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**Epigenetics in hepatocellular carcinoma: An update and future therapy perspectives**

Ma L *et al.*Epigenetics in HCC

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**Abstract**

Hepatocellular carcinoma (HCC), the predominant form of adult liver malignancies, is a global health concern. Its dismal prognosis has prompted recent significant advances in the understanding of its etiology and pathogenesis. The deregulation of epigenetic mechanisms, which maintain heritable gene expression changes and chromatin organization, is implicated in the development of multiple cancers, including HCC. This review summarizes the current knowledge of epigenetic mechanisms in the pathogenesis of HCC, with an emphasis on HCC mediated by chronic hepatitis B virus infection. This review also discusses the encouraging outcomes and lessons learnt from epigenetic therapies for hematological and other solid cancers, and highlights the future potential of similar therapies in the treatment of HCC.

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**Key words:** Hepatocellular carcinoma; Epigenetics; DNA methylation; Histone modification; miRNA; lncRNA

**Core tip:** Hepatocellular carcinoma (HCC) is a global health concern; molecularly targeted therapeutics remains limited to sorafenib. New targets and drugs are urgently needed to broaden the limited treatment options for HCC. Many lines of evidence suggest that epigenetics is associated with the initiation and development of HCC. Here, we review the current state of knowledge on epigenetic deregulation in HCC, and potential therapies that can be exploited for interventions.

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**INTRODUCTION**

Liver cancer is a global health concern. It is the fifth most frequently diagnosed cancer and second most frequent cause of cancer death in men worldwide. Liver cancer is endemic particularly in East Asia and Southeast Asia, where more than half of total cases globally are diagnosed. In addition, Middle Africa, West Africa and South Africa are among the top five regions affected**[**[**1**](#_ENREF_1)**]**. In the United States, the incidence rate of hepatocellular carcinoma (HCC) (the most common histological subtype of liver cancer) has tripled in the past decade, with the rise being related to increased hepatitis C virus (HCV) infection[[2](#_ENREF_2),[3](#_ENREF_3)]. Risk factors for HCC are well characterized, including chronic hepatitis B virus (HBV) infection, HCV infection, excessive alcohol consumption, diabetes, non-alcoholic fatty liver disease, anddietary **exposure to aflatoxin[**[**4**](#_ENREF_4)**].**

**EPIGENETICS AND HCC**

Epigenetics is defined as heritable states of gene expression without altering DNA sequences. Epigenetic mechanisms encompass genomic DNA modifications (methylation of DNA cytosine bases), chemical modifications of histone tails, and non-coding miRNA regulation. During cell division, these epigenetic modifications are passed down faithfully to daughter cells to maintain “cellular memory”[[5](#_ENREF_5)]. DNA methyltransferases (DNMTs) catalyze the addition of methyl groups (CH3) to the 5′ cytosine nucleotides. Mechanistically, DNA methylation leads to transcriptional gene silencing in two ways. First, methylation at CpG sites sterically hinders accessibility of transcription factors to their cognate binding sites on respective gene promoters[[6](#_ENREF_6)]. The second mechanism involves direct binding of methyl CpG binding domain (MBD)-containing proteins to the methylated DNA, causing transcription repression[[7](#_ENREF_7)]. Gene silencing mediated by DNA methylation is observed in many cancer types. Cancers often present with features of global hypomethylation; by contrast, promoters of tumor suppressor genes, in particular, are hypermethylated[[8](#_ENREF_8)]. Several lines of evidence suggest that changes in the epigenome are associated with liver cancer initiation and progression[[9](#_ENREF_9)].

**DNA METHYLATION CHANGES IN HCC**

Thus far, three independent genome-wide methylation profiling studies demonstrated that HCC tumors display differential DNA methylome patterns compared to the respective adjacent normal liver tissues [[10-12](#_ENREF_10)]. Hernandez-Vargas *et al*[[10](#_ENREF_10)] used a bead array to analyze 1505 CpG sites in 30 patients with either HBV- or HCV-associated HCC, and observed that HCC tumors exhibit specific DNA methylation signatures that are correlated with major risk factors and tumor progression stage, implying potential clinical applications in early diagnosis and prognosis. Specifically, a panel of hypermethylated gene promoters (*APC*, *RASSFIA*, *CDKN2A* and *FZD7*) were able to discriminate HCC tumors from paired surrounding non-tumor liver tissues. Another set of hypermethylated genes (*e.g.*, *NAT2, CSPG2* and *DCC*) were exclusively associated with HBV-related HCC[[10](#_ENREF_10)]. In particular, promoter methylation of *DNMT1* was found to significantly correlate with poor tumor differentiation. By using the latest Illumina Methylation450 BeadChip, which allowed measurement of DNA methylation levels at 485577 loci across 99% of RefSeq genes (including 96% of the known CpG islands), Song *et al*[[11](#_ENREF_11)]measured DNA methylation levels in HCC tissues or adjacent normal liver tissues from 27 HCC patients, and found significant enrichment of promoter CpG island DNA methylation loci in the signaling networks of cellular development, gene expression, cell death, and cancer. The genes *BMP4*, *CDKN2A*, *GSTP1,* and *NFATC1* were among the top of the gene list. Shen *et al*[[12](#_ENREF_12)] carried out a genome-wide methylation study using plasma DNA from a cohort consisting predominantly of HBV+ (79%) HCC patients, and found that the top five hypermethylated genes were *DAB2IP*, *BMP4*, *ZFP41*, *SPDY1* and *CDKN2A*, whereas the top five hypomethylated genes were *CCL20*, *ATK3*, *SCGB1D1*, *WFDC6* and *PAX4*.

