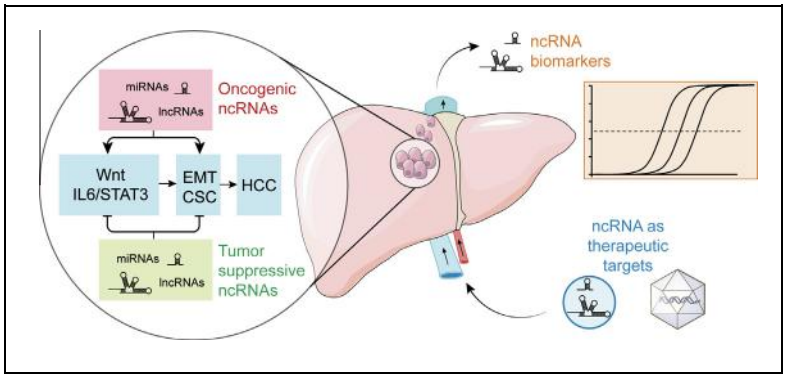
Review

EASL JOURNAL OF HEPATOLOGY

Non-coding RNA in hepatocellular carcinoma: Mechanisms, biomarkers and therapeutic targets

Graphical Abstract



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Non-coding RNA in hepatocellular carcinoma: Mechanisms, biomarkers and therapeutic targets

Marcel Klingenberg[1,2,3], Akiko Matsuda[4,5], Sven Diederichs[1,2,3,6,7,8,*,†], Tushar Patel[4,5,*,†]

Summary

The majority of the human genome is not translated into proteins but can be transcribed into RNA. Even though the resulting non-coding RNAs (ncRNAs) do not encode for proteins, they contribute to diseases such as cancer. Here, we review examples of the functions of ncRNAs in liver cancer and their potential use for the detection and treatment of liver cancer.

Keywords: Hepatocellular carcinoma; HCC; Long non-coding RNA; lncRNA; microRNA; Liver cancer; STAT3; Wnt; Epithelial–to-mesenchymal transition; Cancer stem cell.

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Introduction

The development of high-throughput RNA sequencing technology has led to the discovery of thousands of non-coding RNA (ncRNA) genes. The number of newly identified ncRNA genes is increasing and already outnumbers the number of coding transcripts.[1] ncRNAs are participating in a plethora of physiological and pathological processes.[2,3] This was surprising since most ncRNAs were thought to be the product of random transcription without any intrinsic function. But instead of solely being "junk RNA", there is evidence that ncRNAs are functionally important molecules in the cell.[2] In general, ncRNAs are a heterogeneous group of molecules and a simple sub-classification can be made by dividing the ncRNAs into short (sncRNAs) and long ncRNAs (lncRNAs) with an arbitrary size cut-off at 200 bases of length. The common feature of both groups is their name-giving lack of protein-coding capacity, meaning the functional molecule is the RNA itself. Here, we will focus on the roles of the two most studied classes of the ncRNA family, microRNA (miRNA) and lncRNA.

The heterogeneity of ncRNAs (Fig. 1) can be explained by the diverse biological functions of ncRNAs in the cell, which also require a high structural diversity. Some ncRNAs, especially lncRNAs, show a lot of similarities to messenger RNA (mRNA). This includes RNA-processing (splicing,[4] capping,[5] poly-adenylation,[1] RNA-editing[2]), and differential regulation in cancer and embryogenesis.[1] Like mRNAs, several lncRNAs are localized to specific cellular compartments, depending on their biological function.[6] Nuclear lncRNAs can act as guides for chromatin-modifying-complexes[7] or transcription factors.[8] LncRNAs in the cytoplasm often function as regulators of protein levels, either by directly controlling mRNA stability[9] or by acting as so-called competing endogenous RNA (ceRNA). ceRNAs are thought to function by sequestering miRNAs, acting as molecular miRNA sponges.[10] Well-described examples for functionally important lncRNAs are *HOTAIR*, highly upregulated in liver cancer (*HULC*), and *TERC* (Fig. 2). These lncRNAs function via different molecular mechanisms, involving the interaction with DNA, RNA, and proteins. *HOTAIR* regulates the epigenetic silencing of the *HOXD* locus,[11] *HULC* could function as a ceRNA,[10] and *TERC* is part of the catalytical center of the telomerase enzyme complex.[12] Depending on their biological function and (de-)regulation, lncRNAs can be classified similarly to protein-coding genes, into either oncogenic[7–10,13–19] or tumor suppressive.[20–24]

There are several different classes of sncRNA. Amongst these, the most studied are miRNAs, and piwi-interacting RNAs (piRNAs).[25] Mature miRNAs are short, approximately 22 nucleotides long single-stranded RNA molecules derived either from hairpin or double-stranded RNA precursors.[26,27] The stem-loop structure of pri-miRNAs is cleaved by the enzyme DROSHA within the nucleus and results in precursor miRNAs, pre-miRNAs which are exported from the nucleus into the cytoplasm by Exportin-5 (XPO5) and processed by Dicer (DICER1), an RNase III enzyme, to generate a miRNA duplex. After unwinding, the mature miRNA strand is incorporated into an Argonaute-containing RNA-induced silencing complex (RISC).[28] In the process of siRNA maturation, precursor siRNA is also separated by

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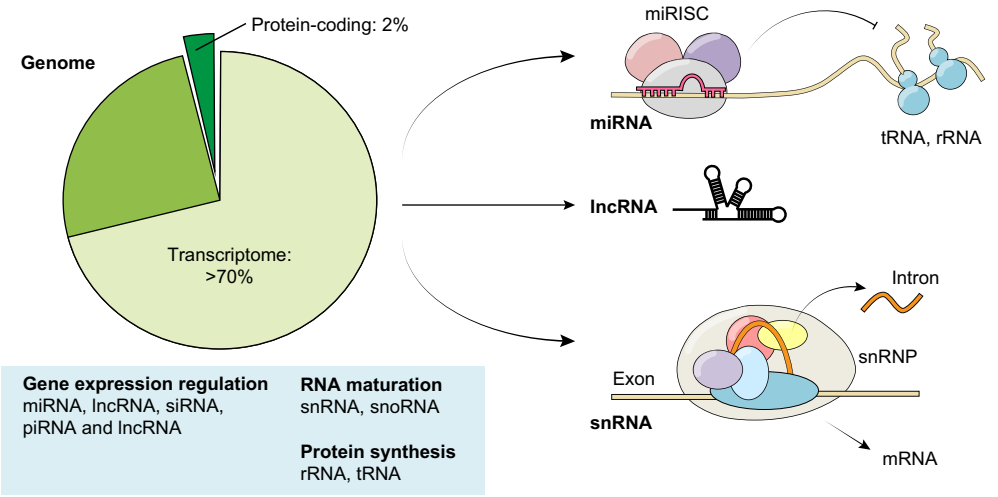

Fig. 1. ncRNA functions. Only 2.0% of the transcriptome encode for proteins. More than 70% of the genome is transcribed to ncRNAs. miRNA, siRNA, piRNA and lncRNA regulate gene expression, small nuclear RNA (snRNA) and small nucleolar (snoRNA) are involved in RNA maturation, and transfer RNA (tRNA) and ribosomal (rRNA) play a role in protein synthesis.

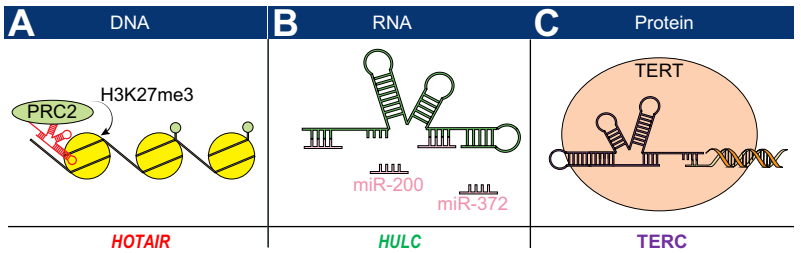

Fig. 2. LncRNAs function via interaction with DNA, RNA, or protein. (A) *HOTAIR* acts as a guide for the recruitment of the PRC2 complex to specific genomic loci. (B) *HULC* could function as a competitive endogenous RNA and sequester miRNAs. (C) TERC is part of the TERT complex and contributes to telomere lengthening.

