Published in final edited form as:
Cancer Lett. 2014 January 28; 342(2): 223–230. doi:10.1016/j.canlet.2012.01.038.

Role of epigenetic aberrations in the development and progression of human hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most lethal and prevalent cancers in humans. The molecular mechanisms leading to the development of HCC are extremely complicated and consist of prominent genetic, genomic, and epigenetic alterations. This review summarizes the current knowledge of the role of epigenetic aberrations, including changes in DNA methylation, histone modifications, and expression of microRNAs in the pathogenesis of HCC. It also emphasizes that identification of the underlying epigenetic alterations that drive cell transformation and promote development and progression of HCC is crucially important for understanding mechanisms of hepatocarcinogenesis, its detection, therapeutic intervention, and prevention.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent life-threatening human cancers that is not only increasing in worldwide incidence in the past decade [1–4], but is also a leading cause of cancer-related deaths worldwide [3–6]. HCC is an aggressive and enigmatic disease, which represents approximately 85% of liver cancers [5,6]. The most prominent etiological factors associated with HCC consist of chronic viral hepatitis B and C infections [4,7–9], nonalcoholic fatty liver disease [10–12], and toxin and alcohol exposure [6,9]. The development and progression of HCC is a multistep and long-term process characterized by the progressive sequential evolution of morphologically distinct preneoplastic lesions (formed as a result of chronic liver injury, necro-inflamation and regeneration, small cell dysplasia, low-grade and high-grade dysplastic nodules) that culminates in the formation of HCC [5,13]. However, the molecular and cellular mechanisms of HCC pathogenesis are still poorly understood [5,6].

Traditionally, the development of HCC in humans has been viewed as a progressive multistep process of transforming of normal cells into malignant driven primarily by the stepwise accumulation of genetic alterations in tumor-suppressor genes and oncogenes [14–16], with mutations in β-catenin and P53 genes being the major genetic alterations [14,15]. However, over the past decade there has been a surge in data indicating the importance of epigenetic processes, which has largely changed the view of HCC as a genetic disease only [17–19]. Presently, HCC is recognized as both a genetic and epigenetic disease, and genetic

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The views expressed in this paper do not necessarily represent those of the U.S. Food and Drug Administration.

and epigenetic components cooperate at all stages of liver carcinogenesis [16,20]. While the sequential accumulation of various genetic changes in hepatocarcinogenesis has been extensively studied, the contribution of epigenetic alterations to HCC development and progression has remained relatively unexplored until recently [17–19].

2. Epigenetic alterations in HCC

The unifying molecular feature of HCC is a profoundly reshaped epigenome that is characterized by global genomic *hypo*methylation, gene-specific DNA *hyper*- or *hypo*-methylation, abnormal expression of DNA methyltransferases and histone modifying enzymes, altered histone modification patterns, and aberrant expression of microRNAs [6,13].

2.1. Global DNA hypomethylation in human HCC

DNA hypomethylation signifies one of the two major DNA methylation states and refers to a condition in which there is a decrease in the number of methylated cytosine bases from the “normal” methylation level. DNA hypomethylation arises mainly from the loss of methylation at normally heavily methylated areas of genome. The molecular events that may lead to hypomethylation of DNA are elusive and it is likely that multiple pathways may be involved. Hypomethylation of DNA can be achieved either passively or actively. Passive loss of methylated cytosines in the genome may be a consequence of either (i) limited availability of the universal methyl donor S-adenosyl-L-methionine (SAM), (ii) compromised integrity of DNA, or (iii) altered expression and/or activity of DNA methyltransferases (DNMTs) [21].

The methyl groups needed for DNA methylation and other cellular methylation reactions are acquired from SAM, which is synthesized from L-methionine and adenosine in an ATP-dependent reaction catalyzed by methionine adenosyltransferase 1 (MAT1) in a one-carbon pathway in the cytosol of every cell. The liver is the primary tissue for SAM biosynthesis and degradation [22]. This indispensably connects faithful DNA methylation to the proper functioning of this metabolic pathway. It is well established that either genetic polymorphism in genes involved in the methyl-group metabolism, or deficiencies in methyl group donors caused by endogenous and exogenous factors, may lead to loss of cytosine methylation in DNA [23,24].