Functional consequences of methylation-mediated silencing of tumor suppressor genes (TSGs) were addressed by recent studies by Nishida *et al*[[13](#_ENREF_13)] and Revill *et al*[[14](#_ENREF_14)]. Using combined genome-wide methylation profiling and “epigenetic unmasking” approaches in primary HCC and gene re-expression in cell lines, Revill *et al*[[14](#_ENREF_14)] narrowed down 13 relevant candidate TSGs to SMPD3 and NEFH. Overexpression of SMPD3 and NEFH led to repression of cell proliferation, with NEFH causing a lesser effect. Nishida *et al* identified a panel of eight TSGs that are highly predictive of progression from chronic HCV infection to HCC[[13](#_ENREF_13)]. However, the biological functions of these TSGs may vary depending on the cellular context, and their roles in HCC remain to be validated in HCC arising from other risk factors. DNA methylation is therefore a significant mechanism in the silencing of these TSGs.

The above studies provide strong evidence that aberrant gene methylation is closely associated with disease stage and clinical outcome in HCC, and suggest that methylation profiling (in particular, using patient plasma) may be a feasible approach for early diagnosis and prognosis of HCC. However, specific gene methylation signatures remain to be validated.

**HISTONE MODIFICATIONS IN HCC**

Within the chromosome, DNA is packaged into chromatin where the DNA coils around an octamer of histones. One hundred and forty-five base pairs of DNA are wrapped around the histone octamer, comprising H2A, H2B, H3 and H4, forming the repeating unit of chromatin, the nucleosome[[15](#_ENREF_15)]. Histone tails protruding out of the nucleosome are targets of post-translational modifications, including acetylation and methylation of lysine (K) and arginine (R) residues, phosphorylation of serine (S) and threonine (T) residues, and ubiquitination of lysine residues[[16](#_ENREF_16)]. These modifications can turn transcription of genes on or off, and are therefore key players in establishing the gene expression patterns of cells by adjusting the tightness of DNA bound to histones, thereby affecting accessibility of transcription factors[[17](#_ENREF_17)].

Histone acetylation is controlled by two families of enzymes: histone acetalytransferases (HATs) that “write” the acetyl mark. Acetylation counteracts the positive charge of histones, thereby loosening the tight interaction between histones and DNA. Conversely, histone deacetylases (HDACs) “erase’ the acetyl group, resulting in tight coiling of DNA around the histones, leading to the transcriptionally inactive or closed chromatin state[[18](#_ENREF_18)]. In contrast, histone methylation is associated with either transcriptionally active or closed chromatin, depending on which histone or which lysine residue is modified. For example, histone 3 lysine 27 trimethylation (H3K27me3) is associated with transcriptional repression, whereas trimethylation of lysine 4 of histone 3 is indicative of gene activation [[19](#_ENREF_19),[20](#_ENREF_20)].

Evaluation of histone methylation status in HCC remains limited to correlative studies with clinicopathlogical features of HCC, using semi-quantitative methods of protein detection such as immunohistochemistry or Western blot. High levels of trimethylated histone H3 lysine 4 (H3K4me3) were correlated with reduced overall survival and poor prognosis in HCC[[21](#_ENREF_21)]. Another study showed that high levels of H3K27me3 predicted worse prognosis, and were additionally closely correlated with aggressive tumor features, including vascular invasion, large tumor size, multiplicity of tumors, and poor differentiation[[22](#_ENREF_22)]. Further studies using more precise detection methods, such as ChIP-sequencing, will be required to analyze these specific DNA-protein modifications in order to fully understand their roles in HCC.

**EPIGENETIC CHROMATIN MODIFIERS IN HCC**

[Polycomb-group proteins](http://en.wikipedia.org/wiki/Polycomb-group_proteins) are chromatin-modifying complexes mediating heritable gene silencing. Polycomb repressive complexes (PRCs) function in the maintenance of cell lineage commitment and stem cell pluripotency. The PRC1 complex comprises the core protein BMI, and RING1A and RING1B, which work as ubiquitin ligases for H2AK119. The other associated protein CBX7 binds to H3K27 *via* its chromodomain[[23](#_ENREF_23)]. The polycomb repressive complex 2 (PRC2) complex consists of SUZ12, EZH1/2, EED1, and RbAp48. EZH2 is a methyltransferase that mediates gene silencing by trimethylating H3K27[[24](#_ENREF_24)]. During embryonic stem cell development, the Suz–/– and Ezh2–/– cells exhibit distinct defects during gastrulation. Loss of Suz12 destabilizes Ezh2, causing a global loss of H3K27me3[[25](#_ENREF_25)].

Elevated expression of EZH2 has been reported in breast [[26](#_ENREF_26),[27](#_ENREF_27)] and prostate [[28](#_ENREF_28)] cancers. EZH2 mRNA transcript [[29](#_ENREF_29)] and protein [[30](#_ENREF_30),[31](#_ENREF_31)] levels were consistently elevated in HCC in comparison to non-tumor liver tissues. Specifically, clinicopatholgical analysis of paired resected tumor and non-tumor tissues showed that high levels of EZH2 were strongly associated with aggressive and metastatic features (including portal vein invasion and lack of tumor encapsulation) [[29](#_ENREF_29)], and with poor prognosis [[30](#_ENREF_30)], although no significant differences were observed in either disease free survival[[29](#_ENREF_29)] or cumulative survival rate between high and low EZH2 expression groups[[31](#_ENREF_31)].

