduced in iCCA tissues and negatively correlated with advanced TNM stage and overall survival. Overexpression of circSMARCA5 was further associated with decreased iCCA cell proliferation.[81] Very recently, circACTN4 has been shown to be induced in iCCA and to promote tumour cell growth and metastasis by regulating the Hippo and Wnt signalling pathways. Interestingly, circACTN4 has been reported i) to act as a sponge for miR-424-5p which could target YAP in the cytoplasm and ii) to recruit YBX1 to initiate FZD7 transcription in the nucleus. This study implies that circACTN4 acts as a signalling nexus allowing for the coordinated activation of Hippo and Wnt pathways in iCCA.[82] However, the dynamic regulation of circRNA shuttling between the nucleus and the cytoplasm, in relationship to their function, is an important feature which remains to be elucidated. A couple of other studies identified aberrantly expressed circRNAs in both CCA cell lines and resected iCCA tumours. Thus, circ-0001649 and circ-0005230 were respectively down- and upregulated in iCCA. These circRNAs were associated with cell proliferation, migration and invasion.[63,67] Very recently, upregulation of circHMGCS1-016 was also reported to contribute to iCCA development and immune tolerance by sponging miR-1236-3p to regulate CD73 and GAL-8 expression.[83] Several circRNAs derived from CASC15, a transforming growth factor beta-induced long non-coding RNA, have also been associated with an inflammatory microenvironment in iCCA.[84] Most of the studies reported so far have focused on circRNAs within iCCA tumours. Nevertheless, circRNA are also found circulating in body fluids (*e.g.* embedded in extracellular vesicles [EVs]) and could therefore provide promising clinical opportunities. For instance, the expression of circ-0000284 was increased in CCA cell lines, tissues and plasma EVs. The circ-0000284-enriched EVs produced by CCA cells were able to enhance migration, proliferation and invasion of the normal surrounding cells both *in vitro* and *in vivo*.[64] These results not only highlight circRNAs as mediators of cellular communication but also as potentially promising biomarkers.

Limitations and challenges in interpreting data on circRNAs

While studies reporting on the functions of circRNAs in liver cancer are promising, it is important to point out that circRNAs' sponge activity is the major mechanism investigated so far (Table 1). Many of these studies rely on the cytoplasmic location of the circRNA of interest as a rationale to investigate miRNA activity, although this is where most exon-encoded circRNAs are located.[7,19] Most studies also look for evidence of sponge activity by searching for any predicted miRNA binding sites in the circRNA and correspondingly expressed miRNAs and/or looking for interaction of the circRNA with proteins from the RNA-induced silencing complex (RISC). Although these strategies suggest interactions, they do not demonstrate function and should be complemented with loss of function or depletion studies to validate a functional effect on miRNAs. It is also important to consider that essentially all of the sequences attributed to circRNAs are contained in the parental mRNA, so unless the circRNA is more abundant than the mRNA or is somehow locking the miRNA into the RISC, it is hard to understand the stoichiometry to explain how circRNAs can effectively act as sponges in many cases. Similarly, a circRNA contains the same mRNA sequence encoded by the same exons (with the exception of the back-splice) so that ectopic expression of circRNA and mRNA from the same gene could have the same effect. This point should be addressed in functional studies given that in many cases, the expression of circRNAs tends to follow the expression of the mRNAs, so increases in expression of circRNAs are usually associated with increased expression of mRNA. However, given that the median half-live of circRNAs is estimated to be at least 2 to 5 times higher than that of linear mRNA,[6,7,85] one can hypothesise that the contribution of circRNA to RNA sponging activity is greater than that of their linear counterpart. Thus, the dynamic of mRNA splicing and translation, the half-life of circRNA *vs.* mRNA, as well as their subcellular location, should be considered, as these features may contribute to the differential sponge activity of circRNA *vs.* mRNA. Additional studies, including Ago-CLIP followed by long read sequencing without RNAse R treatment, will be needed in the future to better understand the stoichiometry of each

Table 2. CircRNA as biomarkers for liver cancer diagnosis and prognosis.