Key point

ncRNAs have an important role in liver carcinogenesis.

DICER and then forms an RISC.[29] A single miRNA can target a broad range of mRNAs and suppress translation via non-perfect pairing with target mRNAs, usually involving a seed pairing of just six to eight nucleotides in length,[30] or (as with siRNAs) cause degradation of target RNAs by the RISC in the case of perfect complementarity to the target site.[31]

Both, lncRNAs and miRNAs, are often deregulated in liver cancer, underlining the importance of ncRNAs in hepatocarcinogenic processes.[1,32] Through a better understanding of how ncRNAs exert their functions in hepatocarcinogenesis, we might find solutions for the most urgent challenges faced in liver cancer management. This includes the need for a detection method which allows an earlier diagnosis of liver cancer and the development of more effective treatment options for advanced liver cancer stages. Mainly due to late diagnosis and poor therapy response, liver cancer causes approximately 750,000 deaths annually. It is the second leading cause of cancer-related deaths worldwide, only outranked by lung cancer.[33] Within the class of liver cancers, hepatocellular carcinoma (HCC) is the most prevalent liver cancer subclass accounting for 70–90% of all cases, followed by cholangiocarcinoma. The five-year survival rate of HCC patients after initial diagnosis has slowly increased over the last years but is still lower than 20%.[34] So far, the only effective HCC therapy, which can be systemically administered, is the multiple kinase inhibitor sorafenib.[35] Other treatment options involve mainly surgical approaches, like partial resection of the liver, image-guided tumor ablation methods, and liver transplantation.[36]

The aim of this review is to give an overview of the different roles of ncRNAs in liver cancer and how these basic research findings could be translated into clinical practice. We will first explain some of the functions and molecular mechanisms of ncRNAs, next discuss the role of ncRNAs as biomarkers, then give some examples for ncRNA targeted therapy approaches, and conclude with an outlook about how ncRNAs might impact HCC management in the future.

Selected functions and molecular mechanisms of ncRNAs in HCC

ncRNAs involved in Wnt signaling

The Wnt signaling pathway is evolutionarily highly conserved. It determines cell fates and is pathologically activated in HCC.[37,38] In brief, activation of Wnt signaling is initiated via soluble Wnt proteins, *e.g.* WNT5A, which bind to the membrane-bound G-protein coupled receptor (GPCR) of the Frizzled family. Upon binding of WNT5A to its receptor, the beta-catenin (CTNNB1) destruction complex gets inactivated and allows CTNNB1 to accumulate. The rise of CTNNB1 levels in the cytoplasm leads to elevated CTNNB1 levels in the nucleus, where CTNNB1 acts as a transactivator of LEF/TCF transcription factors.[39] Protein-coding genes, involved

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in Wnt signaling, are mutated in approximately 30% of liver cancers.[40] Consistently, abnormal activation of the Wnt signaling pathway was found in about half of all HCC cases.[41] *CTNNB1* is one of the most frequently mutated protein-coding genes in HCC, but also genes which are part of the CTNNB1 destruction complex are frequently mutated (*AXIN1*, *APC*).[42,43] *CTNNB1* gain-of-function mutations are often found in a region which is important for CTNNB1 degradation.[44] Nuclear localization of CTNNB1 is associated with poorer prognosis of HCC patients.[45] In contrast, mutations often inactivate *AXIN1* which lowers the activity of the CTNNB1 destruction complex leading to increased CTNNB1 activity.[46]

In line with the mutational pattern of protein-coding genes, the stabilization of CTNNB1 seems to be a ncRNA-mediated mechanism as well (Fig. 3). For instance, the lncRNA *DANCR* binds to the 3′ untranslated region of *CTNNB1* mRNA and blocks by competitive binding the sites for miR-214, miR-320a, and miR-199a. Blocking of the miRNA-mediated suppression of CTNNB1 translation by *DANCR* leads in turn to increased CTNNB1 protein levels and subsequent activation of Wnt signaling. *DANCR* is upregulated in human HCC and significantly correlated with poor prognosis.[13] *In vivo* mouse experiments show that *DANCR* overexpression results in a higher tumor initiation capability of HCC cells and increased liver and lung colonization.[13] Another lncRNA-mediated mechanism which stabilizes *CTNNB1* involves the recruitment of an RNA-binding protein (RBP) to *CTNNB1*. RBPs can have a major impact on the half-life of lncRNA and mRNA molecules.[47,48] Recruitment of the RBP ELAVL1 to *CTNNB1*, mediated via the lncRNA *UFC1*, stabilizes *CTNNB1* levels in the cytoplasm and increases levels of CTNNB1 in the nucleus.[14]

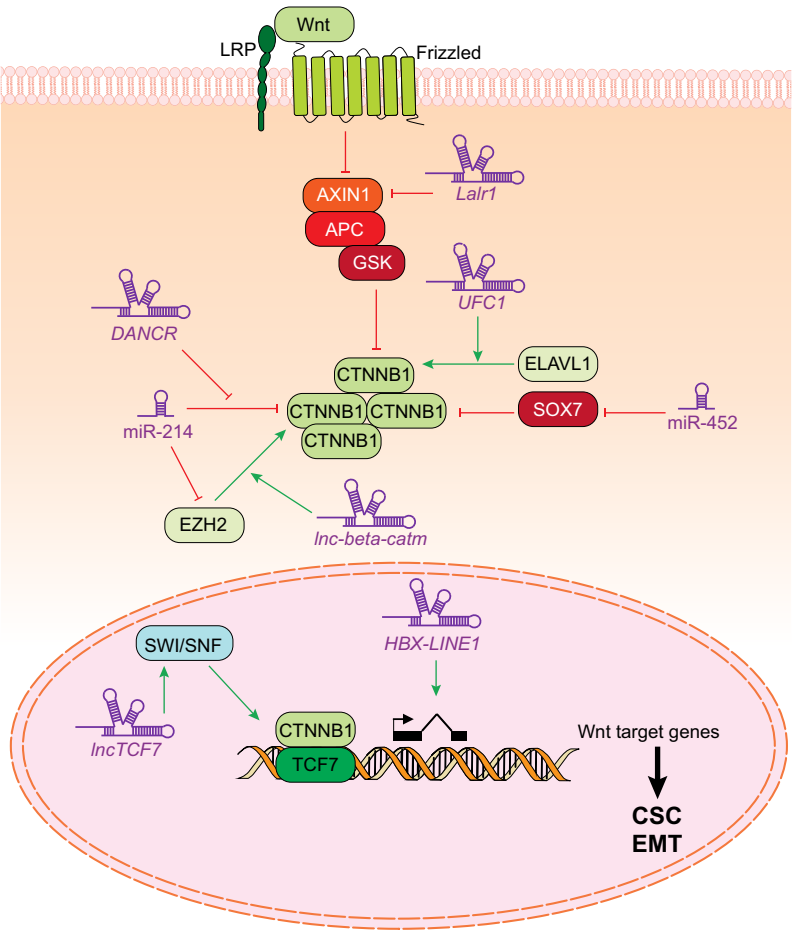

Fig. 3. ncRNAs involved in Wnt signaling. ncRNAs are shown as lilac pictographs. Proteins participating in Wnt signaling are depicted as different geometric shapes in either green (activators) or red (inhibitors) shades. CSC, cancer stem cell; EMT, epithelial-mesenchymal transition.