Detailed mechanistic studies have further elucidated the biological roles of EZH2 in HCC pathogenesis, which support the above clinical correlations.  For example, EZH2 was shown to silence WNT antagonists, thereby activating Wnt/β-catenin signaling to promote cancer progression[[32](#_ENREF_32)]. In contrast,knockdown of EZH2 in liver cancer cell lines reduces the repressive H3K27me3 marker, leading to re-expression of a distinct subpopulation of tumor suppressor miRNAs (miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b), which control motility and adhesion[[33](#_ENREF_33)]. Another study showed that knockdown of EZH2 profoundly inhibited proliferation of Dlk+ hepatic progenitor cells, promoting their differentiation into hepatocytes[[34](#_ENREF_34)]. Additionally, Wang *et al*[[35](#_ENREF_35)] reported that c-Myc together with EZH2 silences the tumor suppressive miRNA-101, which in turn targets the PRC2 complex in a double negative feedback loop fashion to account for the overexpression of EZH2 in HCC. Similarly, overexpression of EZH2 resulting from aberrant genomic loss of miR-101 was also reported in prostate cancer[[36](#_ENREF_36)].Taken together, these findings show the combined regulatory effects of chromatin-modifying activities and miRNA expression in promoting HCC progression, and provide evidence for an essential role of EZH2 in hepatic progenitor cell homeostasis.

In another study, the role of the viral HBx encoded by HBV was studied for its contribution to hepatocyte transformation. HBx is weakly oncogenic, and essential in the HBV life cycle[[37](#_ENREF_37),[38](#_ENREF_38)]. HBx activates mitogenic pathways and increases polyploid cells (> 4 N), which causes genetic instability[[39](#_ENREF_39)]. Moreover, HBx activates mitotic polo-like kinase (PLK)1[[40](#_ENREF_40)], which likely downregulates the PRC2 component Suz12 *via* phosphorylation. Elevated PLK1 and reduced protein levels of Znf198 and Suz12 are also observed in human HCC cell lines, as well as in liver tumors from X/c-myc bi-transgenic mice and woodchucks infected with the woodchuck hepatitis virus[[41](#_ENREF_41)]. Importantly, loss of Suz12 results in de-repression of a subset of PRC2 target genes, specifically those with elevated expression in hepatic cancer stem cells, including EpCAM, BAMBI, DKK2, and DLK1[[41](#_ENREF_41),[42](#_ENREF_42)]. These findings suggest that chronic HBV infection may give rise to a small population of cells with hepatic cancer stem cell properties, which ultimately could contribute to the proliferation and progression of HCC.

To summarize, PRC2 subunits Suz12 and Ezh2 have distinct roles during HCC pathogenesis. Over-expression of Ezh2 is consistently found in advanced HCC. This elevated expression was associated with late stage features such as invasion and metastasis. In contrast, in the setting of chronic HBV infection, the HBx protein modulates Suz12 protein levels, thereby maintaining the “stemness” of a subpopulation of hepatocyte stem/progenitor cells. Depending on how the risk factors interact with the host DNA and epigenetic players, each specific epigenetic modifier component may play a distinct role at different HCC stages.

**MicroRNAs IN HCC**

MicroRNAs (miRNAs) are non-coding small RNA (ncRNA) molecules that are 20–23 nucleotides in length. They play important regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression. More than 1000 miRNAs have been identified to date. Through their roles in post-transcriptional gene regulation, miRNAs regulate diverse cellular functions including proliferation, differentiation, apoptosis, cell fate, and plasticity[[43](#_ENREF_43)]. Pri-miRNAs are transcribed by RNA polymerase II either from their own gene or are located in introns of protein-coding genes. The pri-miRNA is then cleaved in the nucleus to form an [approximately](http://www.iciba.com/approximately)  60–70 nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA, which is further exported from the nucleus to the cytoplasm by Ran-GTP and the export receptor exportin-5. In the cytoplasm, further processing by Dicer, another RNase III endonuclease, generates the 5′ phosphate and [approximately](http://www.iciba.com/approximately) 2 nt 3′ overhang characteristic of an RNase III and produces an siRNA-like imperfect duplex that comprises the mature miRNA. One strand of the duplex is incorporated into the RNA-induced silencing complex, forming a complementary complex with the 3’-untranslated region of the target mRNA, and resulting in mRNA degradation or inhibition of mRNA translation, and hence gene expression silencing[[44](#_ENREF_44)].

It has been increasingly recognized that aberrant miRNA expression profiles are linked to liver cancer development and progression[[45](#_ENREF_45)]. MiRNAs play a role in virus–host interaction, and provide an anti-viral defense mechanism, such as against the HCV and the primate foamy virus type 1[[46](#_ENREF_46),[47](#_ENREF_47)]. In a miRNA library screen, miR-141 was shown to repress HBV expression and replication in HepG2 cells, *via* direct suppression of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α. PPAR-α regulates HBV gene expression through interactions with HBV promoter regulatory elements[[48](#_ENREF_48)]. Another miRNA of interest is miR-122, the most abundant miRNA expressed in the liver, making up as much as 72% of total liver miRNAs[[49](#_ENREF_49)]­. Interestingly, miR-122 exerts opposite functions in HBV and HCV replication[[50](#_ENREF_50),[51](#_ENREF_51)]. It can bind directly to a region of the HBV pre-genomic RNA and negatively regulates HBV replication. An inverse correlation was observed between miR-122 and HBV genome copies in peripheral blood mononuclear cells obtained from HBV+ samples[[50](#_ENREF_50)]. On the other hand, miR-122 is essential for the stability, propagation and replication of HCV RNA[[51](#_ENREF_51)]. Indeed, an antisense oligonucleotide (Miravirsen) with locked nucleic acid (LNA)-modified DNA that sequesters mature miR-122 has shown high efficacy in reducing HCV RNA levels in human clinical trials[[52](#_ENREF_52)]. However, miR-122 is frequently downregulated in HCC[[53](#_ENREF_53),[54](#_ENREF_54)], suggesting its tumor suppressor role[[55](#_ENREF_55)]; therefore, attempts to restore miR-122 expression may inhibit HCC, or theoretically promote HCV replication and HCC, and have to be carefully considered depending on the viral status of HCC patients.