Liver cancer	circRNA ID	Biological sample	Biomarker relevance	Ref.
Upregulated circRNA				
HCC (HBV related)	circRNA-100338	HCC tissue	Prognostic	114
HCC (HBV related)	hsa_circ_0000976 hsa_circ_0007750 hsa_circ_0139897	plasma	Diagnostic	115
HCC (HBV related)	hsa_circ_0027089	plasma	Diagnostic	116
HCC (early stage)	circ-CDYL	HCC tissue	Surveillance biomarker, early diagnostic	95
HCC	circRNA ciRS-7 (Cdr1as)	HCC tissue	Microvascular invasion	117
HCC	circ_0008450	HCC tissue	Prognostic	118
HCC	circRHOT1	HCC tissue	Prognostic	58
HCC	circBIRC6	HCC tissue	Prognostic	119
HCC	hsa_circ_0000517	HCC tissue	Prognostic	120
HCC	hsa_circ_0128298	HCC tissue	Diagnostic, prognostic	121
HCC	hsa_circ_0091579	HCC tissue	Diagnostic, prognostic	122
HCC	hsa_circ_0016788	HCC tissue	Diagnostic, prognostic	123,124
HCC	circPCNX	HCC tissue	Prognostic	125
HCC	hsa_circ_0056836	HCC tissue	Diagnostic, prognostic	126
HCC	hsa_circ_0005075	HCC tissue	Diagnostic, prognostic	127,128
HCC	hsa_circ_0091581	HCC tissue	Prognostic	129
HCC	hsa_circ_0128298	HCC tissue	Diagnostic, prognostic	121
HCC	circ-10720	HCC tissue	Prognostic	130
HCC	circRNA-101368	HCC tissue	Prognostic	131
HCC	circMAT2B	HCC tissue	Prognostic	75
HCC	circ_0000267	HCC tissue	Prognostic	132
HCC	circ_001569	HCC tissue	Prognostic	133
HCC	circRNA-104718	HCC tissue	Prognostic	134
HCC	hsa_circ_0067934	HCC tissue	Prognostic	135
HCC	SCD-circRNA 2	HCC tissue	Prognostic	57
HCC	circRNA_0000502	HCC tissue	Prognostic	136
HCC	circPTPRM	HCC tissue	Prognostic	137
HCC	circPVT1	HCC tissue	Prognostic	138
HCC	circRNA MYLK	HCC tissue	Prognostic	139
HCC	circABCB10	HCC tissue	Prognostic	140
HCC	circ-ZNF652	HCC tissue	Prognostic	141
HCC	hsa_circ_0103809	HCC tissue	Prognostic	142
HCC	circ_0021093	HCC tissue	Prognostic	143
HCC	hsa_circ_0000673	HCC tissue	Prognostic	144
HCC	hsa_circRNA_103809	HCC tissue	Prognostic	145
HCC	circZFR	HCC tissue	Prognostic	146
HCC	circMET	HCC tissue	Prognostic	147
HCC	circ-HOMER1	HCC tissue	Prognostic	148
HCC	circFBXO11	HCC tissue	Prognostic	149
HCC	circ-TCF4.85	HCC tissue	Diagnostic	150
HCC	circBACH1	HCC tissue	Diagnostic, prognostic	151
HCC	circRNA-SORE	HCC tissue	Sorafenib resistance	99
HCC	hsa_circ_0003998	HCC tissue, plasma	Diagnostic, prognostic	152
HCC	circASAP1	HCC tissue, plasma, plasma exosomes	Prognostic	80
HCC	circ-ZEB1.33	HCC tissue, serum	Diagnostic, prognostic	153
HCC	circRNA_101237	HCC tissue, serum	Prognostic, cisplatin resistance	154
HCC	circ-FOXP1	HCC tissue, serum	Diagnostic, prognostic	155
HCC	circ_0000798	Peripheral blood mononuclear cells	Diagnostic, prognostic	156
HCC	circ_104075	Serum	Diagnostic	94
HCC	hsa_circ_000224	Serum	Diagnostic	157
HCC	circPTGR1	Serum exosomes	Prognostic	158
CCA	circCdr1as	CCA tissue	Prognostic	159
Downregulated circRNA				
HCC	hsa_circ_0001649	HCC tissue	Prognostic, diagnostic	160 161
HCC	circMTO1	HCC tissue	Prognostic	47
HCC	circZKSCAN1	HCC tissue	Diagnostic	162
HCC	circSETD3 hsa_circ_0000567	HCC tissue	Prognostic	97
HCC	hsa_circ_0028502	HCC tissue	Diagnostic	163
HCC	hsa_circ_0076251	HCC tissue	Diagnostic, prognostic	163
HCC	circ-ITCH	HCC tissue	Prognostic	65
HCC	hsa_circ_0068669	HCC tissue	Diagnostic, prognostic	164
HCC	hsa_circ_0003570	HCC tissue	Diagnostic	165
HCC	hsa_circ_0078602	HCC tissue	Diagnostic, prognostic	166
HCC	hsa_circ_0004018	HCC tissue	Diagnostic	167
HCC	circC3P1	HCC tissue	Prognostic	168