Similar to *DANCR* and *UFC1*, the lncRNA *lnc-beta-catm* functions via a CTNNB1 stabilization mechanism. In contrast to *DANCR*, *lnc-beta-catm* acts via the stabilization of CTNNB1 protein and not mRNA. *lnc-beta-catm* binds to the methyltransferase EZH2 and recruits it to CTNNB1. EZH2 subsequently methylates a lysine residue of the CTNNB1 protein and thereby suppresses its ubiquitination via the E3 ubiquitin ligase BTRC, leading to enhanced Wnt signaling activity.[19] *LncTCF7* is another lncRNA modulating Wnt signaling. It binds to the DNA in the *TCF7* promoter region as well as the chromatin-modifying SWI/SNF proteins, thereby acting as a guiding co-factor, recruiting SWI/SNF to the *TCF7* promoter. Thereby, TCF7 expression is triggered *TCF7* then which binds to CTNNB1 and Wnt target gene expression is increased.[7]

Additional lines of evidence underline the impact of lncRNAs on Wnt signaling in HCC. Whole-transcriptome sequencing of hepatitis B virus (HBV) positive HCCs identified a preferential genomic integration site for HBV DNA. This specific integration was found in 23% of all HBV-associated HCCs and correlates with the poorer survival of HCC patients. Functionally, the integration at this specific locus gives rise to a human-viral chimeric transcript, named *HBx-LINE1*. *HBx-LINE1* activates Wnt signaling via the enhancement of CTNNB1 transactivity. Furthermore, *HBx-LINE1* transgenic mice are more susceptible to tumor formation in a diethylnitrosamine-induced liver cancer model.[49] Also, *HBx-LINE1* functions as a ceRNA for miR-122.[50] The lncRNA *HOTAIR* is overexpressed in HBx-positive human liver tumors and involved in the regulation of Wnt signaling genes, including *EpCAM*, *Nanog*, *Sox2*, and *Oct4*. Mechanistically, the regulation of gene expression is mediated via an interaction between *HOTAIR* and SUZ12/PRC2, which leads to a de-repression of PRC2 target genes.[51,52] Moreover, the lncRNA *Lalr1* is upregulated during liver regeneration in a 2/3 hepatectomy mouse model. On a molecular level, *Lalr1* recruits the chromatin-architectural component Ctcf to the *Axin1* locus, which results in the subsequent sup-

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pression of *Axin1*. Consequently, Ctnnb1 is stabilized and contributes to cell cycle progression.[53]

Similarly, miRNAs can also act on Wnt signaling (Fig. 3). miR-452 is markedly increased in stem-like HCC cells and human HCC tissues and its expression in HCC patients predicts poor overall survival. Conversely, miR-452 is downregulated in gliomas[48] and breast cancer[49] and its decrease enhances the stem-like characteristics and tumorigenesis of gliomas.[54] Moreover, the tumor suppressor *SOX7*[55,56] is a direct target of miR-452.[57] SOX7 physically binds to CTNNB1 and TCF4 in the nucleus, inhibits Wnt signaling and thereby modulates self-renewal for stem cells and cancer stem cells (CSC).[39,58] Another example of a sncRNA, which influences Wnt signaling, is miR-214. miR-214 is downregulated in HCC[59,60] and is correlated with early recurrence.[61] *EZH2* and *CTNNB1* are identified as two potential targets of miR-214.[62,63] Mutations of *CTNNB1*[64] and overexpression of EZH2, which regulates CTNNB1, have been reported in HCC.[62,65] EZH2 can also function independently of the PRC2 complex and thus could act via other, yet unknown mechanisms, in addition to CTNNB1 methylation.[65,66]

The upregulation of EZH2 and CTNNB1 are correlated with early recurrence and can be an independent predictor of poor survival.[62] The silencing of EZH2 or CTNNB1 expression suppresses the growth and invasion of HCC cells, while the silencing of miR-214 or overexpression of EZH2 increases EpCAM-positive stem-like cells through the activation of CTNNB1.[61] Hence, miR-214 can directly or indirectly modulate the Wnt signaling pathway in HCC. Furthermore, overexpression of miR-214 in HCC cells inhibits proliferation by inducing a G1-S checkpoint arrest. Conversely, silencing miR-214 with siRNA promotes cell cycle progression and accelerates the proliferation of HCC cells.[67] Additionally, loss of PRC2 function results in an overexpression of selected Wnt-regulated genes in HBV-related HCCs.[51]

Key point

ncRNAs are involved in Wnt and STAT3 signaling in HCC.

ncRNAs involved in STAT3 signaling

The STAT3 pathway is another pathway in HCC which is commonly influenced by ncRNAs. STAT3 is a transcription factor which regulates a variety of genes. STAT3 signaling can be induced by the interleukin 6 (IL6) family cytokine receptors, GPCRs or Toll-like receptors. In the case of IL6 receptor (IL6R) activation, the binding of IL6 leads to IL6R dimerization and activation of a receptor associated protein, named Janus Kinase 2 (JAK2). STAT3 gets phosphorylated by JAK2 and subsequently forms the functionally active dimer. STAT3 dimers translocate to the nucleus and activate the transcription of pro-survival, inflammation, epithelial-to-mesenchymal transition (EMT) and CSC associated genes.[68] Interestingly, 87% of the STAT3 target genes are non-coding, highlighting the close relationship between ncRNA and the STAT3 pathway.[69] It is widely accepted that HCC arises often in the context of a chronically inflamed liver, caused for instance by hepatotropic viruses (HBV and HCV) or chemical-induced liver damage. IL6 is one of the early cytokines secreted during an acute phase of inflammatory response to promote liver regeneration, partly by upregulating the expression of the fibrinogenic genes in the liver.[70,71] Chronic IL6 stimulation induces liver tumorigenesis[72] potentially by overactivation of STAT3, which regulates many genes directly involved in the progression of HCC and its preliminary stages.[73,74] In addition, IL6 secretion by stromal cells induces the formation of CSCs[75] via the upregulation of octamer-binding transcription factor 4 (OCT4).[76] Furthermore, a positive feedback loop links IL6 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[77]

Several lncRNAs either activate[9,78] or inhibit[20,22] STAT3 signaling in liver cancer (Fig. 4). The lncRNA activated by TGF-beta (*lncRNA-ATB*) stabilizes interleukin 11 *(IL11)* mRNA via an RNA-duplex formation mechanism. This leads to increased secretion of IL11 and autocrine stimulation of STAT3 mediated transcription. Another lncRNA, urothelial carcinoma associated-1 (*UCA1*, a.k.a. *CUDR*), promotes activation of NF-κB/STAT3 signaling in hepatocyte-like-stem cells. *UCA1* induces also the malignant transformation of hepatocyte-like-stem cells in an experimental mouse model.[78] Moreover, *UCA1* contributes to the proliferation of HCC cells via regulation of *CDKN1B*.[79] *UCA1* is also linked to the development of drug resistances in human cancer cells.[80] The lncRNA *lncSox4* binds directly to STAT3 and recruits it to the promotor region of *SOX4*, where it drives an increased *SOX4* expression. *LncSox4* is upregulated specifically in patient-derived hepatic CSCs.[8] In contrast, the lncRNA *DILC* interferes with the NF-κB-mediated IL6 expression and exerts an inhibitory effect on STAT3 signaling, thereby providing an example for a tumor suppressive lncRNA.[20]

Similarly, miRNAs are involved in the regulation of the STAT3 signaling pathway (Fig. 4). miR-21 expression is increased in many cancers including HCC, and targets tumor suppressor genes such as phosphatase and tensin homolog (*PTEN*),[81] programmed cell death 4 (*PDCD4*)[82] and *TP53*.[83] IL6 can activate miR-21 through direct binding of STAT3 to an upstream enhancer of miR-21.[84,85] In addition, miR-21 is part of a regulatory network involving hepatocyte nuclear factor 1 alpha (HNF1α/HNF1A), PTPN6 (SHP-1), RELA (p65), STAT3, miR-146a and miR-21, which modulates hepatic fibrogenesis.[84] Hepatocytes that are injured or damaged can release IL6 and transforming growth factor beta-1 (TGFβ1/TGFB1) to activate the quiescent hepatic stellate cells (HSCs), and activated HSCs release IL6 and tumor necrosis factor alpha (TNFα/TNF) upregulating miR-21 and miR-146a to further aggravate the