In contrast to miR-122, miR-1 over-expression increases HBV replication *via* induction of the hepatic nuclear receptor farnesoid X receptor, which then enhances transcription of the HBV core protein. This is accompanied by cell cycle arrest in G1 phase and differentiation of hepatocytes, thereby providing a favorable environment for HBV replication[[56](#_ENREF_56)]. Furthermore, in cultured liver cancer cells, the HBx protein represses p53-mediated expression of miRNA-148a, which in turn down-regulates the hematopoietic pre–B cell leukemia transcription factor–interacting protein (HPIP).In HBV-mediated HCC, expression of miR-148a is reduced, whereas that of HPIP is elevated. These observations support that down-regulation of miR-148a has a role in liver cancer pathogenesis[[57](#_ENREF_57)]. Other miRNAs with reported roles in HCC are listed in Table 1, and are discussed in other comprehensive reviews[45, 46].

**MiRNAs AS TUMOR SUPPRESSORS IN HCC**

Profiling of differentially expressed miRNAs and their targets at different disease stages also suggests that miRNAs are associated with disease pathogenesis. In HBV-associated HCC, using TaqMan low-density miRNA arrays, Wang *et al*[[58](#_ENREF_58)] found that miR-138 and miR-199a-5p expression was deregulated. In several studies, miR-138 levels were down-regulated in HCC tissues. MiR-138 exerts tumor suppressor function by directly targeting cyclin D3 and resulting in cell cycle arrest. It was also demonstrated that the use of a miR-138 mimic significantly reduced xenograft tumor growth in nude mice[[59](#_ENREF_59)]. Differential expression levels of miR-199a-5p were also reported in HCC tissues and cell lines[[60](#_ENREF_60),[61](#_ENREF_61)]. In particular, miR-199a-5p inhibits cell invasion by targeting discoidin domain receptor 1, a tyrosine kinase involved in signaling pathways that mediate cell invasion[[60](#_ENREF_60)].

In a cohort of HCC patients characterized with a background of HBV infection ([approximately](http://www.iciba.com/approximately) 90%), miR-26a and miR-26b expression in non-liver tissues was higher in women than in men, suggesting that they have a protective role[[62](#_ENREF_62)]. Moreover, miR-26a and miR-26b expression was down-regulated in tumors compared to paired non-tumor tissues. Patients with low miR-26 expression were associated with shorter survival, but were more likely to respond to interferon α therapy, making it an ideal candidate for predicting therapy response[[62](#_ENREF_62)]. Additional miRNAs with a role in HCC are described below. Another miRNA that is suppressed in human liver cancer is miR-125b, which possesses tumor-suppressive functions such as arresting cell cycle progression, and inhibiting migration and invasion by directly targeting the oncogene LIN28B2[[63](#_ENREF_63)]. MiR-140-5p expression is also decreased in HCC as well as six HCC cell lines. MiR-140-5p suppresses tumor growth and metastasis by targeting TGFBR1 and FGF9[[64](#_ENREF_64)]. Genome-wide miRNA and mRNA profiling during mouse liver development implicates miR-302b and miR-20a in repressing transforming growth factor-β signaling[[65](#_ENREF_65)]. MiR-122, in addition to its anti-HBV role, also functions as a tumor suppressor. Mice lacking the gene encoding miR-122a are viable but develop temporally controlled steatohepatitis, fibrosis, and HCC[[66](#_ENREF_66)]. Restoration of miR-122 in a hepatic cell line reverses its migration and invasion properties[[67](#_ENREF_67)].

**MiRNAs AND LIVER CANCER-INITIATING CELLS**

The hypothesis of liver cancer stem cells (LCSCs) is supported by the identification of subpopulations of cancer cells with antigenic markers, which contribute to cancer origin and chemoresistance[[68](#_ENREF_68),[69](#_ENREF_69)]. To date, the three main LCSC markers are epithelial cell adhesion molecule (EpCAM)[[70](#_ENREF_70),[71](#_ENREF_71)], CD133[[72](#_ENREF_72),[73](#_ENREF_73)] and CD90[[74](#_ENREF_74),[75](#_ENREF_75)]. Cells bearing these markers demonstrate cancer stem cell properties including: (1) ability to form tumorspheres in anchorage-independent assays; (2) ability to initiate tumor growth *in vitro* and *in vivo*; and (3) ability to self-renew. MiR-181 is highly expressed in EpCAM-positive HCC cells isolated from α-fetoprotein-positive tumors, and is important in maintaining the stemness of LCSCs by directly targeting hepatic differentiation transcription factors CDX2 and GATA6[[76](#_ENREF_76)]. MiR-181 levels are transcriptionally induced by the Wnt/beta-catenin signaling pathway[[77](#_ENREF_77)]. In addition, over-expression of miR-150 leads to significant reduction of CD133+ cells, as well as inhibition of cell proliferation and tumorsphere formation[[78](#_ENREF_78)]. MiR-548c-5p, miR-198, miR-375, and miR-874 levels are decreased, whereas miR-155, miR-198, and miR-1289 levels are increased in CD90+ in comparison to CD90– HepG2 cells. Transfection with exogenous miR-548c-5p inhibited proliferation, migration, and invasion of CD90+ HepG2 cells by down-regulating the expressions of β-catenin, Bcl-2, Tg737, Bcl-XL, and caspase 3[[79](#_ENREF_79)].