(*continued on next page*)

Table 2 (*continued*)

Liver cancer	circRNA ID	Biological sample	Biomarker relevance	Ref.
HCC	circTRIM33–12	HCC tissue	Prognostic	77
HCC	hsa_circ_0091570	HCC tissue	Diagnostic, prognostic	169
HCC	circRNA_101505	HCC tissue	Prognostic cisplatin resistance	170
HCC	circHIAT	HCC tissue	Prognostic	61
HCC	circADAMTS13	HCC tissue	Prognostic	171
HCC	circ-EPHB4 hsa_circ_0001730	HCC tissue	Diagnostic	172
HCC	circSMARCA5	HCC tissue, plasma	Diagnostic, prognostic	48,173
HCC	circADD3	HCC tissue, plasma	Diagnostic, prognostic	174
HCC	hsa_circ_0001445	Plasma	Diagnostic	175
HCC	hsa_circ_0051443	Plasma exosomes	Diagnostic	176
HCC	hsa_circ_00156	Serum	Diagnostic	157
HCC	hsa_circ _000520	Serum	Diagnostic, prognostic	157

CCA, cholangiocarcinoma; CircRNA, circular RNA; HCC, hepatocellular carcinoma.

molecule implicated in the sponging of miRNAs. It should also be noted that even if the aforementioned studies performed experimental validations using animal models (subcutaneous injection of cancer-derived cell lines stably over- or under-expressing a specific circRNA), the readout of these experiments is mainly tumour growth. Therefore, even though *in vivo* experiments confirmed the tumour suppressive or pro-oncogenic role of circRNAs, further investigations are needed to validate their involvement in several of the hallmarks of cancer. This aspect could be critical if circRNAs' function is context-dependent and should be considered in view of emerging strategies for specific knockdown of circRNAs *in vivo*.[86]

CircRNAs: clinical opportunities in liver cancer

CircRNAs are promising clinically relevant biomarkers

In addition to the new insights on their regulatory functions, circRNAs also provide good opportunities to improve the management of patients with liver cancer. Indeed, even if most circRNAs are expressed at a low level (roughly <10% of the expression of their linear counterpart),[87] their circular structure provides a higher resistance to exonuclease activity and therefore an expanded half-life. Such intrinsic physical features mean that circRNAs could be promising diagnostic and prognostic biomarkers. In addition, circRNAs are found circulating in liquid biopsies (*e.g.* blood, urine, saliva, bile),[5,88] either freely or embedded into EVs.[89] A recent study demonstrated that circRNAs are more specifically enriched and stable in exosomes compared to host secretory cells.[90] Therefore, exosomal circRNAs could be useful minimally invasive biomarkers. They provide clear benefits over protein biomarkers which are prone to degradation and present limited organ specificity.[91] In addition, methodologies used for circRNA detection are usually more specific and sensitive than the immunoassays commonly used for protein biomarkers.[92] Indeed, as nucleic acids, circRNAs can be easily and specifically amplified by reverse-transcription quantitative PCR using specific primers overlapping the back-splice junction.[34] It was further demonstrated that circRNAs display tissue- and cell-specific expression patterns,[1] a feature which could be helpful for diagnosis. Thus, circRNAs appear as promising molecules for the management of patients with cancer, as they exhibit many of the features expected of a biomarker: they are often more stable than linear RNAs, including miRNAs, and require minimally invasive intervention to be detected in body fluids by specific and sensitive methods. Accordingly, several ongoing clinical trials are exploring circRNAs as biomarkers in cancer, including biliary tract cancers (*e.g.* NCT03334708, NCT04584996, NCT04792437). However, it should be noted that a high level of circRNAs from serum/plasma may also only reflect a high level of circulating cells, as was reported for miRNAs.[3,93]