The integrity of the genome is another critical factor that may affect the DNA methylation status. Specifically, the results of several studies have demonstrated that the presence of unrepaired lesions in DNA, e.g., 8-oxodeoxyguanosine, 5-hydroxymethyl-cytosine, apurinic/apirimidinic sites, and strand breaks, diminishes the ability of DNA methyl transferases (DNMTs) to methylate DNA [25,26]. Additionally, a large body of evidence clearly demonstrates that improper function of DNMTs may compromise the DNA methylation status [27]. Importantly, a number of well-established hepatocarcinogens may perturb the described above processes and trigger loss of DNA methylation in the liver [19,28,29].

Active loss of methylated cytosines, via an active replication-independent DNA demethylation process, was controversial and inconclusive for many years [30,31]. However, recent studies have provided compelling experimental evidence that active loss of DNA methylation is associated with the function of DNA repair machinery [32–35].

The loss of DNA methylation was the first epigenetic abnormality and one of the most common molecular alterations identified in human cancers [36], including HCC [37,38]. Global DNA demethylation in HCC primarily affects stable, methylated areas of the genome composed predominantly of repetitive DNA sequences, such as long interspersed nucleotide

elements 1 (LINE1), retroviral intracesternal A particles (IAP), Alu elements, and body of genes [39]. Indeed, the results of several comprehensive studies have demonstrated extensive hypomethylation of LINE-1, ALU, and SAT2 repetitive elements in HCC [40–42]. More importantly, it has been demonstrated that the serum LINE-1 hypomethylation may be an independent prognostic marker in patients with HCC [43].

There are several molecular consequences of global DNA demethylation that may contribute to the progression of liver carcinogenesis via multiple mechanisms. Specifically, genomic hypomethylation may cause a significant elevation in mutation rates, aberrant activation of "normally" silenced tumor-promoting genes, loss of imprinting, and activation and transposition of repetitive DNA elements leading to chromosomal and genomic instability [44].

Despite the large body of evidence indicating that cancer-associated DNA demethylation is an important early event in the development of HCC, it is still less clear if the loss of DNA methylation is a cause or a consequence of the malignant transformation [45]. The notion that DNA hypomethylation plays a causative role in liver cancer is based on the results of nutritional and genetic studies using "lipogenic methyl-deficient diets" [46–48] or genetically-engineered *Apc*$^{Min/+}$*;Dnmt1*$^{chip/c}$ mice [49], respectively. In contrast, there is also evidence that cancer-linked DNA hypomethylation may be a passive inconsequential side effect of carcinogenesis [45]. The latter is evidenced by the facts that not all liver tumors exhibit DNA hypomethylation [50] and not all carcinogenic processes are accompanied by the loss of DNA methylation [51].

2.2. Cancer-linked gene-specific DNA hypermethylation in human HCC

DNA hypermethylation is the state where the methylation of "normally" undermethylated DNA domains, those that predominantly consist of CpG islands [52], increases. CpG islands are defined as the genomic regions that contain the highest G + C content, have a high frequency of CpG dinucleotides, are at least 400–500 bp long, and are located in intragenic, intergenic, or 5′ ends of genes [53–55]. However, only CpG islands that span the 5′ regions of promoters and first exons are mainly unmethylated. For instance, it is established that less than 3% of CpG islands in gene promoters are methylated [55].

