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Review Article

Hepatoepigenetic Alterations in Viral and Nonviral-Induced Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is a major public health concern and one of the leading causes of tumour-related deaths worldwide. Extensive evidence endorses that HCC is a multifactorial disease characterised by hepatic cirrhosis mostly associated with chronic inflammation and hepatitis B/C viral infections. Interaction of viral products with the host cell machinery may lead to increased frequency of genetic and epigenetic aberrations that cause harmful alterations in gene transcription. This may provide a progressive selective advantage for neoplastic transformation of hepatocytes associated with phenotypic heterogeneity of intratumour HCC cells, thus posing even more challenges in HCC treatment development. Epigenetic aberrations involving DNA methylation, histone modifications, and noncoding miRNA dysregulation have been shown to be intimately linked with and play a critical role in tumour initiation, progression, and metastases. The current review focuses on the aberrant hepatoepigenetics events that play important roles in hepatocarcinogenesis and their utilities in the development of HCC therapy.

1. Introduction

HCC is the most common primary malignancy of hepatocytes that make up 70–80% of the liver mass, and it develops as a result of advanced hepatic disease and cirrhosis [1]. HCC is the third leading cause of cancer-related deaths worldwide, accounting for about 1 million deaths annually [2]. More than 80% of HCC cases occur in people who reside in sub-Saharan Africa, South East Asia, and eastern Mediterranean. HCC primarily occurs due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Nonalcoholic steatohepatitis, aflatoxin exposure, haemochromatosis, obesity, severe alcohol intake, diabetes, and other metabolic factors are additional risk factors that can predispose to liver cancer. Patients with HCC generally present at an advanced stage due to compensated cirrhosis defined by the absence of pathognomonic symptoms, resulting in death within 6 to 20 months, suggesting an urgent need in treatment modalities that will dramatically decrease the mortality rate of HCC [2–4].

Liver diseases are characterised by chronic hepatic inflammation and damage, which appear to be important risk factors for hepatocarcinogenesis. Substantial evidence shows that alteration in the expression of NF-kB-induced proinflammatory cytokine TNF-α and interleukins, oncogenes, and tumour suppressor genes typically follows chronic hepatic inflammation associated with epigenetic aberrations [5]. Hepatocarcinogenesis has been described as CpG island methylator phenotype-positive (CIMP) multistep processes associated with the hallmark of successive accumulation of aberrant genetic and epigenetic alterations which co-operate to drive the malignant phenotype [6]. Deeper understanding of epigenetic aberrations, their interconnectivity, and clinical phenotypes in HCC patients may provide useful insights in the development of novel and more effective biomarkers for HCC treatment and better prognosis. In this review, we will highlight some of the hepatoepigenetic events that occur in response to nonviral and viral aetiologies, but mainly focusing on HBV and HCV infections.

2. Hepatoepigenetics

Hepatoepigenetics refers to activation or silencing in the expression of hepatic genes through chemical markers on DNA that do not involve mutations of the underlying sequence [7–10]. DNA methylation, histone modification, and noncoding miRNA are important epigenetic phenomena that collaboratively regulate gene expression and alter the normal function especially during pathological processes [7–10]. DNA methylation encompasses the attachment of a methyl group to the cytosine, guanine, or amino acids of histones wrapped with DNA, often leading to either normal or aberrant modification in gene function. DNA methylation often targets the CpG island promoter regions, which are a small (0.5–2 Kb) stretch of DNA with considerable quantity of CpG-rich regions as compared to the rest of the sequence [11, 12].

Addition or maintenance of methyl groups on the nucleotide sequence is usually catalysed by various DNA methyltransferases (DNMT) including DNMT1, DNMT3A, and DNMT3B. Aberrant DNA methylation is a common and well-described phenotype in HCC, and it can be defined as hypo- or hypermethylation depending on the targeted gene and alteration status [13]. Cancer-related hypermethylation denotes increased methylation in the CpG islands that is normally devoid methylation in normal cell and often results in the suppression of tumour suppressor genes [8, 14]. In contrast, hypomethylation signifies loss of DNA methylation and leads to activation of oncogenes in cancerous cells [7, 8]. Methyl groups can also be removed in the process known as DNA demethylation, a key regulator in tumour progression. Active DNA demethylation is governed by a group of ten-eleven translocation (Tet1, Tet2, and Tet3) enzymes that utilise oxygen to decarboxylate α-ketoglutaric acid and iron leading to oxidation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC) [15, 16].

Histone modifications involving acetylation and methylation also contribute to hepatocarcinogenesis. Histone acetylation involves addition of an acetyl group from acetyl coenzyme via histone acetyltransferase [17]. Histone methylation uses histone methyltransferases (HMTs) to transfer 1 to 3 methyl groups from S-adenosyl-L-methionine to arginine (R) or lysine (K) of the histone proteins that package and order DNA into structural units called nucleosomes [18–22]. Histone methylation regulates gene transcription and is implicated in carcinogenesis. Several histone variants associated with either activation or silencing in gene transcription are well characterised, including histone (H) 2A, H2B, H3K4, H3K9, H3K27, H3K36, H3K79, H4K5, H4K8, H4K12, H4K16, and H4K20. Methylation of H3K4, H3K36, and H3K79 is associated with transcriptional activation whereas methylation of H3K9 and H3K27 leads to transcriptional repression. Histone methylation can be either mono-, di-, or trimethylated [23, 24]. Altered histone methylation or acetylation targeting H3K4, H3K9, and H3K27 has been well-described in HCC and will be unpacked later in this review. Methylation of H3K4 is activated by SET-1 family enzymes SET1A, SET1B, MILL1, MILL2, MILL3, and MILL4. Set domain bifurcated 1 (SETDB1) regulates epigenetic repression of euchromatic genes via H3K9me3 by recruiting heterochromatin protein 1-(HP1-) related proteins to methylated histones [25, 26].