***Long non-coding RNAs in HCC***

Long non-coding RNAs (lncRNA) are transcripts longer than 200 nt, constituting a subpopulation of ncRNAs. They exert molecular regulatory functions *via* diverse modes of mechanisms[[80](#_ENREF_80)]. Accumulating evidence indicates that lncRNAs are implicated in many cellular functions and play a role in carcinogenesis of multiple cancer types[[81](#_ENREF_81)]. It has been shown that 20% of lncRNAs are associated with PRC2, through which they recruit and guide chromatin modifying complexes to specific genomic regions to regulate gene expression[[82](#_ENREF_82)]. They behave like transcription co-activators/repressors by directly binding with various interaction partners; for example, lncRNA *TERRA* can directly bind to human telomerase and inhibit telomerase activity[[83](#_ENREF_83)]. Alternatively, they act as decoys competing for miRNAs to modulate the expression of target genes[[84](#_ENREF_84)].

LncRNA *HULC* (highly up-regulated in liver cancer) was the first lncRNA with highly specific up-regulation detected in the blood of HCC patients[[85](#_ENREF_85)]. Du *et al*[[86](#_ENREF_86)] further revealed that HBx could regulate the promoter of HULC to promote hepatoma cell proliferation *via* down-regulating the tumor suppressor p18. HBx was also found to downregulate an lncRNA termed lncRNA-Dreh, which can inhibit HCC growth and metastasis *in vitro* and *in vivo*, and acts as a tumor suppressor in the development of HBV-HCC[[87](#_ENREF_87)]. The downregulation of lncRNA-Dreh additionally correlated with poor survival of HCC patients.

LncRNA *MALAT-1* (metastasis-associated lung adenocarcinoma transcript 1) was initially reported to be closely associated with non-small cell lung cancer (NSCLC) metastasis[[88](#_ENREF_88),[89](#_ENREF_89)]. Likewise, lncRNA MALAT-1[[90](#_ENREF_90)] and lncRNA HOTAIR ([HOX](http://en.wikipedia.org/wiki/Hox_gene) antisense [intergenic](http://en.wikipedia.org/wiki/Intergenic) RNA)[[91](#_ENREF_91)] have been shown to be upregulated in large cohorts of HCC patients. In particular, lncRNA HOTAIR was implicated as a prognostic biomarker for tumor recurrence after liver transplantation. Both studies demonstrated that siRNA-mediated reduction of lncRNA MALAT-1 and lncRNA HOTAIR suppressed cell viability and cell invasion, sensitized TNF-α induced apoptosis, and increased the chemotherapeutic sensitivity of cancer cells to cisplatin and doxorubicin[[91](#_ENREF_91)]. LncRNA MVIH (microvascular invasion in HCC) is elevated in HCC, and as its name suggests, was associated with microvascular invasion in HCC[[92](#_ENREF_92)]. Another lncRNA overexpressed in HCC, lncRNA HEIH (high expression in HCC), was found to be significantly associated with recurrence and is an independent prognostic factor for survival[[93](#_ENREF_93)]. Additionally, lncRNA-HEIH was found to physically interact with EZH2 - an interaction which is fundamental for repressing target genes such as p16 (lncRNA-HEIH can enhance the binding of EZH2 and H3K27me3 levels across the p16 promoters)[[93](#_ENREF_93)].

Given the key regulatory functions of lncRNAs in cancers including HCC, and the evidence of dysregulated lncRNA expression in HCC, the targeting of lncRNAs offers a novel exciting opportunity to treat HCC. In principle, targeting of lncRNA can be achieved using the following approaches: (1) siRNA-mediated silencing; (2) functional block using small molecules or oligonucleotide inhibitors to prevent interactions of lncRNAs with proteins such as PRC2; and (3) structure disruption using small molecules or oligonucleotide inhibitors to change or mimic their secondary structure to compete for their binding partners. Since targeting lcnRNAs is still in its infancy, no investigational agents are currently available[[94](#_ENREF_94)]. The complex epigenetic regulatory networks in HCC are summarized in Figure 1.

**CHALLENGES IN HCC MANAGEMENT**

HCC is a heterogeneous disease whose management requires a multidisciplinary approach. One of the major clinical challenges is the inability to detect HCC at its early stages; patients are often diagnosed at advanced stages, which limits therapeutic options and leads to poor prognosis and unfavorable outcome[[95](#_ENREF_95)].Current treatment strategies for HCC include: (1) surgical removal of the tumor and liver transplantation; (2) minimal invasive surgery with application of radiofrequency ablation or cryoablation; and (3) chemoembolization by injecting drugs directly into the liver[[96](#_ENREF_96)]. However, these approaches are limited by shortage of organ donors, small percentage of patients suitable for surgical removal, high post-operative recurrence rate, and underlying complications such as cirrhosis, HBV and HCV infections[[97](#_ENREF_97)].

To date, only a number of molecularly-based therapeutics are available in the clinical management of HCC. In 2007, sorafenib (Nexavar) was approved by the US Food and Drug Administration (FDA) for treatment of advanced primary HCC. This multi-tyrosine kinase inhibitor works by interfering with vascular endothelial growth factor signaling pathways in tumor angiogenesis. Clinical trials showed modest prolonged median survival and time to progression of 3 mo[[98](#_ENREF_98)]. Similarly, in a cohort of patients from the Asia–Pacific region, the median overall survival increased from 4.2 to 6.5 mo[[99](#_ENREF_99)]. Ongoing clinical trials are underway to test the efficacy of sorafenib in combination with other drugs[[100](#_ENREF_100)]. Studies are also in progress for other drugs targeting the epidermal growth factor receptor (EGFR), hepatocyte growth factor/c-Met, platelet-derived growth factor receptor (PDGFR), and mammalian target of rapamycin, all involved in molecular pathways of growth[[101](#_ENREF_101)]. These drugs all show variable outcomes in the treatment of HCC.