The focus of studies aimed to explore the role of DNA methylation in HCC has been on global epigenomic alterations in HCC, abnormal gains of DNA methylation (hypermethylation) of typically unmethylated CpG island-containing promoters, transcriptional repression, and loss of gene function. Specifically, gene-specific DNA hypermethylation has gathered most attention as a critical event in liver carcinogenesis. Several epigenetically inactivated genes, as evidenced by association between diminished mRNA levels with highly methylated promoters, have been identified in HCC. Among genes frequently methylated in HCC are tumor suppressors, including *RASSF1A* [56], *p16*^{INK4A} [57,58], *p15*^{INK4B} [59], *RB1* [60], *SOCS1* [61,62], *SOCS3* [63], *SYC* [64], *GSTP1* [65], *NQO1* [66], *PROX1* [67,68], *NORE1B* [69], *RIZ1* [70], *RELN* [71], *FBLN1* [72], and *PAX5* [73]. These genes are involved in the regulation of vital biological processes, including cell-cycle control, apoptosis, cell proliferation, and xenobiotic metabolism.

In addition, there is growing evidence of the importance of non-CpG island-containing promoter coding region hypermethylation in gene inactivation. For instance, hypermethylation of the p53 promoter region and the *MAT1A* coding region is associated with inhibition of gene expression in human HCC [74,75]. The fact that the aberrant gene-specific hypermethylation of the aforementioned genes occurs not only in HCC, but also in premalignant pathological conditions, including chronic viral hepatitis B and C and liver

cirrhosis, suggests the importance of gene-specific hypermethylation event in pathogenesis and progression of HCC.

2.3. Cancer-linked gene-specific DNA hypomethylation in human HCC

Until recently, the majority of the studies in the field of cancer research, including liver cancer, have focused on alterations in DNA hypomethylation, mainly hypomethylation of repetitive sequences, and epigenetically-driven gene silencing, as the main mechanisms favoring the development of HCC. However, mounting evidence indicates that the hypomethylation of "normally" methylated genes is significant in the pathogenesis of HCC [76]. Currently, a number of hypomethylated tumor-promoting genes, including *uPA* [77], *HPA* [78], *SNCG* [79], *TFF3* [80], *MAT2A* [81], HKII [82], CD147 [83], and *VIM* [84] have been identified in primary human HCC.

Importantly, gene-specific DNA methylation changes, both hyper- and hypomethylation, in HCC are associated with well-established hallmarks of cancer, including the acquisition of persistent proliferative signaling, resistance to cell death, evasion of growth suppression, replicative immortality, inflammatory response, deregulation of energy metabolism, induction of angiogenesis, and activation of invasion [85]. However, while gene-specific promoter DNA hypermethylation changes are associated predominantly with deregulation of pathways important for the initiation of HCC, such as cell-cycle control, apoptosis, and cell proliferation, gene-specific promoter DNA hypomethylation changes are related to biological processes critical for tumor progression, including cell growth, cell communication, adhesion and mobility, signal transduction, and drug resistance.

The existence of two opposing hyper- and hypomethylation events in the same functional pathways complement or enhance each other in the disruption of cellular homeostasis favoring progression of HCC. For instance, hypermethylation and transcriptional inactivation of the E-cadherin (*CDH1*) gene [86] and hypomethylation-induced up-regulation of the Vimentin (*VIM*) gene [84] in HCC may exaggerate invasion and escalate further progression of HCC.

2.4. Alterations of DNA methyltransferases in human HCC

Abnormal patterns of DNA methylation in HCC is closely related to the disruption in the functioning of DNA methylation machinery. Several reports distinctly established the major role of altered gene expression of DNA methyltransferase DNMT1, a main enzyme involved in the maintenance of genomic methylation patterns, the *de novo* DNA methyltransferases DNMT3A and DNMT3B, and methyl-binding proteins in the development and progression of HCC [27,87–89]. This is evidenced by a progressive marked up-regulation of DNMT1, DNMT3A, and DNMT3B in premalignant non-cancerous liver tissues and in full-fledged HCC [27] and by the fact that over-expression of these DNMTs significantly correlated with CpG-island hypermethylation of tumor-related genes [90]. Additionally, it has been demonstrated that over-expression of DNMT3B4, a splice variant of DNMT3B, causes DNA hypomethylation in pericentromeric satellite regions [44].