H3K27 methylation is catalysed by one of the two classes of polycomb-group proteins (PcGs) [27]. PRC2 represses the transcriptional activities of genes involved in cell cycle regulation, differentiation, and proliferation [27]. It is activated by its subunit, a famous enzyme enhancer of zester homolog-2 (EZH2), which requires its binding partners, suppressor of zeste 12 protein homolog (SUZ12), and embryonic ectoderm development (EED) for proper function [28]. EZH2 has been described as a useful marker for aggressive stages and its upregulation leads to HCC malignant progression signifying poor prognosis [29, 30]. PRC1, another PcG, is an E3 ubiquitin ligase that transfers monoubiquitin to the C-terminal tail of H2A at K118/119 [31, 32]. It is catalysed by RING class heterodimer, chromobox homolog 8 (CBX8), B-Lymphoma moloney murine leukemia virus insertion regional-1 (BMI1), and MEL18 paired with RING1A/B [30, 33]. Lysine specific demethylase 1 (LSD1) and Jumonji domain containing proteins (JMJD1A, JMJD2, JMJD3/UTX, and JARID1B) are two major histone demethylases (HDMTs) that have been identified to erase methylation from histone proteins in HCC [34].

Noncoding miRNA (miR) is another epigenetic mechanism that primarily regulates gene transcription at the post-transcriptional level and also contributes to hepatocarcinogenesis [35, 36]. miRs are a large class of 22 nucleotide long small RNAs processed from long endogenous transcripts that usually form local hairpin structure through Watson-Crick pairing (e.g., A-U and G-C) [37, 38]. Three key steps required for miR biogenesis [38] include nuclear processing of primary miR by DROSHA, nuclear export of precursor miR by Export in 5, and finally cytoplasmic processing of pre-miR by DICER [38, 39]. miRs do not encode for proteins, however, they can still alter gene transcription by base-pairing with complementary miR response element sequences located at the 3′-untranslated region of their target genes. miR-mediated gene regulation plays an important role in a variety of cellular processes such as cell differentiation and organ development, cell cycle progression, growth, proliferation, and apoptosis [40]. In HCC patients, miRs have been shown to alter gene transcription leading to tumour inhibition, progression, and poor prognosis [41]. This suggests that miRs may serve as transcription factors, oncogenes, or tumour suppressor genes contributing to hepatocarcinogenesis.

3. Nonviral-Induced HCC Hepatoepigenetic Aberrations

Substantive meta-analysis studies show that HCC tumours exhibit consistent CpG island promoter hypermethylation of classical tumour suppressor genes such as *ras association domain family 1A* (*RASSF1A*), *p16$^{ink4\alpha}$/cyclin dependent kinase inhibitor 2A* (*CDKN2A*), *p15*, *suppressor of the cytokine signalling 1* (*SOCS1*), *E-cadherin* (*CHD1*), and *glutathione-S-transferase Pi 1* (*GSTP1*) [42, 43] (Figure 1). CpG island promoter hypermethylation-mediated inactivation of these tumour suppressor genes especially *RASSF1A*, *E-cadherin*,