CircRNAs for liver cancer diagnosis and prognosis

Recent profiling studies support the utility of circRNAs as potential diagnostic and prognostic biomarkers in patients with liver cancer (Table 2). However, these studies currently suffer from limited sample sizes; hence, a robust signature of circRNAs commonly deregulated in patients with liver cancer remains elusive. Despite this weakness, merging the results of 3 independent studies identified circ_104075 as a circRNA recurrently upregulated in tissues and sera of patients with HCC compared to healthy individuals.[94] Its expression is particularly high in HCC compared to other types of cancer and liver disease, suggesting that it could represent a biomarker for HCC diagnosis.[94] Further statistical analyses demonstrated that circ_104075 exhibited a better sensitivity and specificity than biomarkers commonly used to diagnose HCC, such as alpha-fetoprotein.[94] Such observations could be practice changing given that alpha-fetoprotein serum dosage and ultrasound, which are routinely used to diagnose early stage HCC, sometimes lack precision.[54] Thus, patients could benefit from these innovative specific biomarkers if they enable the early detection of HCC. Similarly, circCDYL was expressed during the early stages of HCC, and thus might be a clinically relevant biomarker for early diagnosis and surveillance in high-risk populations, including patients with hepatitis B or C infection and/or cirrhosis.[95]

The prognostic potential of circRNAs has also been reported in liver cancer. A recent meta-analysis in HCC highlighted a tight correlation between an elevated expression of pro-oncogenic circRNAs and poor clinical outcomes such as reduced overall survival.[96] Two other studies reported that low expression of circMTO1 and circSETD3 in HCC was significantly associated with poor prognosis.[47,97] In the same vein, it was shown that low levels of circSMARCA5 in iCCA correlate with poor overall survival. Interestingly, overexpression of circSMARCA5 in iCCA cell lines improves chemosensitivity to gemcitabine and cisplatin.[81] Likewise, downregulation of circ-0003418 resulted in cisplatin resistance in HCC cells through activation of the Wnt/β-catenin pathway.[98] Lately, circRNA-SORE was reported to mediate sorafenib resistance in HCC by stabilising YBX1, a master oncogenic factor. CircRNA-SORE acts by trapping YBX1 and thus, preventing its degradation by the E3 ubiquitin system. More interestingly,

