Epigenetic Mechanisms in Hepatitis C Virus-Associated Hepatocellular Carcinoma: A Potential New Link Between Stem Cells, Virology and Cancer

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ABSTRACT

Recent studies suggest that epigenetic mechanisms are not only essential for the dynamic transcriptional regulation in embryonic and somatic stem cells, but are also actively involved in tumorigenesis: genes important for pluripotency are epigenetically regulated and aberrant epigenetic changes have been detected in virtually all human malignancies studied, including Hepatocellular Carcinoma (HCC). Infection with Hepatitis C Virus (HCV) is a major risk factor for the development of HCC. Despite the fact that HCV is a RNA virus without a DNA intermediate, recent studies demonstrate that HCV viral proteins may actively participate in epigenetic regulation of hepatic cancer stem cell phenotypes and induce HCC-specific epigenetic changes. Identification of host epigenetic alterations induced by HCV infection and epigenetic differences between hepatic cancer stem cells and the bulk non-tumorigenic cancer cells, may yield potential biomarkers for early detection, as well as therapeutic targets for HCV associated HCC.

Keywords: HCV, Epigenetics, Epithelial Mesenchymal Transition (EMT), Cancer Stem Cells (CSC)

1. INTRODUCTION

1.1. HCV Infection Associated HCC is Increasing

Hepatocellular Carcinoma (HCC) is the fifth most common solid tumor worldwide and the third leading cause of cancer-related death, accounting for approximately 600,000 deaths per year worldwide (Bosch et al., 2005; El-Serag and Rudolph, 2007; Schutte et al., 2009; Thomas and Zhu, 2005; Tsai and Chung, 2010). Infection with either HBV or HCV, is the major risk factor for HCC worldwide, with at least one of the two viruses present in over 80% of HCC cases (Perz et al., 2006). More than 80% of HCC cases occurring in developing countries are due to HBV infection, which are preventable through effective childhood HBV vaccination (Kane, 2003). On the other hand, the recent increase in the incidence of HCC in Western countries is largely due to the HCV endemic. About 200 million people are infected with Hepatitis C Viruses (HCV) worldwide. More than two thirds of people with acute HCV infection will develop persistent HCV infection, leading to chronic hepatitis, liver cirrhosis and ultimately HCC (Grebely and Dore, 2011;
Micallef et al., 2006). It has been estimated that age-adjusted HCC incidence rates have doubled between 1985 and 2002 (El-Serag and Rudolph, 2007) and the incidence and mortality rates of HCC in the United States are likely to double again over the next 10-20 years (El-Serag, 2002). Surgical resection or liver transplantation remains the most effective treatment options for HCC; however, very few patients are suitable for these treatments. Despite recent advances in HCV antiviral therapies, these therapies are only effective to prevent HCC in a small proportion of highest risk patients, as sustained viral clearance is difficult to accomplish among patients with liver cirrhosis (Tai and Chung, 2009). Therefore, it is imperative to elucidate the molecular mechanisms underlying HCV caused hepatocarcinogenesis, in order to identify early detection biomarkers as well as effective targeted therapies and thus improve clinical outcomes of HCV associated HCC.

1.2. Cancer Stem Cells
1.2.1. Potential Cancer Stem Cells

Recent studies support the hypothesis that cancer is derived from a small proportion of tumorigenic cells, called Cancer Stem Cells (CSCs), which are responsible for cancer initiation, proliferation, heterogeneity, as well as invasion and metastasis (Vlerken et al., 2012) (Fig. 1A). Similar to normal Embryonic Stem Cells (ESCs), CSCs possess the capability of self-renewal and differentiation (Wu, 2008). They express established ESC markers, pluripotency transcription factors (Oct4, Sox2 and Nanog) and activate the embryonic signaling pathways, Hedgehog, Notch and Wnt (Harris et al., 2012; Takebe and Ivy, 2010). Similar to ESCs, Epithelial-Mesenchymal Transition (EMT) plays an important role in CSCs, increasing the capacity for tumor invasion and metastasis (Ksiazkiewicz et al., 2012; Mani et al., 2008) (Fig. 1B). CSCs are also highly influenced by signals in their microenvironment and often reside in specialized niches within tissues (Li and Neaves, 2006). Finally, similar to ESCs, polycomb repressive protein complexes (PRC1 and PRC2) are also involved in CSCs to establish dynamic epigenetic profiles (Takebe and Ivy, 2010; Tysnes, 2010; Vlerken et al., 2012).