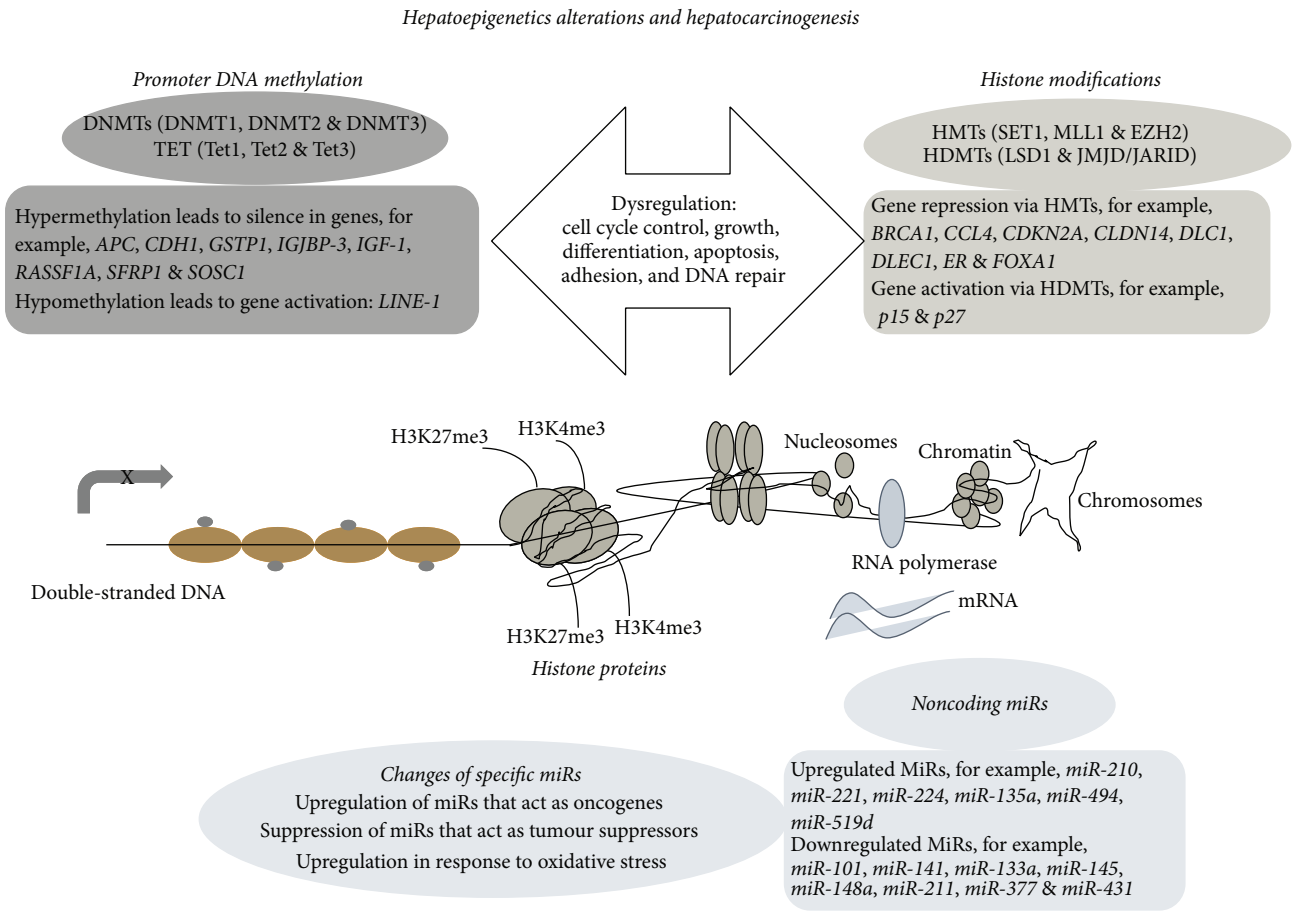

Figure 1: Epigenetic alterations in hepatocarcinogenesis. DNA methylation, histone modification, and noncoding miRs cooperate in altering gene transcription and hepatic architecture leading to perturbed cellular processes associated with tumour initiation and metastases. Alteration in gene transcription is governed by several enzymes, including DNA methyltransferases (DNMTs), group of ten-eleven translocation (Tet1, Tet2, and Tet3) enzymes, histone methyltransferases (HMTs), and histone demethylases (HDMTs).

GSTP1, and *SOCS1* genes is associated with either increased risk of HCC and more aggressive clinical phenotype with high risk of metastasis [43, 44]. Promoter hypermethylation of *RASSF1A* was demonstrated to be a valuable diagnostic marker that can be used to complement the alpha fetoprotein (AFP) in screening for HCC [45]. As an important regulator of epithelial-mesenchymal transition (EMT), E-cadherin impedes cell adhesion within hepatic tissue resulting in loss of cell polarity and acquisition of mesenchymal phenotype. This promotes tumour cell infiltration, an essential feature associated with migration and metastasis into the surrounding or distant tissues and organs [46]. For example, aberrant expression of Notch1, which is an important silencer of E-cadherin through activation of Snail1, was associated with tumour-node-metastasis stages III-IV, tumour venous invasion, and poor prognosis in HCC patients [47]. Hypermethylation of *GSTP1* gene also allows intrahepatic metastases of HCC by enhancing the expression of β-catenin molecules that mediate epithelial cell adhesion [48]. *SOCS1* stimulates hepatocyte regeneration by negatively regulating the Janus kinase-Signal transducer and activator of transcription (JAK-STAT) signalling pathway. Aberrant epigenetically silenced *SOCS1* leads to uncontrolled cell differentiation and proliferation associated with HCC aggressiveness [49, 50]. *Ras association* (*ralgds/af-6*) *domain family member 1* (*RASSF1A*), *insulin-like growth factor 2* (*IGF-2*), and *adenomatous polyposis coli* (*APC*) have also recently been shown to be hypermethylated in HCC patients in which they act as potential candidate epidrivers that predict poor clinical outcome [51].

Global DNA hypomethylation on pericentromeric satellite regions is a frequent and early event associated with chromosomal instability induced via heterochromatin decondensation and enhanced recombination enhancement in hepatocarcinogenesis [52]. DNA hypomethylation of chromosome 1 heterochromatin coincides with Q-arm copy gain in HCC. More than 90% of HCC tissues exhibit loss of heterozygosity (LOH) on different chromosomes in various tumour suppressor genes, and these genetic alterations further inactivate gene transcription to initiate tumour development [53, 54]. LOH located on chromosome 16 encoding *axis inhibition protein 1* tumour suppressor gene was observed more frequently in poorly differentiated or metastatic tumours and therefore rather contributes to tumour aggressiveness than initiation [54, 55]. HCC-related DNA hypomethylation is

commonly observed in promoter regions of transposable elements such as *long interspersed nuclear element-1* (*LINE-1*) and monoacylglycerol acyltransferase-2 (*MGAT3*) [56]. Active demethylation mediated by Tet proteins was shown to play an important role in global DNA methylation and HCC. For instance, Tet 2 and Tet 3 proteins are abnormally regulated in HCC and result in the reduction of 5hmC, suggesting a new therapeutic modality for HCC [57].