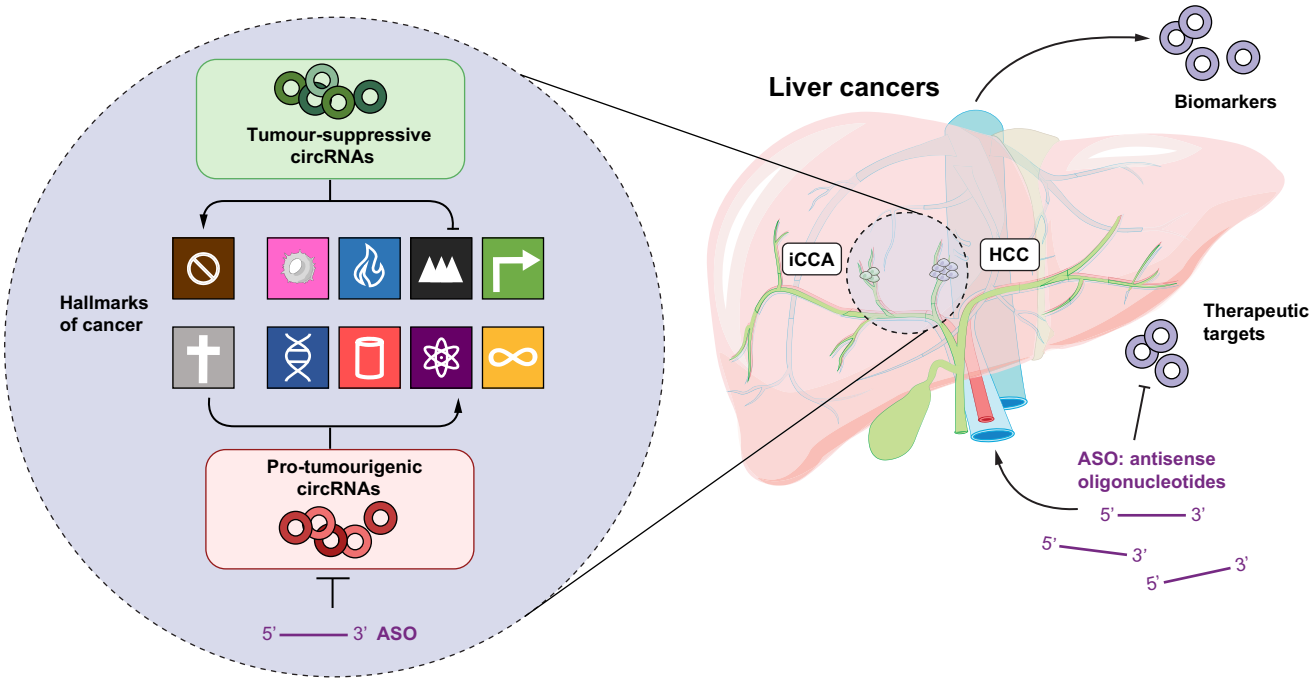

Fig. 4. CircRNAs as innovative biomarkers and therapeutic targets in liver cancer. The left panel highlights tumour-suppressive and pro-tumourigenic circRNAs acting on well-known cancer hallmarks. The right panel highlights circRNAs as innovative biomarkers and therapeutic targets using ASOs. ASO, antisense oligonucleotide; circRNA, circular RNA; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma.

circRNA-SORE-related features spread across cancer cells through exosomal communication.[99] These observations raise the possibility of developing innovative circRNA biomarkers to predict response to therapy.

CircRNAs as companion biomarkers for targeted therapies in liver cancer

Several targeted therapies are currently under clinical evaluation in liver cancer.[100] In this context, the development of robust diagnostic tools to select the most suitable personalised therapy could be valuable. In HCC, nivolumab, an immune checkpoint inhibitor targeting PD-1, was approved by the FDA in 2017. However, objective anti-tumour response rates were only 15-20%.[54,101] Therefore, a circRNA signature predictive of anti-PD1 response might be relevant. This innovative concept involving the development of companion biomarker tests from circRNA signatures may represent a breakthrough to guide targeted therapies. So far, to the best of our knowledge, there is no published report of circRNAs as companion biomarkers and/or theranostic targets that can predict response to a specific therapy, either in HCC or in iCCA.

Future directions: circRNAs and therapeutic opportunities

As mentioned above, there is growing evidence showing that circRNAs are deregulated in cancer and contribute to the regulation of oncogenic processes (Fig. 3). Thus, circRNAs might be considered as relevant therapeutic targets in cancer. The singularity of circRNA regulatory functions offers various opportunities to regulate their action during tumour progression and therefore, to develop innovative therapeutic strategies.