2.5. Dysregulation of histone modifications in human HCC

DNA methylation changes in HCC are not isolated events; they occur in an environment of large-scale disruptions of the cellular epigenome that also include alterations in histone modification patterns [91]. Similarly to alterations in DNA methylation, changes in histone modifications in HCC occur genome-wide and on gene-specific scales. At least eight different classes of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitynation, sumoylation, biotinylation, and ADP-ribosylation have been identified on the core histones H2A, H2B, H3, H4, and the H1 family of linker histones

[92]. With respect to HCC, acetylation and methylation of histone lysine residues are the best studied histone modifications so far. Typically, histone acetylation is associated with an active transcription, whereas methylation may be associated with either active or repressive states, depending on the modified site [91,92].

Currently, it is well-documented that transcriptional silencing of cancer-related genes in HCC is associated with DNA methylation and/or histone modifications at their promoters. For instance, a number of transcriptionally repressed genes in human HCC are associated either with hypoacetylation of lysine residues at histone H3 and H4 [66,93]. Likewise, transcriptionally silenced *RIZ1*, $p16^{INK4A}$ and *RASSF1A* tumor-suppressor genes in human HCC are characterized by an increased level of repressive histone H3 lysine 9 and histone H3 lysine 27 methylation marks at their promoters [70,94,95] In addition to aberrations in histone modifications at promoters of individual genes, HCC also displays genome-wide changes in histone modifications, particularly a loss of trimethylation of histone H4 lysine 20 and increase of histone H3 lysine 27 trimethylation and histone H3 phosphorylation [96,97].

2.6. Alterations of histone modifying enzymes in human HCC

It is well-documented that HCC is characterized by a prominent dysregulation of several histone-modifying enzymes, including histone deacetylase (HDAC) class I isoforms HDAC1, HDAC2, and HDAC3, SIRT1, a class III HDAC, and histone methyltransferases (HMTs) SMYD3, RIZ1, and EZH2 [98–102]. Histone deacetylases are responsible for removal of an acetyl group from lysine residues of target proteins and display multi-faceted roles in coordinating the interaction of intracellular signaling pathways through chromatin remodeling. Several recent studies have demonstrated an elevated expression of HDAC1, HDAC2, and HDAC3 [97,99] and SIRT1 in human HCC [103,104] that correlated with clinicopathological features and recurrence of HCC.

Histone methyltransferases SMYD3, RIZ1, and EZH2 are responsible for methylation of lysine residues 4, 9 and 27 at histone H3, respectively [99,100]. The up-regulation of the SMYD3 HMT promotes proliferation of HCC via increasing H3 lysine 4 methylation, a mark of transcriptional activation, and thus subsequent activation of downstream genes, including *NKX2-8* gene [99], which is frequently up-regulated in human HCC [105]. In contrast, over-expression of the EZH2 in HCC may facilitate the progression of HCC through increasing trimethylation of H3 lysine 27 and enhancing heterochromatin formation at promoters of transcriptionally silenced genes [100]. Indeed, a recent report by Cheng *et al.* [106] has demonstrated that EZH2 inserts its oncogenic activity in HCC through EZH2-mediated epigenetic silencing of the growth-suppressive Wnt antagonists, which causes a constitutive activation of Wnt/β-catenin signaling and proliferation of HCC cells. Additionally, EZH2 recruits DNA methyltransferases to the EZH2-containing Polycomb complex leading to *de novo* methylation of cancer-related genes [107,108].

In contrast, down-regulation of RIZ1 HMT that is frequently found in HCC [70,109] may promote progression of hepatocarcinogenesis via reduction of the level of histone H3 lysine 9 trimethylation, followed by chromatin decondensation, and genomic instability.