Several histone posttranslational modifications also play an important role in altering the transcription of cellular genes including tumour suppressors that are commonly known to epigenetically promote malignant transformation [58, 59]. MacroH2A1 is a variant of histone H2A protein that correlates with suppressed gene transcription in the chromosomal region, and it has been found to be heavily upregulated in HCC [60]. The interaction of macroH2A1 and DNA hypermethylation was found to intercept HCC progression by attenuating chemotherapy-induced senescence in rodents and human livers [61]. This synergistic effect results in the suppression of a subset of tumour suppressor genes (CDKN2A, DLEC1, and RUNX2) and enhanced HCC cell growth in Hep G2 and Huh-7. Treatment with DNA hypomethylating agent guadecitabine reversed this effect leading to inhibition of HCC cell growth [62]. EZH2 is an important component of PRC2 that interacts with EED and SUZ12 to establish trimethylation of H3K27 (H3K27me3) leading to tumour initiation and progression [63, 64]. H3K27me3 is a well-established marker of transcriptionally silent chromatin implicated in hepatocarcinogenesis [59]. Gao and coauthors identified *CDKN2A* as a target of repression by H3K27me3 mediated by upregulation of EZH2 and SUZ12 enzymes in HCC human samples [64]. *CDKN2A* encodes proteins for $p16^{ink4\alpha}$ and $p14^{arf}$, two important tumour suppressor genes that prevent tumour formation by regulating cell growth, division, and apoptosis. The $p16^{ink4\alpha}$ protein attaches to cyclin dependent kinase- (CDK-) 4 or CDK-6 to inhibit cell cycle progression. The $p14^{arf}$ protein protects p53 from degradation and promotes p21 activation leading to controlled cell division and enhanced apoptosis. Epigenetic repression of *CDKN2A* resulted in the obstruction of CDKN2A-TP53-P21 pathway leading to HCC initiation and aggressiveness. Importantly, reduced expression of PRC2 protein via H3K27me3 inhibitor restored CDKN2A-TP53-P21 pathway and effectively blocked the aggressive phenotype of HCC cells [63]. Epigenetic silencing by EZH2-mediated with H2K27me3 in HCC was also observed with other several tumour suppressor genes including *deleted in lung cancer 1* (*DLC1*) and *chromodomain helicase DNA binding proteins 5* (*CHD5*), and this correlates with metastasis and poor prognosis [58, 65]. Simultaneous activation of H3K27me3 and acetylation in association with aberrant expression of p53 gene was also reported in HCC with aggressive phenotype [66]. G9a, GLP, and suppressor of variegation 3-9 homolog 1 (SUV39H1) are another group of HMTs responsible for H3K9 methylation in association with p53. Increased expression of G9a-induced H3K9 methylation was also associated with poor prognosis in HCC patients [67].

Claudin 14 (CLDN14) was recently labelled a novel prognostic biomarker of HCC [68]. Integrative genome-wide analysis of H3K27me3 and gene expression profiling using chromatin immunoprecipitation with high-throughput sequencing and gene expression microarray showed that CLDN14 was another target for EZH2-mediated H3K27me3. Negative regulation of CLDN14 was associated with increased expression of EZH2 and H3K27me3. CLDN14 is a cell adhesion tight junction molecule that belongs to the claudin family proteins found in all epithelial and endothelial cells, and it plays an important role in selective paracellular permeability. Altered expression of CLDN14 was associated with activation of Wnt/β catenin signalling pathway leading to EMT of HCC cells. Low levels of CLDN14 expression consistently correlates with tumour aggressiveness and poor prognosis, suggesting that it is an effective prognostic marker and therapeutic target for HCC therapy. Silencing of CLDN14 protein expression with similar consequences was also observed in HCC tissues as a result of the CpG island promoter hypermethylation [69]. This suggested an intimate link of epigenetic mechanisms in synergistically dysregulating gene transcription [66].

LSD1 and JmjC domain containing H3K4 histone demethylase Jumonji AT-rich interactive domain 1B (JARID1B) are erasers for mono-, di-, and trimethylation of H3K4 (Figure 1). LSD1 is frequently overexpressed in HCC cells and promotes tumorigenesis by epigenetically dysregulating EMT and glycolytic and mitochondrial metabolism [70, 71]. JMJD1A is an important regulator of hypoxia-inducible transcription factor and also stimulates tumour growth. Upregulation of JMJD1 was significantly associated with HCC cell growth and recurrence, supporting the notion that histone demethylation plays an important role in hepatocarcinogenesis [34, 72]. JARID1B, also known as lysine demethylase 5B (KDM5B), acts as an oncogene and contributes to hepatocarcinogenesis by promoting cell migration and invasion. Most recently, it was demonstrated that knockdown of KDM5B blocks HCC cell proliferation and arrests cell cycle progression at G1/S-phase by upregulating p15 and p27 expression via H3K4 tri-methylation. Bone morphogenetic protein 7 (BMP7) is a secreted ligand for transforming growth factor-β that binds to and phosphorylates the SMAD family member proteins to activate gene transcription [73–75]. BMP7 is also a KDM5B target protein, and its aberrant inactivation is associated with HCC invasiveness with phenotypic features of TGF-β-induced cell migration, invasion, and EMT [76]. KDM5B has been labelled a potential target for cancer treatment because it has also been shown to promote tumorigenesis by targeting and silencing tumour suppressors such as *p21*, *breast cancer 1* (*BRCA1*), *estrogen receptor* (*ER*), *forkhead box 1* (*FOXA1*), *carbon tetrachloride* (*CCL4*), and *caveolin 1* in some human malignancies [77].