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hepatic damage. Additionally, miR-124 can directly inhibit the expression of STAT3 and phosphoinositide 3-kinase catalytic subunit alpha (PIK3CA) to induce a G1-arrest and suppress proliferation. Hence, downregulation of the tumor suppressive miR-124 in HCC activates proliferation via STAT3 and PIK3CA.[86,87] Transient inhibition of hepatocyte nuclear factor 4 alpha (HNF4α/HNF4A), which is essential for liver development and hepatocyte function, is sufficient to initiate malignant transformation through a network including miR-124, IL6R, STAT3, miR-24 and miR-629.[88] Furthermore, systemic administration of miR-124 prevents and suppresses hepatocellular carcinogenesis by inducing tumor-specific apoptosis without toxic side effects *in vivo*. A network comprised of HNF4A, miR-124, miR-7, RELA and miR-21, which modulates HCC initiation and progression, might be useful to predict the prognosis of HCC patients.[89]

ncRNAs involved in the determination and maintenance of hepatic CSCs

The liver is a highly complex organ with a variety of functions in human physiology. Despite its complex structure, the liver is able to regenerate after partial hepatectomy. This rare replicative capacity is a beneficial feature for patients recovering from a massive liver injury or a partial hepatectomy. However, this feature is also a double-edged sword since even mature hepatocytes are still able to proliferate.[90] This may explain how the liver can quickly compensate cell loss with hyperplasia but it also explains the stem-cell-like features in liver cancer cells.[91] Consistently, several signaling cascades have been linked to both liver regeneration and the hepatic CSC phenotype.[92,93] CSCs are a population of cancer cells that can form tumors and possess the properties of self-renewal, and differentiation.[94] The existence of CSCs has been postulated in many solid tumors including HCC.[95] Epidemiological data suggest that up to 40% of HCCs develop from clonal populations originated from hepatic CSCs.[96] CSCs are hypothesized to be involved in tumor resistance to therapy, recurrence, relapse and metastasis.[97]

Apart from influencing Wnt signaling and STAT3, there are additional examples of how lncRNAs contribute to the formation and maintenance of hepatic CSCs. The ICAM-1-related *(ICR)* lncRNA was identified in a screen for differentially regulated RNAs in portal vein tumor thrombus (PVTT) *vs.* corresponding primary tumors. *ICR* contains a sequence complementary to *ICAM1* mRNA and regulates *ICAM1* stability by RNA-duplex formation. *ICR* contributes to self-renewal of hepatic CSC and is regulated by the pluripotency-associated transcription factor NANOG.[18] Plasmacytoma variant translocation 1 (*PVT1*) is another lncRNA which contributes to the CSC phenotype of liver cancer cells. *PVT1* is upregulated in different cancer entities including ovarian,

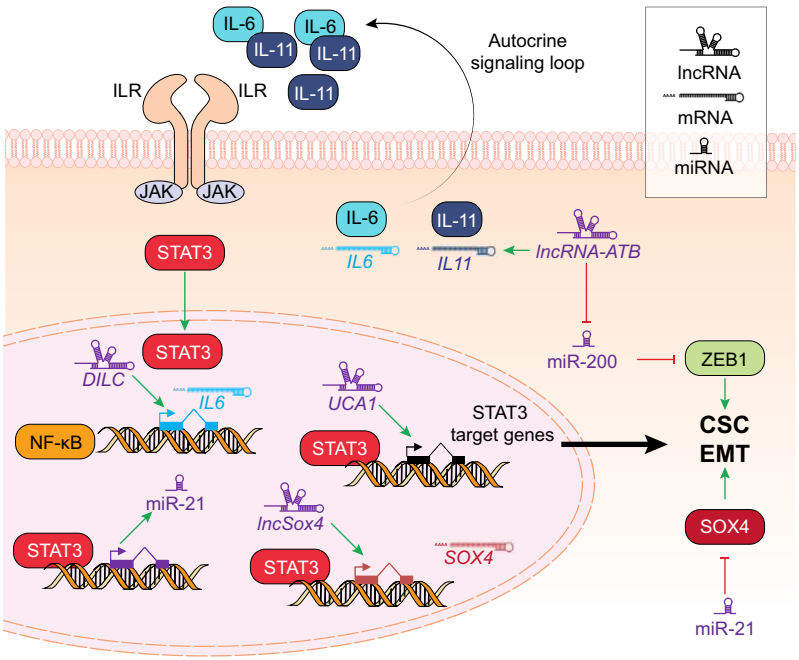

Fig. 4. ncRNAs involved in STAT3 signaling. ncRNAs are shown as lilac pictographs. Proteins participating in STAT3 signaling are depicted as different geometric shapes. CSC, cancer stem cell; EMT, epithelial-mesenchymal transition.

breast and liver cancer.[17,98] On a molecular level, *PVT1* interacts with the NOP2 protein and regulates its stability, resulting in altered rRNA modification and augmented stem-cell-like properties of liver cancer cells.[17] Moreover, *PVT1* is discussed as a ceRNA for miR-152 and as a positive regulator for MYC stability.[99,100]

Several lines of evidence also link sncRNAs to hepatic CSCs. miR-122 is a marker of hepatocyte-specific differentiation and accounts for 70% of the total adult liver miRNA content.[101,102] The physiological role of miR-122 is maintaining the homeostasis of hepatocyte differentiation.[103] In HCC tissue, miR-122 expression is decreased in more than 70% of the cases.[104] The mechanisms of this deregulation are unknown, but many targets of miR-122 in HCC have been elucidated, including cut homeobox 1 *(CUTL1)*, cyclin-G1 *(CCNG1)*, proto-oncogene Wnt1 (*WNT1*) and Myc proto-oncogene protein (*MYC*),[104–107] which are implicated in proliferation, apoptosis, and metastasis of HCC cells. miR-122 has various roles in the pathogenesis of liver diseases such as HCC, including the regulation of CSCs. Restoration of miR-122 in human HCC-BCLC9 cells with a solid stem-like cell profile, high tumor initiating ability and undetectable miR-122 expression decreases cell proliferation and reduces significantly the tumor size *in vivo*.[108] Moreover, restoration of miR-122 expression can reduce tumor growth by inducing tumor cell dormancy in a human HCC cell line with a consistent stem-like profile. Delivery of miR-122 to an MYC-driven HCC mouse model strongly inhibits tumorigenesis.[109]

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miR-122 also targets Krueppel-like factor 6 (*KLF6*), a pro-fibrogenic factor.[110] Re-establishing the tumor suppressive miR-122 expression is a promising therapeutic strategy that would work concurrently to reduce tumor aggressiveness and recurrence. Another miRNA that could contribute to a CSC phenotype in HCC is miR-155. The downregulation of miR-155 decreases the subpopulation of CD90- and CD133-positive cells, both are specific surface markers of CSCs,[111,112] the formation of spheres, and suppresses the expression of OCT4, one of the markers of an undifferentiated cell.

Key point

ncRNAs are involved in CSC and EMT signaling in HCC.

ncRNAs involved in the regulation of epithelial-to-mesenchymal transition

EMT is mediated by genetic and epigenetic changes in the cells, for example, activation of transcription factors such as Snail (SNAI1), TWIST, and ZEB1/2, upregulation of mesenchymal-related proteins such as vimentin (VIM), fibronectin (FN1), and N-cadherin (CDH2) and downregulation of intercellular junction proteins such as E-cadherin (CDH1), coxsackievirus and adenovirus receptor (CXADR), and claudins.[113,114] As a result, cells lose polarity and adhesion to the surrounding cells or tissue transitioning into a mesenchymal phenotype. Physiologically, EMT occurs during embryogenesis and wound healing. HCC cells undergoing EMT can subsequently gain invasive, migratory and stem cell-like properties allowing them to disseminate and metastasize.[115,116] Within an inflammatory environment, EMT of HSCs and other cells can promote deposition of extracellular matrix, lead to liver fibrosis, and a suitable environment for cancer progression and migration.