**EPIGENETIC DRUGS FOR TREATING HEMATOLOGICAL CANCERS**

In contrast to conventional or molecularly-targeted therapies for inhibiting dysregulated genes or signaling pathways, epigenetic drugs provide an alternative approach by reversing the methylation status and histone modifications of aberrantly expressed genes[[102](#_ENREF_102)]. There are currently four FDA-approved epigenetic drugs, including two DNA demethyltransferase (DNMT) inhibitors, 5-azacytidine and decitabine, and two histone deacetylation (HDAC) inhibitors, vorinostat and valporic acid. These drugs have been successful in treating hematological cancers, specifically myelodysplastic syndrome, a blood cancer characterized by inability to generate blood cells in the bone marrow[[103](#_ENREF_103)]. Interestingly, low doses of 5-azacytidine and decitabine show anti-tumor effects on cultured and primary leukemia cells[[104](#_ENREF_104),[105](#_ENREF_105)], as well as primary cells isolated from luminal-type breast cancer[[106](#_ENREF_106)]. More importantly, theycan reduce the number of CD34+ stem cells in leukemia, and mammosphere-forming breast cancer cells.Importantly, CD34+ cells are the origin and cause of tumor recurrence and chemoresistance[[106](#_ENREF_106)].

**DNA METHYLATION INHIBITORS FOR TREATING HCC**

Studies using cell lines[[107](#_ENREF_107)] and pre-clinical mouse models[[108](#_ENREF_108)] reveal promising results that may open up new avenues for the intervention and management of HCC. Drugs targeting epigenetic changes in HCC are summarized in Table 1. Andersson *et al*[[108](#_ENREF_108)] showed that treatment with the DNMT inhibitor zebularine can prevent and effectively inhibit tumor growth in xenograft mouse models that are sensitive to this drug. Zebularine-resistant cell lines, however, showed up-regulation of oncogenic activities that instead promote liver cancer growth. These findings suggest that this drug may only benefit a specific sub-population of HCC patients.Treatment with zebularine was also shown to inhibit cell proliferation and to induce apoptosis of the HepG2 cell line. However, the DNA methylation levels of tumor suppressor genes p53 and p21 were not affected by zebularine treatment, while the anti-apoptotic protein BCL-2 is down-regulated, indicating that a DNA-methylation-independent pathway exists for increased p53 and p21 protein levels[[107](#_ENREF_107)]. Additional cell-based studies show that 5-aza-2′-deoxycytidine exerts anti-tumor effects by inhibiting telomerase activity, accompanied by reactivation of p16 and c-Myc expression[[109](#_ENREF_109)].

**HDAC** **INHIBITORS FOR TREATMENT OF HCC**

The use of HDAC inhibitors in HCC has been investigated in preclinical and clinical studies. In preclinical studies, belinostat inhibited cell growth in a HCC cell line [[110](#_ENREF_110)], while suberoylanilide hydroxamic acid sensitized HCC cells to acetylation of p53[[111](#_ENREF_111)] and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[[112](#_ENREF_112)]. In a multi-center phase I/II clinical trial, belinostat was found to stabilize unresectable advanced HCC[[113](#_ENREF_113)]. Another important aspect of this clinical trial is the identification of HR23B as a potential biomarker for predicting the response to belinostat[[114](#_ENREF_114)]. The combination of suberoylanilide hydroxamic and dihydroartemisinin significantly halted the growth of liver cancer tumor xenografts[[115](#_ENREF_115)]. Of note, treatment with HDAC inhibitors was observed to induce cell death while simultaneously activating tumor-progression genes[[116](#_ENREF_116)]. These observations indicate that a more in-depth understanding of epigenetic mechanisms is needed to obtain further insights into the *in vivo* determinants of responses to epigenetic drugs.

A recent addition to the family of epigenetic drugs is GSK-1, the first chemical inhibitor synthesized for targeting histone demethylases JMJD3/UTX (KDM6A/KDM6B) in a selective and potent manner[[117](#_ENREF_117)]. These histone demethylases (JMJD3/UTX) catalyze removal of the trimethylation marker of H3K27. JMJD3 is induced *via* nuclear factor-κB exclusively in macrophages upon lipopolysaccharide (LPS) stimulation, providing an important link between inflammation and epigenetic reprogramming [[118](#_ENREF_118)]. This small molecule specifically inhibits the LPS-induced pro-inflammatory response by sustaining the repressive effect of H3K27 on the TNF-α gene, as well as by inhibiting recruitment of RNA polymerase II during transcription, thereby reducing TNF-α expression[[117](#_ENREF_117)]. Chronic inflammation due to chronic HBV and HCV infection, or obesity, gives rise to liver injury that slowly progresses to HCC, with elevated levels of pro-inflammatory cytokines TNF-α and interleukin (IL)-6 in HCC[[119](#_ENREF_119),[120](#_ENREF_120)]. By suppressing TNF-α expression, GSK-1 may serve as a potential candidate drug for HCC.