Regulation of noncoding RNAs by aberrant DNA methylation and histone modifications also provided some insights in the pathogenesis of HCC [78]. Bioinformatics studies revealed an array of several epigenetic-regulated miRs that emerged to be differentially pivotal in hepatocarcinogenesis. For instance, *miR-101*, *miR-141*, *miR-133a*, *miR-145*, *miR-148a*, *miR-211*, *miR-377*, and *miR-431* reduce HCC progression by inducing apoptosis and suppressing cell proliferation,

migration, and invasion. These *miRs* target various genes such as *vascular endothelial growth factor C*, *hepatocyte nuclear factor-3β*, *fascin 1*, *sphingosine-1-phosphate receptor 1*, *secreted protein acidic and rich in cysteine*, *T-cell lymphoma invasion and metastases 1*, and *zinc finger E-box binding homeobox 1* [79–84]. In particular, *miR-145* was found to function as a tumour suppressor gene and inhibited HDAC2 oncogenic effects by restoring the expression of G1/S cell cycle proteins in HCC [85]. In contrast, *miR-135a* and *miR-494* promote tumour growth and increase sorafenib treatment resistance by epigenetically regulating genes such as *forkhead box O1* and *phosphatase and tensin homolog* (PTEN) [86, 87]. Additionally, upregulation of *miR-210, miR-221*, *miR-224*, and *miR-519d* was shown to promote hepatocarcinogenesis by targeting *CDK1*, *CDKN1C/p57*, *CDKN1B/p27*, *CDKNA/p21*, *AKT serine/threonine kinase 3* (*AKT3*), *TIMP metallopeptidase inhibitor 2* (*TIMP2*), and *PTEN* [88–90]. Attenuated and noninfectious lentiviruses, adenoviruses, adenoassociated viruses, and herpes simplex viruses are commonly used as delivery vehicles for miR antagonists or mimics in various cancers [91]. However, there are limited data on miR-based therapeutics for HCC.

4. HBV-Induced HCC Hepatoepigenetic Alterations

Hepadnaviruses are the primary cause of hepatitis in both humans and animals. They are nonsegmented and very small genomes of 3.2 kb relaxed circular (rc) partially double-stranded DNA that, upon infection, are transported into the nucleus by nucleocapsid [92]. Inside the nucleus, covalently closed circular DNA (cccDNA) is converted from rcDNA and serves as a template for viral replication through RNA intermediates using reverse transcriptase. *Orthohepadnavirus* and *Avihepadnavirus* are two well-characterised hepadnavirus genera [93, 94].

During persistent infection, an *Orthohepadnavirus* HBV genome integrates in the host DNA leading to accumulation of profound genetic and epigenetic signatures that subsequently leads to HCC development [95]. Considerable data demonstrate that more than 90% of HCC cases exhibit integrated HBV genome within the host DNA [96]. HBV integration induces *trans*- and *cis*-activation in HBV and host genome, respectively. It has been shown that, following integration into the host DNA, HBV genome undergoes methylation induced as part of innate immune defense mechanisms to protect the host from increased viral replication [95–97]. Vivekanandan and his coauthors demonstrated that long-term upregulation of DNMTs in the host may also become detrimental by methylating the surrounding hepatocyte CpG islands promoter regions. This may lead to activation of oncogenes or silencing in immunoregulatory and tumour suppressor genes that are critical in hepatocarcinogenesis [98]. Numerous studies showed that HBx gene, transcriptional activator, and oncogenic protein encoded by HBV manipulate DNMTs and induce promoter hypermethylation of wide array of cellular tumour suppressor genes. HBx-mediated methylation phenotype of these tumour suppressor genes was associated with tumour progression, aggressiveness and poor prognosis as a result of disrupted host cellular signalling pathways that regulate DNA repair, cell growth, proliferation, and apoptosis [99–102].

Retinoic acid receptor β2 (*RARβ2*) is one of the isoforms encoded by *RARβ,* which is a nuclear receptor gene that was first identified in HCC where it flanks a HBV integration site. It binds to and inactivates the E2F1 transcription factor, which is essential for cell cycle progression [103–105]. HBx protein was shown to induce the hypermethylation of *RARβ2* promoter region by upregulating DNMT1 and DNMT3A activities leading to repression in the expression of RARβ2 protein [104, 106]. This *cis*-activation with *RARβ2* gene was associated with activation of E2F1 transcription factor, which abolishes the ability of retinoic acid to regulate expression of G_1 checkpoint regulators such as p16, p21, and p27 proteins. Under the influence of HBx transcriptional activities in the cytoplasm, *RAR* may also promote hepatocarcinogenesis by interacting with other proteins such as AFP. It was recently shown that interaction of HBx-induced AFP with *RAR* resulted in perturbed *RAR* signalling pathway, leading to repression of growth arrest and DNA damage 45*α* (GADD45*α*) protein expression [107]. GADD45*α* is an 18.4 kDa RNA-binding acidic protein that is expressed in response to DNA damage for repairing and induction of apoptosis by inhibiting G2/M transition of cell cycle. Downregulation of GADD45*α* in HCC was associated with uncontrolled HBV-infected hepatocytes growth and hepatocarcinogenesis, suggesting its effects in allowing the cells to evade senescence and apoptosis [104, 107]. Although the role of GADD45*α* in promoting instability through DNA demethylation has been reported previously, it has not been explored in HBV-induced HCC and therefore warrants investigation [108, 109]. Caveolin- 1, encoded by *caveolin-1* gene, is an integral membrane protein abundantly expressed in adipose, fibrous, and endothelial tissue [110]. HCC cells expressing high levels of Caveolin-1 protein are associated with uncontrolled cell growth, motility, in vivo tumour aggressiveness, and metastasis [111]. Conversely, HBx-induced methylation of *caveolin-1* gene promoter region suppresses its transcriptional activities leading to reduced tumour aggressiveness and metastasis, indicating a good prognostic marker of the disease [110].