LncRNAs play a pivotal role in EMT and metastasis formation in several cancer entities including HCC.[3,117] The above mentioned Wnt and STAT3 signaling pathways are not only involved in hepatic CSC formation[37,92] but also in the induction of EMT.[68,115] The following ncRNAs function independently of Wnt and STAT3 signaling pathways in EMT induction:

During the rapid growth of cancer cells, parts of the tumor are often deprived of oxygen. This leads to a hypoxic condition in which hypoxia-inducible factor 1 alpha (HIF1A) signaling is activated. HIF1A has been linked directly to EMT[118] and contributes also indirectly to the metastatic cascade via tumor vascularization.[119] Several lncRNAs have been implicated in the modulation of HIF1A signaling. The long intergenic non-coding RNA-ROR (*linc-ROR*), a stress-responsive lncRNA, is highly enriched within HCC-derived extracellular vesicles in hypoxic conditions. Knockdown of *linc-ROR* decreases tumor cell viability and the expression of HIF1A and increases the expression of miR-145.[120] The lncRNA, low expression in tumor (*LET*) negatively regulates HIF1A signaling in HCC, acting as a tumor suppressive lncRNA. *LET* was significantly downregulated in HCC, lung squamous and colon carcinoma. Mechanistically, *LET* binds to interleukin enhancer-binding factor 3 (ILF3/NF90) and causes its degradation.[21] Subsequently, protein levels of HIF1A and CDC42 are downregulated leading to a suppression of the metastatic phenotype.[21] CPS1 intronic transcript 1 (*CPS1-IT*) is another tumor suppressive lncRNA, which exerts its function via negatively regulating HIF1A signaling and is also downregulated in HCC and correlated with overall and disease-free survival. Mechanistically, *CPS1-IT* impacts on HSP90 and HIF1A complex formation. *CPS1-IT* overexpression leads to a decreased HIF1A activity and downregulation of VIM, SNAI1, and TWIST.[121]

The lncRNA *HULC* is overexpressed in HCC.[122] Silencing *HULC* in HCC cells increases miR-200a expression and decreases *ZEB1* mRNA expression significantly. This correlation is also found in clinical HCC tissues. Thus, *HULC* induces HCC cells to activate EMT and then promotes tumor progression and metastasis through the miR-200a/ZEB1 signaling pathway.[123] Beyond regulation of EMT, *HULC* also controls HCC cell proliferation. Moreover, *HULC* is regulated by the RBP IGF2BP1, which itself proved to be essential for liver cancer cell proliferation.[47,124] *LncRNA-ATB* is another lncRNA, which is upregulated in PVTT. It acts via the same molecular mechanism as *HULC*, by functioning as a ceRNA for miR-200a and regulating ZEB1.[9,123] Similarly, ZNFX1 Antisense RNA 1 *(ZFAS1)* exerts its function by sequestering miR-150, which also targets the EMT regulator ZEB1.[15] ZEB1 levels are also influenced by the *ZEB1-AS1* lncRNA, which is upregulated in metastatic HCC tissue. *ZEB1-AS1* is sharing a bidirectional promoter with its target gene *ZEB1* and positively regulates ZEB1 expression and EMT.[125] In bladder cancer, the paralog *ZEB2* is similarly regulated via its corresponding antisense transcript *ZEB2-AS1*.[126]

Several miRNAs, such as miR-155, miR-194, and miR-200a, have been implicated in EMT during HCC initiation and progression. At its physiological level, miR-155 regulates the homeostasis of the immune system and differentiation of the hematopoietic lineage.[127] However, miR-155 is overexpressed in several types of human cancers including HCC.[128,129] miR-155 expression can be induced by the activation of macrophages through the NF-κB pathway[130] and a broad range of pro-inflammatory cytokines like TNF, interferon γ (IFNγ/IFNG), and interferon β (IFNβ/IFNB1),[131] functioning as a critical link between inflammation and hepatocarcinogenesis. miR-155 is upregulated by the TGFβ pathway, which plays an important role in EMT induction in breast cancer cells.[132] *TP53INP1* is a pro-apoptotic stress-induced p53 target gene and a tumor suppressor inhibiting cell migration and invasion.[133] *TP53INP1* is a direct target of miR-155.[134] Loss-of-function of *TP53INP1* reduced CDH1

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expression and increased the levels of CDH2 and VIM in liver cancer cells, all hallmarks of EMT.[135]

Overexpression of miR-194 in liver cancer cells, which express mesenchymal markers such as N-cadherin (*e.g.* SK-Hep-1 and SNU475), reduced the expression of CDH2 and suppressed invasion and migration *in vitro* and *in vivo*.[136] A network activated upon TNF stimulation involves NF-κB, HNF1A, miR-194, tripartite motif containing 23 (TRIM23), and chromosome 21 open reading frame 91 (C21ORF91). Upon knockdown of miR-194, its repressive effect on *TRIM23* and *C21ORF91* is relieved, rendering the activation of NF-κB and promoting HCC cell migration, invasion, and tissue colonization.[137] Moreover, miR-194 can target several genes involved in EMT and cancer metastasis such as *CDH2*, *RAC1*, Heparin-binding epidermal growth factor–like growth factor *(HBEGF)* and type 1 insulin-like growth factor receptor *(IGF1R)*.[136]

ncRNA biomarkers

Currently, the diagnosis of early HCC relies mainly on imaging methods. Ultrasonography is used to detect tumor nodules and has a sensitivity of 60–80% and a specificity of 94%.[138] Serum alpha-fetoprotein (AFP) is one of the most tested biomarkers for HCC. However, AFP lacks the necessary specificity for use as the standard diagnostic tool for HCC. The combination of AFP with ultrasonography showed only a minor increase in sensitivity but produced a lot of false positives and additional costs.[139] Other biomarkers, *e.g.* des-gamma carboxyprothrombin (DCP) and fucosylated AFP, have been investigated for their clinical usefulness as well, but showed equally low accuracy as AFP.[140] Therefore, none of these biomarkers is considered a clinically useful tool for HCC diagnosis. As a consequence, only ultrasonography is recommended by the EASL-EORTC for HCC diagnostic and surveillance guidelines.[141]

The lack of appropriate biomarkers for early detection combined with the lack of pathognomonic symptoms often results in the late diagnosis of a cancer only at higher stages, which is correlated with a poorer survival.[36] The only potentially curative therapeutic options for HCC, which include liver transplantation, ablation and resection methods, are recommended only for early (BCLC score 0 + A) stages.[36] Thus, there is apparently a need for the development of reliable tests for early HCC diagnosis. Since most protein-based assays lack the desired accuracy, ncRNA-based assays could be considered as an alternative diagnostic tool for HCC.

Several studies suggested circulating ncRNAs[142–155] and tumor tissue derived ncRNAs[14–17,24,156] for HCC diagnosis or survival prediction. While tissue derived ncRNAs might be functionally relevant in the tumor, they are not necessarily good biomarkers for diagnosis. To obtain a tissue sample, a liver biopsy is needed, which is an invasive procedure with potential side effects.[157] Therefore, the detection of circulating ncRNAs in body fluids instead of tumor tissue is advantageous for HCC diagnosis and surveillance.