**MiRNA-BASED TREATMENT OF HCC**

MiRNAs with tumor suppressor roles in HCC, such as miR-26a, are feasible anti-cancer agents. Specifically, reduced expression levels of miR-26a have been reported in human HCCs[[62](#_ENREF_62)], whereas systemic restoration of miR-26a expression by an adeno-associated virus suppresses cancer cell proliferation in a liver-specific Myc transgenic mouse model[[121](#_ENREF_121)]. MiR-26a exerts these effects by directly targeting and down-regulating cyclins D2 and E2, consequently inducing cell cycle arrest. Importantly, as reported in that study, the cytotoxic effect of this miRNA is minimal in major organs. In another mouse model, in which liver cancer was induced by administration of the chemical hepatocarcinogen diethylnitrosamine, restoration of miR-124 by systemic injection significantly reduced liver tumor size[[122](#_ENREF_122)]. Likewise, no cytotoxic effects on vital organs were detected.

**COMBINATION OF EPIGENETIC DRUGS WITH EXISTING THERAPEUTIC MODALITIES IN HCC**

Aberrant expression of several HDACs and copy number gains of HDAC3 and HDAC5 were detected in HCC patients[[123](#_ENREF_123)], providing the rationale for treating HCC with HDAC inhibitors. In preclinical models of HCC, the combination of the pan-HDAC inhibitor panobionostat and sorafenib significantly decreased tumor growth and improved survival in murine xenograft models, compared to either drug used alone. Detailed molecular mechanisms include induction of apoptosis, acetylation of histone 3, down-regulation of BIRC5, or up-regulation of CDH1. This observation implies that such combination treatment may also achieve favorable clinical outcome for HCC patients.

Besides reversing aberrant epigenetic modifications in diseased conditions, epigenetic drugs can also induce host immunogenicity by increasing tumor antigen presentation. Systemic administration of the DNMT inhibitor 5-aza-2’-deoxycytidine induces the cancer/testis antigen (CTA) and augments adoptive immunotherapy by making cancer cells more visible to immunotherapy[[124](#_ENREF_124)]. Under the inflammatory microenvironment of melanoma, melanocytes exhibit cell plasticity by converting between differentiated and undifferentiated status caused by TNF-α. Gradually, cells acquire resistance to adoptive cell transfer therapy[[125](#_ENREF_125)]. The immunomodulatory activity of 5-aza-2’-deoxycytidine *in vivo* suggests its clinical use to design novel strategies of CTA-based chemo-immunotherapy for melanoma patients[[126](#_ENREF_126)]. Such a concept may also be extended to the treatment of HCC, especially since HCC routinely occurs on a background of inflammation resulting from chronic HBV or HCV infection, where increased levels of IL-6[[119](#_ENREF_119),[127](#_ENREF_127)] and TNF-α[[120](#_ENREF_120)] are readily detected.

**CONCLUSION**

Like other types of cancers, HCC is associated with multiple genetic mutations and epigenetic aberrations. Whereas gene mutations are not easily amenable for therapy, epigenetic aberrations that appear frequently in HCC may serve as new targets[[128](#_ENREF_128)]. To date, global DNA hypomethylation, promoter methylation, aberrant expression of miRNAs, and dysregulated expression of other epigenetic regulatory genes such as EZH2 are the best-known epigenetic abnormalities in HCC. Epigenetic drugs targeting these abnormalities may reverse the expression of dysregulated genes that are important in apoptosis and cell cycle arrest, thereby controlling the development and/or progression of HCC. Concerns of using epigenetic drugs are their off-target effects, which may activate genes having the opposite effects. Detailed mechanisms of action should be further investigated in multiple cancer types. Future research should aim at understanding how best to identify patient groups that would benefit most from the prescribed therapy, depending on the clinical setting, for example, disease stage, disease background, which drugs will give the best outcome, and when they should be administered. Useful biomarker(s) should provide guidance in identifying patients who may have optimal responses and reduced likelihood of relapse. Last but not least, most epigenetic drugs are used mainly in advanced tumors. Whether these drugs can be used as a preventative measure or in high risk patients who may benefit from future therapy remains to be explored.

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**Table 1 MicroRNAs in hepatocellular carcinoma and their characteristics**