Deleted in Lung and Esophageal Cancer 1 (*DLEC1*) is a functional tumour suppressor gene silenced by promoter hypermethylation in several human malignancies including HBV-induced HCC through activation of DNMT1 and HATs [112–114]. *Insulin-like growth factor binding 3* (*IGJBP-3*) is another potential tumour suppressor gene which was shown to be methylated and histone deacetylated in The Chang Liver, HuH-7, and HEP-G2 cells expressing HBx-construct [115]. Downregulation of *IGJBP-3* gene expression following hypermethylation of CpG island within *IGJBP-3* promoter regions was observed in HBV-induced HCC tissues, and the expression was restored by azacytidine-2′-deoxycytidine [116].

In mouse and cell line models, repression of $p16^{ink4\alpha}$ gene via H3K27me3 was found to be an early aberrant epigenetic event associated with HCC initiation and progression [117].

Several studies showed similar effects through HBx-induced hypermethylation within the promoter region of $p16^{ink4\alpha}$ gene via DNMT1/3A, and this was associated with advanced HBV infection [118–120]. These data may suggest synergistically relationship between H3K27me3 and hypermethylation in promoting HBV-related hepatocarcinogenesis via alteration of $p16^{ink4\alpha}$ gene. Cooperation in the activity of epigenetic mechanisms has also been observed with *deleted in liver cancer 1* (*DLC1*) gene. EZH2 and other demethylating agents also play an important role in HBV-related hepatocarcinogenesis. Synchronous epigenetic regulation targeting classic tumour suppressor genes known to be altered in HBV-related HCC was also demonstrated with aberrant acetylation and trimethylation of H3K27 catalysed by CBX8, BMI1, EZH2, and SUZ12 enzymes [66]. It was recently shown that epithelial cell adhesion molecule (EpCAM) was upregulated via active DNA demethylation catalysed by EZH2 and Tet2 in conjunction with nuclear factor kappa B (NF-Kβ) and RelA [121]. EZH2 promotes HCC motility and metastasis by epigenetically silencing the expression of multiple tumour suppressor miRs including *miR-99a*, *miR-101*, *miR-125b*, *miR-139-5p*, and *let-7c* [122]. Downregulation of *miR-99a* in HCC is associated with suppressed tumour growth [123]. Downregulation of *miR-125* promotes proliferation and migration of HCC by upregulating SUV39H1 [124]. *MiR-101* is negatively regulated by c-Myc-EZH2 complex, and its epigenetic silencing is associated with HCC poorer prognosis [65, 125]. Serum *miR-150* was found to be a promising novel noninvasive diagnostic and prognostic biomarker for HBV-related HCC [126]. *SETDB1* is an oncogene that marks the transcriptional repression of euchromatic gene via H3K9me3. In the absence of HBx, *SETDB1* represses HBV-cccDNA transcription by regulating chromatin organization via methylation of H3K9me3 [127]. *SETB1* is habitually upregulated in HBV-related HCC through multiple complementary acting mechanisms that occur at certain chromosomal (gain of *SETB1* copy number at chromosome 1q21), transcriptional (hyperactivation of SP1 transcription factor), and posttranscriptional levels (methylation of p53 and loss of *miR-29*). Anomalous regulation of *SETB1* correlates with tumour growth, aggressiveness, and poorer prognosis in HBV-related HCC patients [128–130].

5. Hepatoepigenetic Alterations Elicited by HCV-Induced HCC

HCC and liver failure are the life-threatening conditions associated with untreated chronic HCV infection. Development of HCV-induced HCC is a multistep process that involves chronic liver inflammation and repetitive-cycles of hepatic fibrosis, which may occur over years leading to hepatic failure or cirrhosis and/or malignant transformation [131]. The recent development of directly acting antiviral agents (DAAs) has been an important revolution and exciting progressive era in the field of HCV therapy. Excellent clinical outcomes from increased SVR rates to curing HCV were observed in more than 90% of HCV-patients including those previously regarded as difficult-to-treat. This raised hope not only for the HCV eradication in the near future but also for the dramatic decline in the risk of developing HCV-induced HCC or recurrence of disease in previously HCC-treated patients [132, 133]. Unfortunately, recent data shows that DAAs may promote tumour development in patients with hepatic cirrhosis and recurrence of HCC in patients who had previously been cured, suggesting the need for effective HCV-related HCC treatment with better response and a higher safety profile [134].