Additionally, prominent epigenetic abnormalities found in HCC may be induced by alterations in intracellular metabolism. For example, the status of DNA and histone methylation in HCC may be compromised by deficiency of intracellular SAM caused by cancer-related transcriptional silencing of the liver-specific *MAT1A* gene [110,111]. This gene encodes methionine adenosyltransferase, which is an essential enzyme for the biosynthesis SAM [112]. Likewise, expression of several other genes involved in methionine metabolism, e.g., *BHMT* and *CBS*, is reduced in HCC [110. This corresponds to

a recently emerged hypothesis that alteration of cellular epigenome in cancer is associated with cancer-linked metabolic disturbances, specifically with aberrant levels of variety of small molecules derived from intermediary intracellular metabolism, including glucose, glutathione, flavin adenine dinucleotide and nicotine adenine dinucleotide, folate, acetyl coenzyme A, and α-ketoglutarate [113,114].

2.7. MicroRNAome alterations in human HCC

Extensive studies in the past decade have indicated the existence and importance of another epigenetic mechanism of regulation of gene function by means of small non-coding microRNAs (miRNAs). Currently, miRNAs are recognized as one of the major regulatory gatekeepers of protein-coding genes in the human genome [115]. MiRNAs are small 16–29 nucleotide-long non-coding RNAs that primarily function as negative gene regulators at the post-transcriptional level [116]. MiRNAs are generated by RNA polymerase II or RNA polymerase III as long primary transcripts, primary miRNAs. Following transcription, primary miRNAs form a stem-loop structure, which is recognized and processed by the RNase III-type enzyme Drosha creating precursor miRNAs. These precursor miRNAs are transported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNAs are further processed by Dicer, an RNase III enzyme, generating miRNA:miRNA hybrids. After unwinding, one strand of the duplex is degraded, and another strand becomes a mature miRNA. MiRNAs can induce mRNA cleavage if their complementary to the 3′-untranslated region of mRNA targets are perfect or miRNAs can induce translational repression if complementarity is imperfect [115].

Currently there are more than 700 mammalian miRNAs that can potentially target up to one-third of protein-coding genes involved in development, cell differentiation, metabolic regulation, signal-transduction, cell proliferation, and apoptosis. As the deregulation of these very same biological processes is a hallmark of cancer [85], it has been suggested that changes in miRNA expression might have significance in cancer [117–119], including HCC [120,121]. In recent years, a number of comprehensive studies have documented an aberrant expression, both up-regulation and down-regulation, of miRNAs that have been associated with virtually every aspect of HCC biology, including tumor progression, e.g., up-regulated miR-21, miR-17-92, miR-155, miR-191, and miR-221/miR-222 and down-regulated miR-122 [122–126], invasion and metastasis, e.g., up-regulated miR-21 and miR-151 and down-regulated miR-200 family [122,127,128], and acquisition of resistance of malignant cells to various chemotherapeutic agents, e.g., up-regulated miR-21 and down-regulated miR-122 and miR-199a-3p [129–131].

Growing evidence indicates a direct and interdependent link between epigenetic and miRNA expression alterations illustrating the complexity of epigenetic abnormalities in HCC. For instance, high expression of miR-191 in HCC is associated with hypomethylation of the *mir-191* gene [132], whereas down-regulation of miR-1, miR-124, miR-125b, and miR-203 is attributed to DNA hypermethylation [133–135]. On the other hand, altered expression of miR-29, miR-152, and miR-200a causes substantial alterations in DNA methylation and histone modifications [136–138].

3. Molecular diagnosis of HCC: epigenetic biomarkers

Understanding the molecular mechanisms involved in neoplastic hepatocyte transformation, promotion, and progression of hepatocarcinogenesis are crucial for the diagnosis, prognosis, and determination of treatment strategies of HCC. The post-genomic era molecular tools, e.g., transcriptomic and genome-wide association studies, have been used succesfully for the identification of new diagnostic and prognostic biomarkers of HCC [139–142]. Mapping the patterns of DNA methylation has also been proposed recently as a valuble molecular

diagnostic tool for HCC. Here we show the proposed chronology of epigenetic events in the progression of human HCC (Figure 1).