Several publications evaluated lncRNAs alone or in combinations as candidate biomarkers for HCC diagnosis, as summarized in Table 1. The lncRNA urothelial carcinoma associated-1 (*UCA1*) is one example for a single lncRNA-based HCC diagnostic approach.[144,145] The reported sensitivities were between 91.4% and 92.7% and the specificites between 82.1% and 88.6%. In both studies, *UCA1* performed better than AFP. In a combinatorial approach of *UCA1* lncRNA with *JUN* mRNA, the sensitivity and specificity were 97.1% and 80%. For early-stage HCC detection, the combination of *UCA1* and *JUN* achieved 100% sensitivity and 80% specificity. These values underline the utility of RNA-based detection methods for early HCC diagnostic.

However, major limitations of most studies suggesting lncRNAs as HCC biomarkers are the limited cohort sizes and focusing on only one, sometimes two different HCC etiologies. Only four studies analyzed a total of more than 200 patients[142,151,153,154] and very few studies compared more conditions than HCC *vs.* healthy control.[142,151,154,155,158] Regarding the heterogeneity of HCC, comparisons between HBV/HCV carriers without HCC, cirrhotic liver condition, early HCC, alcohol-induced HCC, and HBV/HCV-associated HCC are necessary to achieve a comprehensive analysis. A bigger patient cohort including patients from different liver disease conditions and different centers could be the basis for the evaluation of the clinical usefulness of the suggested lncRNA biomarkers. Still, these first studies are encouraging enough to consider lncRNAs as potential biomarkers for HCC, particularly given the lack of serum biomarkers for (early) HCC. Considering the high tissue specificity of lncRNAs, some of the lncRNA biomarkers might be highly specific for a single liver disease condition, allowing a fast diagnosis and better HCC management.[159]

Aberrant expression of circulating miRNAs has also been reported in liver inflammation, chronic liver injury or HCC (Table 2). Although these miRNAs may be useful to distinguish HCC from healthy controls,[160–163] it is still difficult to differentiate HCC from chronic liver injury with a high accuracy.[164–166] miR-122 could represent a sensitive biomarker for liver injury.[110] Studies of HCC related miR-122 report contradictory results: one study shows that serum miR-122 was significantly downregulated in mainly HBV-related HCC patients compared with healthy adults,[167] whereas another study found it significantly upregulated.[164] Expression of miR-122 might vary due to the underlying etiology and active ongoing necroinflammatory changes. A decrease in

Key point

ncRNAs can be used as biomarkers in HCC.

Review

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Table 1. Circulating lncRNA biomarkers in HCC.

	lncRNA	Alteration	Study sample	Sensitivity (%)	Specificity (%)	References
Single	*AF085935*	Up	HCC *vs.* HL + HBV carrier			[142]
	DANCR	Up	HCC *vs.* CHB + LCB	80.8	84.3	[146]
		Up	HCC *vs.* HL + CHB + LCB	83.8	72.7	[146]
	HULC	Up	HCC *vs.* HL			[147]
	JPX	Down	HCC *vs.* HL	52.4	100	[148]
	LINC00974	Up	HCC *vs.* HL	51.1	95.6	[149]
	LINC01225	Up	HCC *vs.* HL	76.1	44.3	[150]
	SPRY4-IT1	Up	HCC *vs.* HL	87.3	50.0	[151]
	UCA1	Up	HCC *vs.* HL	92.7	82.1	[145]
		Up	HCC *vs.* HL	91.4	88.6	[144]
	uc003wbd	Up	HCC *vs.* HL + HBV carrier			[142]
	WRAP53	Up	HCC *vs.* HL	85.4	82.1	[145]
Combination (lncRNAs)	*PVT1, uc002mbe.2*	Up	HCC *vs.* HL	60.6	90.6	[152]
	AX800134, uc001ncr	Up Up	HBV-HCC *vs.* HL + HBV carrier eHBV-HCC *vs.* HL + HBV carrier	95.0 95.7	88.1 88.1	[153]
	LOC149086, RP11–160H22.5, XLOC_014172	Up	HCC *vs.* HL	85.0	95.0	[154]
	UCA1, WRAP53	Up	HCC *vs.* HL	95.1	82.1	[145]
	HULC, LINC00152	Up	HCC *vs.* HL			[143]
Combination (lncRNA + miRNA/mRNA)	*CTBP*, *LAMP2*, miR-16–2, miR-21-5p	Up: CTBP, miR-16–2, miR-21-5p Down: LAMP2	HCC *vs.* HL	79.5	100	[155]
	JUN, UCA1	Up Up	HCC *vs.* HL eHCC *vs.* HL	97.1 100	80.0 80.0	[144]
Combination (lncRNA + AFP/DCP)	AFP, DCP, MALAT1	Up	HCC *vs.* HL	88.6	75.0	[158]
	AFP, UCA1, WRAP53	Up	HCC *vs.* HL	100	62.8	[145]
	AFP, SPRY4-IT1	Up	HCC *vs.* HL	87.3	65.0	[151]
	AFP, JPX	Up:AFP Down:JPX	HCC *vs.* HL	72.2	97.1	[148]
	AFP, UCA1	Up	HCC *vs.* HL	100	74.2	[144]

AFP, alpha-fetoprotein; CHB, HBV chronic hepatitis; DCP, des-gamma carboxyprothrombin; eHBV-HCC, early HCC related with HBV infection (early BCLC stage); HBV-HCC, HCC related with HBV infection; HCC, hepatocellular carcinoma; HL, healthy liver; LCB, HBV liver cirrhosis; lncRNA, long non-coding RNAs; miRNA, micro RNA; mRNA, messenger RNA.

miR-122 levels has been described in liver tissues of non-alcoholic steatohepatitis (NASH) patients[168] and increased serum miR-122 has been noted in non-alcoholic fatty liver disease.[169] Furthermore, alterations in circulating miR-122, miR-192 and miR-375 have been correlated with disease severity in NASH patients.[169] Alterations in serum and plasma miR-122 correlate with alanine aminotransferase increases in the liver damage caused by alcohol or acetaminophen.[170] Deregulation of miR-122 in HCC patients is linked to AFP elevation and a more aggressive phenotype in HCC, correlating with shorter recurrence-free and overall survival due to increased expression of *CUX1*, a direct target of miR-122.[105]

miR-200a suppresses HCC proliferation by induction of a G1 phase arrest and is associated with overall survival in HCC.[171] miR-214 is a suppressor of HCC proliferation and its low expression correlates with portal vein invasion and early recurrence in HCC patients.[172] miR-155 expression levels are significantly higher in HCC patients with post-orthotopic liver transplantation recurrence than in those patients with non-recurrence. Additionally miR-155 levels are correlated with microvascular invasion of HCC tissue samples.[173] The overexpression of serum miR-221 is correlated with tumor size, tumor stage, poor overall survival and shorter time to local recurrence.[174,175] Meanwhile, patients with higher serum miR-1 and miR-122 levels showed longer overall survival than individuals with lower levels.[176]

A plasma miR-21 level could differentiate HCC from chronic hepatitis (AUC 0.773 with 61.1% sensitivity and 83.3% specificity).[165] Moreover, this value is superior to AFP and further improved by the combination of miR-21 and AFP. One systematic meta-analysis of circulating miRNAs for diagnosis of HCC suggested that circulating levels of miR-21 and miR-122 could be used either alone or in combina-

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Table 2. Circulating miRNA biomarkers in HCC.