HCV is an icosahedral blood-borne RNA virus of 9.6 kb genome and a member of the Flaviviridae family [135]. HCV genome encodes for a large polyprotein processed by viral and cellular proteinases to produce structural and nonstructural (NS) proteins [136, 137]. Amongst NS proteins are NS3, NS4A, NS4B, NS5A, and NS5B. The NS3-5B coding region serves as the HCV replicase, and it is therefore required for proper RNA replication in cell culture and chimpanzees [135, 138]. In particular, NS3, NS5A, and NS5A play an important role in HCV pathogenesis and potentiate oncogenic transformation. Unlike HBV, HCV genome is unable to integrate into the human genome. However, it is able to cause epigenetic changes that favour its own replication through NS3, NS5A, and NS5B oncogenic events that are associated with the development of liver cancer [139]. Apolipoprotein E, which is required for the replication and infectivity of HCV, is known to be hypermethylated in chronic HCV infection. This is associated with increased viral replication and an increased risk of developing malignancy [140, 141].

Tumour suppressor genes such as *E-cadherin* and *p16* are implicated in HCV core protein transcriptional activities and epigenetically dysregulated via DNMTs [142]. For instance, HCV core protein stimulates immortalization of hepatocytes by silencing p16 expression and E-cadherin (CDH1) via upregulation of DNMT1 and DNMT3B [142, 143]. Upregulation of DNMT1 and DNMT3B was associated with suppressed CDH1 mRNA in Huh-7 cells expressing HCV core protein of genotype 1b but not in genotypes 2a, 3a, 4h, and 5a. However, CDH1 protein expression level was downregulated in cells expressing HCV core of genotypes 1b, 2a, and 3a [144, 145]. Downregulation of *secreted frizzled-related protein 1* (*SFPR1*) gene via HCV core protein-mediated DNMT was associated with aberrant activation of Wnt-signalling pathway leading to HCC aggressiveness by EMT [146]. Silent SFPR1 was induced by the HCV core protein-induced methylation of SFRP1 promoter region via DNMT1, and this enhanced cell proliferation, migration, and invasiveness. Inhibition of HCV core-activated ECM by either knockdown of DNMT1 and HDAC1 or restoration of SFPR1 disrupted Wnt/β-catenin-c-Myc-cyclin D1 pathways leading to abolished tumour growth and aggressiveness [146].

The epigenetic alterations in HCV-induced HCC may also implicate *CDKN2A* gene. Several histone posttranslational modifications also play an important role in altering the transcription of cellular genes, including classic tumour suppressors that are commonly known to epigenetically promote malignant transformation. EZH2 is an important component of PRC2 that interacts with EED and SUZ12 to establish trimethylation of H3K27 (H3K27me3) leading to HCV-related HCC tumour initiation and progression. H3K27me3 is a well-established marker of transcriptionally silent

chromatin implicated in hepatocarcinogenesis [59]. *Cyclin dependent kinase inhibitor 2A* (*CDKN2A*) was identified as a target of repression by H3K27me3 mediated by upregulation in EZH2 and SUZ12 enzymes in HCC human samples [64]. *CDKN2A* encodes for $p16^{ink4\alpha}$ and $p14^{arf}$, two important tumour suppressor genes that prevent tumour formation by regulating cell growth, division, and apoptosis. The $p16^{ink4\alpha}$ protein attaches to cyclin dependent kinase (CDK) 4 or CDK6 to and inhibit cell cycle progression. The $p14^{arf}$ protein protects p53 from degradation and promotes p21 activation leading to controlled cell division and enhanced apoptosis. Epigenetic repression of *CDKN2A* resulted in the obstruction in CDKN2A-TP53-P21 pathway leading to HCC initiation and aggressiveness. Importantly, reduced expression of PRC2 protein via H3K27me3 inhibitor restored CDKN2A-TP53-P21 pathway and effectively blocked the aggressive phenotype of HCC cells [64]. Epigenetic silencing by EZH2-mediated H2K27me3 in HCC was also observed with other several tumour suppressor genes including *DLC1* and *chromodomain helicase DNA binding proteins 5* (*CHD5*) [58]. Reduced levels of *CHD5* in HCC cells coincide with metastasis and poor prognosis [65].

6. Epigenetic Drug Therapy and Immunotherapy of HCC

The primary objective of epigenetic therapy is to pharmacologically reverse the aberrant epigenetic alterations and to restore altered gene expression by specifically targeting abnormal cells while leaving normal cells unaffected. There are four epigenetic reversals currently approved for cancer treatment by the US Food and Drug Administration (FDA). Decitabine, azacytidine, vorinostat, romidepsin, and belinostat inhibitors are well-known epigenetic inhibitors that were developed to treat several haematopoietic cancers and explored in HCC [147, 148]. Decitabine (5-aza-2′-deoxycytidine) and azacytidine (5-azacytidine) are potent epigenetic reversals that incorporate into DNA and inhibit DNMTs activities leading to DNA hypermethylation and reactivation of gene transcription. Although these DNA methylation inhibitors were both approved to treat acute leukemia and myelodysplastic syndrome, azacytidine is more potent than decitabine and provides better clinical outcome [149, 150]. However, recent phase I/II studies showed that lower-dose decitabine based therapy exhibited antitumour activities and tolerance in patients with advanced HCC [151]. Decitabine and azacytidine also differ in the manner in which they mediate apoptosis and senescence in solid tumours [152]. In squamous cell carcinoma, senescence is mediated by decitabine-induced overexpression of *p16ink4a* [153]. Silencing of *p53* and upregulation of *GADD45* genes through azacytidine is associated with caspase activation and enhanced apoptosis in HCC and colon tumour cells [152, 154].