Many studies provide evidence that cancer-linked DNA methylation alterations may be used as early indicators of liver carcinogenesis, as well as prognostic markers of cancer progression and response to chemotherapy [143–149]. Specifically, using a DNA methylation profiling approach it is possible to differentially diagnose HCC from preneoplastic lesions, e.g. low-grade and high-grade dysplastic nodules and cirrhosis [143,146]. This is evidenced by different pattern and magnitude of gene-specific DNA methylation changes in preneoplastic livers and full-fledged HCC. Recently, Nagashio *et al.* [144] was able to predict HCC with 95.6% sensitivity and 100% specificity using a quantification of DNA methylation level approach in preneoplastic liver tissue. These authors also demonstrated that the methylation status of gene-specific DNA regions significantly correlates with the outcome of patients with HCC. More importantly, the results of several comprehensive studies have demonstrated clearly that aberrant gene-specific DNA methylation patterns discriminate HCC, with an etiology associated with viral hepatitis B and C infection and alcohol intake [146–151]. Additionally, several reports have indicated that gene-specific methylation, e.g, *RIZ1* gene, in surgically resected non-tumorous tissue significantly associated with reoccurrence of HCC [152,153].

The steadiness and specificity of cancer-associated DNA hypo- or hypermethylation changes offer substantial advantages over other molecular markers for cancer diagnostics. Firstly, DNA hypermethylation is a positive signal not observed in normal cells, and therefore it is independent of contamination with normal cells. Secondly, because DNA methylation changes are stable, the same aberrant DNA methylation patterns specific for the primary tumor can be identified in plasma and other body fluids. Indeed, several studies have shown that promoter hypermethylation of cancer-related genes, including $p16^{INK4A}$, *RASSF1A*, *GSTP*, and *RUNX3* genes [154–159], as well as LINE-1 hypomethylation [43] were detected in the plasma of the majority of patients diagnosed with HCC. Such observations indicate that cancer-linked aberrant DNA methylation may be a potential valuable noninvasive diagnostic and prognostic biomarker for HCC.

Little evidence has been presented to clarify whether or not miRNA alterations may distinguish HCC from preneoplastic lesions; however, similar to DNA methylation changes, emerging evidence indicates that hepatic miRNA profile may predict the recurrence of HCC after resection [160] and circulatory blood miRNA levels may be used as a potential biomarker for noninvasive diagnosis of HCC [161–163].

4. Animal models of HCC

In humans, most of the research on HCC is conducted on patients who have already developed the disease. This limits the scope of the investigation to tumor biology and does not allow extensive study into the mechanisms of disease progression, which is critical for early diagnostic and successful treatment. On the contrary, relevant animal models of liver carcinogenesis provide a unique opportunity to understand the underlying molecular mechanisms involved in pathogenesis of HCC and substantially complement many shortcomings of humans-only studies [164–166]. The application of animal models to study process of hepatocarcinogenesis is further supported by a recent evidence [167] showing that despite major differences in etiology of mouse and human HCC (human HCC arises predominantly within an environment of chronic inflammation, hepatocellular degeneration, necrosis and regeneration, fibrosis and cirrhosis [168]) there are great similarities in the molecular landscape between human and spontaneous HCC in B6C3F1 mice.