	miRNA	Alteration	Study sample	Sensitivity (%)	Specificity (%)	References
Single	miR-1	Up	HBV-HCC *vs.* HL			160
	miR-15b	Up	HBV-HCC *vs.* HL + HBV carrier	98.3	15.3	161
	miR-18a	Up	HBV-HCC *vs.* HL HBV-HCC *vs.* CHB + LCB	86.1 77.2	75.0 70.0	162
	miR-21	Up	HBV-HCC *vs.* HL + CHB + LCB			164
		Up	HBV-HCC *vs.* HL	89.4	71.19	161
		Up	HCC *vs.* HL HCC *vs.* CH	87.3 61.1	92.0 83.3	165
	miR-25	Up	HBV-HCC *vs.* HL			160
	miR-26a	Down	HBV-HCC *vs.* HL + CHB + LCB			166
	miR-27a	Down	HBV-HCC *vs.* HL + CHB + LCB			166
	miR-92a	Up	HBV-HCC *vs.* HL			160
	miR-122	Up	HBV-HCC *vs.* CHB			164
		Down	HCC *vs.* HL	70.6	67.1	167
	miR-130b	Up	HCC *vs.* HL	87.7	81.4	161
	miR-206	Up	HBV-HCC *vs.* HL			160
	miR-223	Up	HBV-HCC *vs.* HL			160
		Up	HCC *vs.* HL			164
		Down	HBV-HCC *vs.* HL + CHB + LCB			166
	miR-375	Up	HBV-HCC *vs.* HL	96.0	100.0	160
	miR-801	Up	HBV-HCC *vs.* HL + CHB + LCB			166
	miR-885-5p	Up	HBV-HCC *vs.* HL			163
	Let-7f	Up	HBV-HCC *vs.* HL			160
Combination	miR-375, 23b, 423, 23a, and 342-3p	Up	HBV-HCC *vs.* HL	96.9	99.4	160
	miR-10a and 125b	Up	HBV-HCC *vs.* CHB + LCB	98.5	98.5	160
	miR-375, 25 and let-7f	Up	HCC *vs.* HL	100	96	160
	miR-122, 192, 21, 223, 26a, 27a and 801	Up: miR-192, 21, 801 Down: miR-122, 223, 26a and 27a	HCC *vs.* HL	83.2	93.9	166
	miR-206, 141-3p, 433-3p, 1228-5p, 199a-5p,122-5p, 192-5p and 26a-5p	Up: miR-206, 141-3p, 433-3p and 1228-5p Down: miR-199a-5p, 122-5p, 192-5p and 26a-5p	HBV-HCC *vs.* HL HBV-HCC *vs.* LCB	82.3 81.6	83.3 84.6	178
	miR-15b and 130b	Up	HBV-HCC *vs.* HL + CHB	98.2	91.5	161

CHB, HBV chronic hepatitis; HBV-HCC, HCC related with HBV infection; HCC, hepatocellular carcinoma; HL, healthy liver; LCB, HBV liver cirrhosis.

tion with other diagnostic tools as a first-line detection method of HCC.[177] This review included a total of 3,423 cases of HCC, 2,403 chronic hepatitis patients, and 1,887 healthy controls. Data from 50 studies was included in this meta-analysis and showed that two individual miRNAs, miR-21 and miR-122, were able to distinguish between HCC and healthy controls. miR-21 showed a sensitivity of 86.6% and specificity of 79.5%. miR-122 had a slightly lower sensitivity of 68.0% and specificity of 73.3%. This indicates that miR-21 and miR-122 are promising biomarkers for the diagnosis of HCC.[177]

A miRNA panel may provide a high diagnostic accuracy of HCC regardless of disease status, and it can also differentiate HCC from healthy controls and chronic liver injury.[178] Multiple circulating miRNAs and/or a combination with other biomarkers, such as AFP may further increase the specificity and sensitivity for HCC diagnosis or for prediction of prognosis. Several extensive genome-wide ncRNA profiling studies have been reported, and have identified new insights into molecular classifications of HCC.[179] For instance, the expression level of the miR-517a and miR-520a cluster was used to sub-classify a more aggressive type of HCC in a small patient cohort.[179] Extending these studies to evaluate the potential role of individual or clusters of ncRNAs associated with these classifications as biomarkers

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could be useful. Several profiling studies have reported miRNA profiles based on sequencing microarrays[160,163,166,178] to examine circulating miRNAs as HCC associated biomarkers.

Key point

ncRNAs are potential targets for treatment of HCC.

Targets and therapy

A deeper understanding of lncRNA mechanisms will identify new targets for therapeutic intervention. Commonly faced problems in HCC treatment include poor therapy response to classical chemotherapeutics and the emergence of resistances. Curative treatment options are only effective in the case of early HCC diagnosis and options for the treatment of advanced HCC are very limited. Therapeutic approaches which directly target ncRNAs or make use of ncRNA molecules might help to improve HCC treatment.

One advantageous feature of the liver regarding the delivery of (RNA-based) drugs is its microanatomical structure. Blood enters the liver via the portal vein (or the liver arteries) and passes the liver sinusoids before draining into the inferior vena cava. Liver sinusoids are highly specialized blood vessels with a fenestrated endothelium, with a discontinuous or partially lacking basal lamina.[180] Particles, which are smaller than 100 nm in diameter, can therefore directly enter the perisinusoidal space (Space of Disse) and make contact with hepatocytes. Thus, hepatocytes are easily accessible for transduction or transfection *in vivo*. This has been proven by several studies with various techniques and model organisms. For instance, a 90% knockdown of Apolipoprotein B (*APOB*) in the liver of *Macaca fascicularis* was noted 48 h after a single intravenous siRNA injection.[181]

LncRNA, ribozyme, and aptamer delivery or inhibition in cancer therapy

One obvious way to use lncRNAs in HCC treatment could be the direct delivery of tumor suppressive lncRNA molecules to the target cells. The delivery of lncRNAs can be achieved by injection of "naked" RNA, viral vectors, or in combination with nanoparticles of different compositions.[182] A mechanism of interest, exerted by the delivered tumor suppressive lncRNA, could be the sequestering of oncogenic miRNAs, as described before.[10,50] An artificially designed lncRNA as a sponge for twelve oncogenic miRNAs was delivered by a viral vector and reduced tumor growth in a xenograft HCC mouse model.[183] The modulation of transcription by lncRNAs is another powerful mechanism which could be exploited for HCC therapy. Growth-arrest specific 5 *(GAS5)* is a cytoplasmic lncRNA, which regulates transcription. *GAS5* acts as a decoy binding to the DNA-binding domain of the glucocorticoid receptor (GR) and mimicking the glucocorticoid response element motif.[184] As a consequence, *GAS5* induces apoptosis by suppressing GR-mediated survival signaling.[185] *GAS5* is downregulated in HCC and therapeutic delivery could induce apoptosis in HCC cells.[186] Hypothetically, a deeper mechanistic understanding of the *GAS5*-GR interaction could result in the development of RNA molecules, which function in a similar way but target other transcription factors. Other therapeutic strategies, utilizing lncRNA-mediated transcriptional regulation would include the delivery of epigenetic regulatory lncRNAs or lncRNAs guiding transcription factors or chromatin modification complexes.[7,8,20] This would be useful for a targeted modification of the cancer cell transcriptome.

The knockdown of oncogenic lncRNAs would be an alternative strategy to the delivery of tumor suppressive lncRNAs. This approach could exploit the high cell type- and cancer-specificity of lncRNAs and the good accessibility of the liver for transduction and transfection.[1,159,180,181] In recent years, RNAi has gained interest in gene silencing and drug development because of its potential specificity, significant effect, and ease of synthesis. However, several challenges remain for the clinical use of siRNA for cancer therapy.[187] The current RNAi technology can modify siRNA with 2′-O-methyl and 2′-deoxy-2′-fluoro groups, locked nucleic acids (LNAs), or phosphorothioate linkages[188] to avoid the degradation of the siRNA by serum nucleases. The nanoparticle surface can be shielded with polyethylene glycol (PEG) to minimize interaction between the delivery nanoparticles and serum proteins.[189] Polymer-mediated anti-cancer siRNA delivery including cyclodextrin and polyethyleneimine (PEI) has been used successfully for nucleic acid delivery.[190] Furthermore, exosomes are natural carriers of coding and non-coding RNA with the ability to induce *de novo* transcriptional and translational changes in target cells. The siRNA delivery via exosome has little or no toxicity or immunogenicity.[191] AMD3100, a CXCR4 antagonist, modified nanoparticles can efficiently deliver vascular endothelial growth factor A (*VEGFA*) siRNAs into HCC and downregulate *VEGFA* expression *in vitro* and *in vivo*.[192] Targeting *VEGFA* via transarterial embolization mediated siRNA delivery can reduce tumor growth in a rabbit VX2 liver-transplanted HCC model.[193] A lipid-nanoparticle formulation with siRNA directed against polo-like kinase 1 *(PLK1)*, namely TKM-080301, showed antitumor activity in xenograft animal models as well. In a clinical phase I/II study with a small cohort, it showed encouraging results in patients with advanced HCC (NCT02191878).[40] This proof-of-principle study documents the effective use of a sncRNA to target an mRNA, which also should be transferable to targeting lncRNAs in HCC. Multiple studies showed that the knockdown of lncRNAs had tumor growth-inhibiting and anti-metastatic effects in animal models.[13,117] However, the knockdown of nuclear lncRNAs is difficult to achieve with siRNAs. For this purpose, antisense