The pro-oncogenic activity of numerous circRNAs relies on sponging of tumour suppressor miRNAs.[47,48] Thus, several strategies could be developed to reduce this pro-oncogenic activity, including the development of specific target site blockers (TSBs). TSB antisense oligonucleotides (ASOs) could be designed to target miRNA response elements carried by circRNAs. Thus, by competitive binding, TSBs could restore the tumour suppressor activity of a given miRNA. Although this strategy has not been applied to circRNAs yet, it showed great efficacy when applied to linear RNA sponges *in vitro* and *in vivo*.[102] Another strategy aiming at directly decreasing circRNA abundance could be even more efficient. For that purpose, antisense locked nucleic acid gapmers specifically targeting the back-splice junction of circRNAs could switch off the expression of a specific circRNA without affecting the expression of the parental linear RNA. Several studies already demonstrated the efficacy of this approach not only with gapmers, but also with small-interfering RNAs and shRNAs. Besides, it was shown that circHIPK3 silencing in mice *via* adeno-associated virus shRNA could alleviate diabetic proliferative retinopathy.[103] CRISPR/Cas13-based RNA editing systems[104] could also be used to specifically silence circRNA activity. In addition, the abundance of pro-oncogenic circRNAs could be modulated at an upstream level by interfering with the splicing machinery to control the occurrence of back-splicing events. Indeed, it was already reported that circRNA biogenesis during EMT is tightly regulated by the RBP splicing factor QKI5.[23] Thus, one could hypothesise that modulating the expression level of a specific RBP could control the expression of circRNAs. Otherwise, as indicated above, there is strong evidence that circRNA biogenesis is mediated by hybridisation of complementary

intronic regions flanking circRNA sequences.[21,22] Thus, one might anticipate that targeting these complementary sequences on pre-messenger RNAs using ASOs may block circRNA biogenesis by preventing the interaction of back-spliced regions. On the contrary, ASOs targeting key splice domains could be designed to enhance circRNA biogenesis. Recently, a base editing strategy targeting key back-splice domains showed great efficacy to specifically repress circRNA expression without affecting the linear counterpart RNAs (bioRxiv; https://doi.org/10.1101/2021.08.05.455347). This study provides an efficient method to deplete circRNAs for functional studies.

Nucleic acid-based therapeutics represent a promising approach in cancer. To date, several RNA-based drugs like ASOs are already FDA approved, and many are under clinical evaluation,[105] paving the way for circRNA-based therapeutics. Indeed, circRNAs are often more stable than mRNA or miRNA molecules, and methods have already been developed to produce synthetic circRNAs.[106] The advantage of this approach is the possibility of designing specific circRNAs. For example, a synthetic circRNA containing 5 binding sites for miR-21, an overexpressed onco-miRNA, was shown to effectively suppress miR-21 activity and to induce apoptosis in gastric cancer cell lines.[107] Similarly, synthetic circRNAs could be designed to sequester onco-proteins. Indeed, a specific artificial circRNA able to sequester and inactivate the RBP hnRNP has already been engineered.[108] Considering that RBPs participate in cancer progression, especially via splicing deregulation, this proof-of-concept study opens new avenues for promising circRNA-based therapeutics. In addition, it was demonstrated that exogenous circRNA can efficiently produce proteins *in vitro*.[109] Thus, circRNA carrying IRES could be specifically engineered to express tumour suppressor proteins.

Conclusions

A plethora of therapeutic strategies are being developed to target circRNAs in liver cancer. Although proof-of-concept studies have reported promising results, it must be taken into account that circRNAs are still newly described protagonists in cancer onset and progression. Indeed, a great deal of effort is needed to fully understand the physiological roles, the regulatory functions and the biogenesis of circRNAs. A better understanding of these molecular mechanisms will provide a deeper insight into the specific role of circRNAs in the whole RNA regulatory network governing cancer hallmarks. This knowledge could eventually pave the way for circRNA-mediated molecular therapies and for clinically relevant biomarkers in liver cancer (Fig. 4). Thus, as an emerging field, circRNAs represent a wealth of opportunities for future research.

Abbreviations

ASO, antisense oligonucleotide; CCA, cholangiocarcinoma; circRNA, circular RNA; CLIP, cross-linking immunoprecipitation; EMT, epithelial-to-mesenchymal transition; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; HN1, haematopoietic- and neurologic-expressed sequence 1; IRES, internal ribosome entry sites; miRNA, microRNA; NGS, next-generation sequencing; QKI, Quaking; RBP, RNA-binding protein; RISC, RNA-induced silencing complex; shRNA, small-hairpin RNA; snRNP, small nuclear ribonuclear proteins; TAM, tumour-associated macrophage; TSB, target site blockers.

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Conflict of interest

The authors declare no competing interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

All the authors contributed to all aspects of the work. CL and DL contributed equally as co-first authors.

Supplementary data

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Author names in bold designate shared co-first authorship

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