The most commonly used animal models of HCC consist of those that are induced by different genotoxic chemical carcinogens, e.g., diethylnitrosamine, aflatoxin B_1, and 2-acetylaminofluorene, non-genotoxic chemical agents, e.g., peroxisome proliferators, transgenic mouse models of hepatitis B and C viral infection, and constitutive and conditional transgenic mice with over-expression of oncogenes, e.g., c-myc and β-catenin, or growth factors, e.g., TGFα, TGFβ, EGF, and PDGF-C [164–166]. One of the most extensively studied models of rodent HCC that is relevant to humans is endogenous liver carcinogenesis induced by dietary methyl deficiency [169]. This model is unique because dietary omission of sources of methyl groups, rather than xenobiotic addition, leads to tumor formation. In addition, the sequence of pathological and molecular events is remarkably similar to the development of human HCC that is associated with viral hepatitis B and C infections, alcohol exposure, and metabolic liver diseases, all of which are currently considered major risk factors of HCC worldwide.

5. Perspectives

It is clear that epigenetic alterations are critical determinants of human hepatocellular cancer. The progressive accumulation of epigenetic changes during development of HCC gives a unique opportunity to use them as biomarkers in cancer detection. However, not all aberrations may be equally important for the tumorigenic process [170]. Specifically, it is highly unlikely that all epigenetic aberrations play a significant role in hepatocarcinogenesis. For example, some epigenetic changes may drive other events that contribute to the formation of a transformed phenotype, while others may be passenger events that accompany the transformation process. In this respect, the identification of alterations that drive cell transformation and promote hepatocarcinogenesis is crucially important for understanding mechanisms of HCC progression and prevention. Additionally, the presence of epigenetic abnormalities in premalignant and non-tumorous livers unequivocally indicates the involvement of an “epigenetic field cancerization” effect in the pathogenesis of HCC [90,143,152,153,171–174]. This emphasizes the role and the usefulness of epigenetic biomarkers for early detection and prediction of HCC development. More importantly, the potential reversibility of epigenetic alterations opens a novel mechanism-based approach to molecular-targeted HCC treatment and prevention.

Acknowledgments

This work was supported, in part, by grants from NIH (R01 ES015241 and P42 ES005948).

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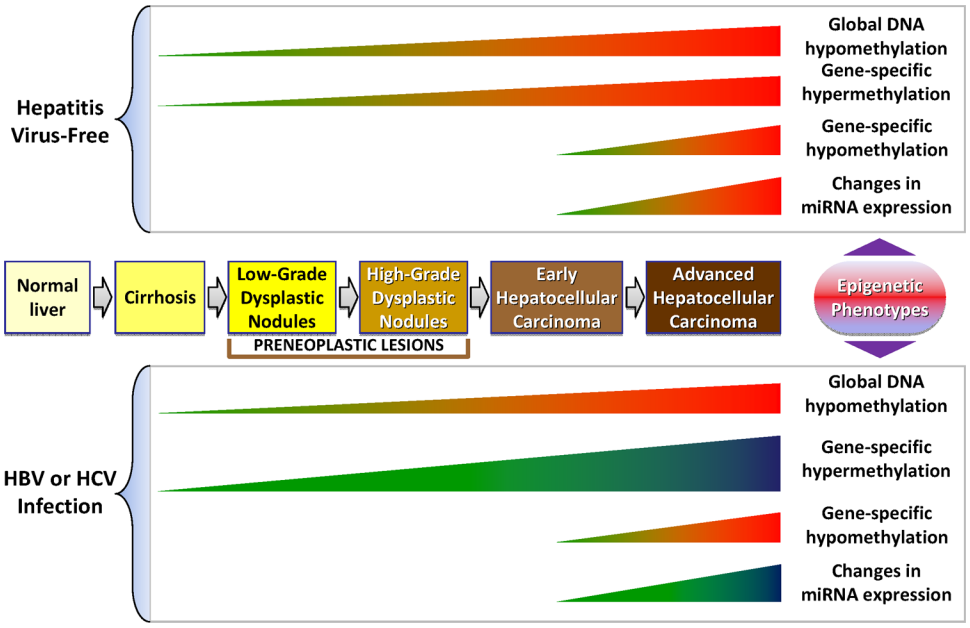

Figure 1.
The chronology of DNA methylation and miRNA alterations in human multistage hepatocarcinogenesis.