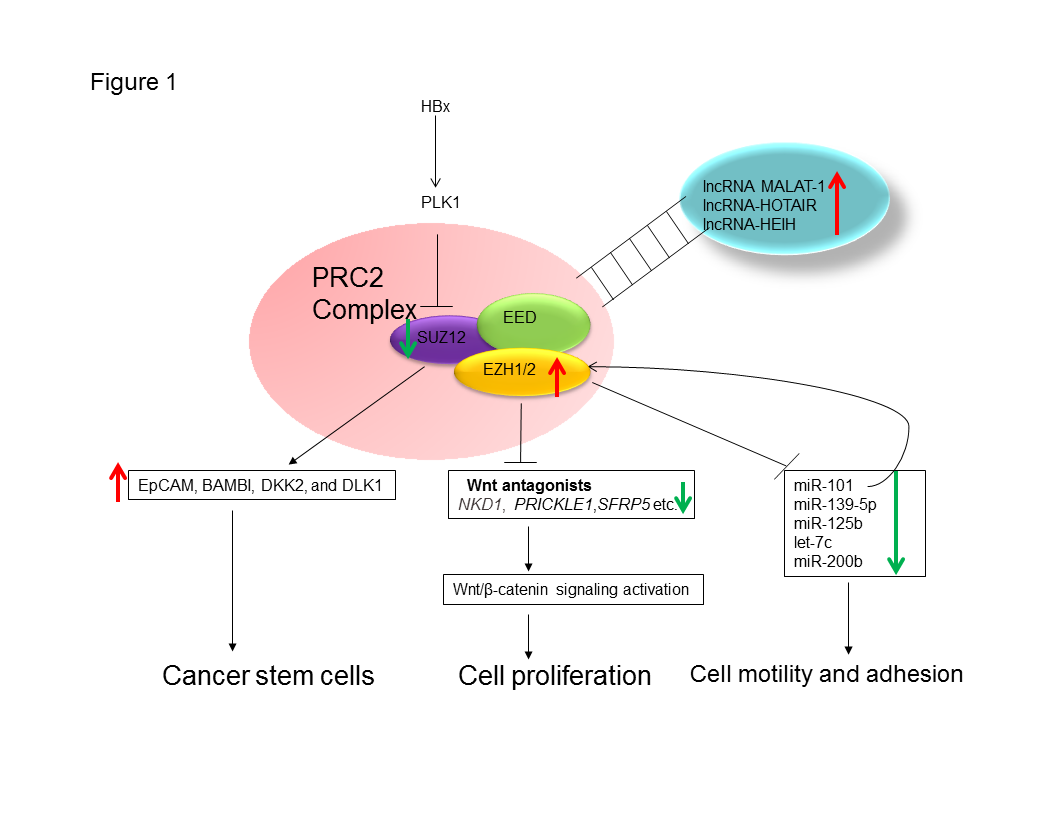
|  |  |  |  |
| --- | --- | --- | --- |
| Role | miRNA | Characteristics | Ref. |
| Viral replication | miR-141 | Represses HBV expression and replication | [[48](#_ENREF_48)] |
|  | miR-122 | Inhibits HBV replication |  |
| miR-122 | HCV RNA stabilization, propagation and replication | [[50](#_ENREF_50)] |
| miR-1 | Increases HBV replication | [[56](#_ENREF_56)] |
| Tumor supressor | miR-138 | Down-regulated in HCC tissues. miR-138 can directly target cyclin D3 | [[58](#_ENREF_58)] |
| miR-26a and miR-26b | Down-regulated in tumors compared to paired non-tumor tissues | [[62](#_ENREF_62)] |
| miR-125b | Arrests cell cycle progression, and inhibits migration and invasion by directly targeting the oncogene LIN28B2 | [[63](#_ENREF_63)] |
| miR-140-5p | Suppresses tumor growth and metastasis by targeting TGFBR1 and FGF9 | [[64](#_ENREF_64)] |
| miR-122a | Mice lacking the gene encoding miR-122a are viable but develop temporally controlled steatohepatitis, fibrosis, and HCC | [[66](#_ENREF_66)] |
| Target tumor initiating cells | miR-181 | Maintains the stemness of liver cancer stem cells, target liver differentiation transcription factors CDX2 and GATA6 | [[76](#_ENREF_76)] |
| miR-150 | Overexpression led to reduction of CD133+ cells | [[78](#_ENREF_78)] |
| miR-548c-5p | Ectopic overexpression inhibited proliferation, migration, and invasion of CD90+ HepG2 cells by down-regulating the expressions of β-catenin, Bcl-2, Tg737, Bcl-XL, and caspase 3 | [[79](#_ENREF_79)] |

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

**Table 2 List of drugs targeting epigenetic modifications in hepatocellular carcinoma**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Epigenetic modification** | **Targets** | **Drug(s)** | **Cell line/Animal model/Clinical trial phase** | **Results** | **Ref.** |
| DNA methylation | DNA methyltransferase | Zebularine | Huh7 and KMCH cell lines.  Human xenograft models | Zebularine-sensitive cell lines (Huh7 and KMCH) showed preferential demethylation of genes for tumor suppression, apoptosis, and cell cycle regulation.  *In vivo* inhibition of tumor growth in xenograft model. | [[108](#_ENREF_108)] |
|  | DNA methyltransferases | Zebularine | HepG2 cell line | Zebularine treatment inhibited cell proliferation and induced apoptosis in HepG2 cell line. | [[107](#_ENREF_107)] |
|  | DNA methyltransferases | 5-aza-2′-deoxycytidine | MMC-7721 and HepG2 cell lines | Inhibited telomerase activity, accompanied by reactivation of p16 and c-Myc. DAC synergized with cisplatin on growth inhibition. | [[109](#_ENREF_109)] |
| Histone acetylation |  |  |  |  |  |
|  | Histone deacetylase | Belinostat | PLC/PRF/5, Hep3B and HepG2 cell lines | Inhibited cell growth and induced apoptosis. | [[110](#_ENREF_110)] |
|  | Histone deacetylase | Belinostat | Multi-center phase I/II clinical trial | Stabilized tumor in non-resectable advanced HCC. | [[114](#_ENREF_114)] |
|  | Histone deacetylase | Suberoylanilide hydroxamic acid | HepG2, Hep3B and SK- Hep1 cell lines | Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. | [[112](#_ENREF_112)] |
| Combination | Histone deacetylase+ TKIs | Panobinostat +Sorafinib | Huh7, Hep3B and HepG2 cell linesHCC xenograft model | Induction of apoptosis.  Combined panobinostat and sorafenib decreased vessel density, tumor volume and increased survival in HCC xenografts. | [[123](#_ENREF_123)] |

HCC: Hepatocellular carcinoma.

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**Figure 1 Epigenetic regulatory networks in hepatocellular carcinoma.** Thediagram depicts the composition of PRC2, which consists of core components EZH1/2, EED, and SUZ12. The PRC2 complex intersects multiple layers of epigenetic regulators to maintain cancer stem cell features, cell proliferation, cell motility and adhesion. The red arrows indicate upregulation of genes, and the green arrows indicate downregulation of genes.