Immunotherapy involves the use of substances either produced by the body or in the laboratory to boost the host's innate and adaptive immune responses to fight infectious diseases and cancer. Although this method has shown to elicit impressive response rates in eradicating the transformed hepatocytes and controlling tumour growth/remission, the emergence of resistance and drug toxicity has been problematic [155, 156]. Combination therapy with decitabine and suberoylanilide hydroxamic acid (SAHA) significantly reduces cell growth and proliferation in a xenograft hepatoma model [157]. On the other hand, azacytidine induces apoptosis by inhibiting protein biosynthesis when used in combination with tumour necrosis factor related apoptosis inducing ligand (TRAIL) [158]. Guadecitabine is a hypomethylating agent that has been formulated as a dinucleotide antimetabolite of a decitabine that is linked to a guanosine through a phosphodiester bond. Preclinical studies showed that the combination of guadecitabine and cytotoxic agent oxaliplatin yielded a synergistic response by inhibiting cell growth and proliferation through disruption of Wnt/EGF/IGF signalling pathways in HCC models, suggesting that the combination of epigenetic inhibitors and immunotherapies may deliver a better response to HCC [159]. Combination of azacytidine and alendronate also elicited cytotoxic effects in Huh-7 HCC cells [160]. Recently, it was demonstrated that azacytidine promotes not only passive demethylation pathway of DNA but also TET2/3-mediated generation of 5hmC via active demethylation pathway in [57]. Combination of vitamin C and azacytidine enhances Tet activities in HCC cells leading to induction of active demethylation that suppresses the expression of Snail, a key regulator of EMT and cell cycle process [57].

Vorinostat (also known as SAHA) and romidepsin are two HDAC inhibitors (HDACIs) that were developed to treat a heterogeneous group of lymphoproliferative cutaneous T-cell lymphomas [161–163]. These HDACIs alter gene transcription by interfering with class 1 and II HDACs, leading to cell cycle arrest and apoptosis in a wide variety of transformed cells [13]. Vorinostat or SAHA perturbs the ERK/NF-κB signalling pathway, which is an important inducer for cell proliferation/and invasiveness and suppressor for apoptosis [164]. Sorafenib is a tyrosine kinase inhibitor targeted therapy for metastatic HCC, thyroid and renal cell cancers. A recent study showed that combination of SAHA and sorafenib has potential as a novel strategy for treating metastatic HCC (Table 1). This study demonstrated that prolonged treatment with sorafenib boosted therapeutic efficacy of SAHA against HCC in both cell line and animal models through inhibition of ERK/NF-κB signalling pathway [165]. Belinostat is another HDACI that was approved by FDA in 2014 for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma [166, 167]. Phase I and II-based study presented in 2012 Gastrointestinal Cancers Symposium reported disease stabilization and favourable safety profile in advanced HCC patients treated with belinostat [168]. Bortezomib is a potent proteasome inhibitor approved for the treatment of patients with mantle cell lymphoma [169]. Combination of belinostat and bortezomib exhibited significant antiproliferative and proapoptotic activities in HCC cell lines [170]. Thus the combination of epigenetic drugs and immunotherapy may provide highly promising, feasible, and intriguing therapeutic benefit to HCC patients (Table 1) [155, 156, 171].

Table 1: Strategies of HCC Treatment by Combining Epigenetic Drugs and Immunotherapy.

Combination	Outcome	References
Guadecitabine plus oxaliplatin	Inhibition in cell growth and proliferation	[159]
Azacytidine plus alendronate	Increased cell cytotoxicity and suppression of tumour growth	[160]
Azacytidine plus vitamin C	Suppression of cell proliferation and induction of cell cycle arrest	[57]
Decitabine plus SAHA	Reduces cell growth and proliferation	[157]
Azacytidine plus TRAIL	Prevents protein biosynthesis and induces apoptosis	[158]
Vorinostat plus sorafenib	Inhibition of tumour growth	[165]
Belinostat plus bortezomib	Suppression of cell proliferation and induction of cell cycle arrest	[170]

miRs are also important targets for epigenetic therapy. Substantiating data show that inhibition of miRs that act as oncogenes may eradicate tumour growth. For instance, inhibition of miR-221 blocks hepatocarcinogenesis, suggesting the potential of miRs as potential therapeutic targets for treating HCC [88]. It is evident that miRs play an important role in hepatoepigenetics and related tumorigenesis enlightening their utility to serve as potential biomarkers for HCC prediction, diagnosis, prognosis, and therapeutic targets.

7. Conclusion

Understanding the manner in which epigenetic mechanisms alter gene transcription and genome architecture is currently a major challenge but one which should yield better cancer therapeutic approaches [172]. Hepatoepigenetics is becoming increasingly visible with a growing understanding of the roles of specific epigenetic aberrations in the liver and their utilities in the development of cancer therapy. Substantiating data shows that DNA methylation, histone modifications, and noncoding miRs synergistically cooperate in aberrantly altering gene transcription and critical cellular processes that lead to hepatocarcinogenesis and metastasis. Epigenetics alterations are pharmaceutically reversible with various inhibitors, offering an opportunity for therapeutic intervention. Combination of epigenetic inhibitors and immunotherapies emerge as a better therapeutic approach for HCC and may help in eradicating cancer cells especially those that are refractory to standard treatment.

Competing Interests

The authors declare no conflict of interests.

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