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oligonucleotides (ASOs) are the better strategy.[194] ASOs are short (15–20 nucleotides long) single-stranded molecules of DNA and other specialized nucleotides, which form a partial DNA: RNA hybrid and then degrade the RNA via RNase H1. The chemical modification of ASOs with trivalent N-acetylgalactosamine (GalNAc) targets hepatocytes with a high specificity.[195] An ASO targeting *STAT3* (AZD9150) showed antitumor activity in pre-clinical model systems and in a phase I trial (NCT01563302) with treatment-refractory lymphoma and lung cancer patients.[196]

Further, ribozymes and aptamers are therapeutically interesting classes of RNA molecules. Ribozymes can be used to perform trans-splicing reactions between specific cellular RNAs and exon-like regions present in the ribozyme. This reaction can produce a cancer-specific therapeutic transgene with impact on tumor growth *in vivo*.[197] Aptamer-siRNA chimeras can specifically bind to molecules of different complexity, ranging from simple cations to complex proteins. In a xenograft mouse model of prostate cancer, an siRNA-aptamer showed a cell type specific knockdown of the target gene *PLK1* after systemic application.[198]

sncRNA delivery or inhibition in cancer therapy – miRNAs, miRNA mimics, and anti-miRNAs

A number of studies have suggested miRNAs as attractive therapeutic targets for HCC. A single miRNA could regulate multiple targets involved in diverse cellular pathways - hence, if these targets are uniformly oncogenic, a single siRNA could target multiple oncogenic pathways. In turn, if the targets are heterogeneous with oncogenes, tumor suppressor genes and essential genes, this may also cause adverse effects.

The adeno-associated virus (AAV) 8 serotype has a strong tropism for the liver, can be administered intravenously[199] and hence is appealing for targeting the liver. Recombinant AAV8 harboring a human miR-122 minigene encoding a pri-miRNA fragment inhibits tumor development in a *Myc*-induced liver cancer model, which exhibits silencing of miR-122.[109] Non-viral systems are considered safer because AAV can elicit several adverse effects, such as activation of the immune response, mutation of the host genome and possible liver toxicity,[200] as well as integrations into driver genes that can lead to overexpression of affected genes.[201] An advantage of the positively charged lipid nanoparticles (LNPs) is that they can easily encapsulate anionic oligonucleotides forming lipo-plexes that stabilize oligonucleotides in blood circulation, facilitate uptake of anionic oligonucleotides through the negatively charged cell membranes and promote the release of encapsulated miRNAs to the cytoplasm of target cells.[202] LNP-DP1-formulated miR-122 effectively suppresses subcutaneous tumor growth in xenografts in immune-compromised mice.[203] As already described, miR-124 can suppress HCC progression through the STAT3 signaling pathway. The systemic administration of miR-124 could suppress HCC development by inducing tumor-specific apoptosis without toxic side effects in a murine liver cancer model.[88]

Alternatively, miRNAs can be delivered via exosomes. miR-122 can be transferred via exosomes between human HCC cells. Adipose-derived mesenchymal stem cells (AMSC)-derived exosomes have been used for miRNA delivery.[204] The sensitivity of HCC cells to chemotherapeutic agents such as 5-fluorouracil or sorafenib increases with AMSC transfected with a miR-122 expression plasmid. The increase in sensitivity depends on exosome-mediated miR-122 transfer and downregulation of miR-122-target genes, leading to higher antitumor activity of sorafenib *in vivo*.[205] Delivery of miR-122 via AMSC exosomes in combination with chemotherapeutic agents enhances cell apoptosis and cell cycle arrest at G0/G1, which represents a promising strategy for HCC chemotherapy.

The silencing of aberrantly expressed miRNAs *in vivo* has been achieved using ASOs with various nucleic acid analogs involving LNAs, 2′-O-methyl oligonucleotides (antagomiRs), and peptide nucleic acids (PNAs).[206,207] However, the *in vivo* efficacy of current anti-miRNA technologies is hindered by physiological and cellular barriers to delivery into targeted cells.[208] miR-122 can be effectively depleted *in vivo* by systemic delivery of a chemically-modified antisense oligonucleotide. Miravirsen, an anti-miRNA targeting miR-122, was the first miRNA-based therapeutic agent that was successful in clinical trials in HCV-infected patients.[209] In contrast, miR-122 had been described earlier as a tumor suppressor in HCC raising the possibility that an anti-miR-122 therapy may inhibit HCV but foster HCC. However, loss of miR-122 is not universal in HCC and is more prominent with an HBV-dependent etiology. Additional studies to clarify the role of miR-122 in HCC of different etiologies are needed. However, adoption for use in HCC has not occurred. Fibrosis and tumor incidence were reduced in a transgenic mouse with overexpression of platelet-derived growth factor C (PDGF-C) treated with LNA-anti-miR-214.[210]

Conclusions

ncRNAs contribute to hepatocarcinogenesis by regulating Wnt and STAT3 signaling. They function in Wnt signaling mainly via modulation of CTNNB1 stability.[7,13,14,19,23,53] The Wnt and STAT3 pathways are crucial regulators of the hepatic CSC phenotype and are also involved in EMT induction and

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metastasis.[37,68,92,115] Further ncRNAs are important factors in hepatic CSCs and EMT, *e.g.* modulating the ZEB1 and HIF1A pathways.[9,21] Given the huge number of uncharacterized ncRNAs, the ncRNAs reviewed here are probably only the tip of the iceberg of functionally relevant ncRNAs in HCC.

ncRNAs can be detected in serum and have remarkably high tissue specificity.[143,159,211,212] A lot of HCC related clinicopathological parameters have been linked to ncRNAs, including HBV/HCV status, survival, recurrence, and metastasis formation.[3,9,49,125,213] These features render ncRNAs as suitable biomarkers for HCC diagnosis.

Despite rapid progress in ncRNA and RNA biopharmaceutical research, RNA-based therapies have not yet reached clinical practice. However, there are encouraging studies regarding experimental RNA-based therapies. The anatomical structure of the liver provides the possibility to easily target the intended cell types *e.g.* via RNAi.[180,181] Hence, ncRNA centered treatments should be considered as a promising alternative to surgical methods, especially for advanced HCC, where the treatment options are limited so far.

Taken together, ncRNA research has already added an additional layer of complexity to the understanding of HCC although the mechanistic understanding of their function is only beginning to emerge. The lack of appropriate therapeutic targets and detection systems for HCC are still major challenges. These challenges may be faced by ncRNA centered HCC research and the translation into clinical applications in the near future.

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Conflict of interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest regarding this manuscript. S.D. is co-owner of siTOOLs Biotech, Martinsried, Germany.

Authors' contributions

MK drafted the manuscript. MK and SD wrote the lncRNA part. AM and TP wrote the sncRNA part. All authors read and critically revised the manuscript. All authors designed and prepared the figures. SD and TP contributed equally.

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