Several lines of evidence suggest that HCV infection is intimately linked to the presence of hepatic stem cells, such as Hepatic Progenitor Cells (HPCs) (Ali et al., 2011; Machida et al., 2012; Wu et al., 2012). HPCs are small periporal cells capable of proliferation and differentiation into both hepatocytes and bile ductular epithelium (Clouston et al., 2005; Roskams, 2003). They express stem cell, hepatocyte and bile duct cell markers, including CD133, Nanog, α-Fetoprotein (AFP), CK19, Lin29 and c-Myc (Clouston et al., 2005; Oliva et al., 2010). Their frequency increases with the severity of the liver disease and inversely correlated with response to treatment (Oliva et al., 2010; Tsamandas et al., 2006). Further, there is evidence that HCV infection directly induces HPCs and the presence of HPCs facilitates HCV replication. For example, HCV infection in vitro induces both cancer stem cell markers (DCAMKL-1, CK19, α-fetoprotein, active c-Src) and a distinct tumor phenotype (Ali et al., 2011); expression of HCV NS5A gene coupled with alcohol intake induces stem cell regulator Nanog expression through the TLR4 signaling pathway (Machida et al., 2012; 2009). Finally, it has been shown that HCV can infect Differentiated Human Hepatocyte-like cells (DHHs) from human ESCs and induced Pluripotent Stem Cells (iPSCs) and clinical HCV isolates reportedly infect HPCs with a higher efficiency than infection of mature hepatocytes (Wu et al., 2012).

We have previously characterized the gene expression pattern of a HCV replicon-containing hepatoma cell line using the Agilent whole human genome oligo microarrays 4×44k (Miltonic Biotech, Germany) (Blight et al., 2002). The Agilent Feature Extraction Software (FES) was used for initial processing of the microarray image files and Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware) was used to build pair-wise ratios and for data normalization. We have detected up regulation of Octamer-binding protein 3 (Oct3, transcription factor, essential for embryonic stem cell pluripotency), Sex determining region Y-box 2 (Sox2, transcription factor required for stem-cell maintenance in the central nervous system) and suppressor of zeste 12 homolog (SuZ12, a core component of Polycomb Group (PcG) proteins) in HCV replicon cells compared to parental HCV naïve cells (Table 1). Similarly, we have also detected the expression of EMT markers, such as down regulation of E-cadherin (CDH1) and cytokertatin 19 (KRT19) and up regulation of Vimentin (Vim). Using immunohistochemistry analysis, we have detected over expression of Enhancer of Zeste Homolog 2 (EZH2, histone lysine methyltransferase, a member of the PcG proteins) in HCC samples compared to normal liver tissues. These results provide further evidence that HCV infection might be directly involved in inducing EMT and hepatic stem cell phenotypes, which in turn may facilitate HCV replication.
Fig. 1. Cancer Stem Cells (CSC) and Epithelial Mesenchymal Transition (EMT) in hepatocarcinogenesis. (A) The stochastic model suggests that each cancer cell has the ability to generate the bulk tumor, while in cancer stem cell model, only a few of CSCs have the capability to generate the bulk tumor. (B) EMT and CSC are intimately connected. Epithelial cells undergoing EMT generate CSCs, which in turn are responsible for tumor growth (self-renewal), heterogeneity (bulk tumor) and invasion (metastasis).

1.3. Epigenetic Regulation and Cancer Stem Cells

Normal stem cells involve complex molecular networks to achieve a flexible but precise transcription regulation of genes important for pluripotency and differentiation and epigenetic mechanisms are a key component of this dynamic transcriptional program. It is known that genes important for pluripotency are epigenetically regulated. For example, Oct 4 and Nanog genes are active in normal stem cells, their promoters are enriched with H3K4me3 marks and not methylated, they become silenced upon differentiation and their promoters are then enriched with H3K27me3 marks and undergo DNA methylation. On the other hand, developmentally regulated genes are characterized by the presence of both active and repressive histone marks in normal stem cells, which ensures their rapid transcriptional activation upon differentiation.
Table 1 Gene expression alteration in HCV replicon cells

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold change</th>
<th>Entrez gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes involved in stem cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI1</td>
<td>-1.9</td>
<td>BMI1 polycomb ring finger oncogene</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>-1.8</td>
<td>cyclin-dependent kinase inhibitor 2A (p16)</td>
</tr>
<tr>
<td>CDX2</td>
<td>2.9</td>
<td>caudal type homeobox 2</td>
</tr>
<tr>
<td>DLX1</td>
<td>5.5</td>
<td>distal-less homeobox 1</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>1.7</td>
<td>DNA (cytosine-5-)-methyltransferase 3 beta</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>2.5</td>
<td>DNA (cytosine-5-)-methyltransferase 3-like</td>
</tr>
<tr>
<td>ISL1</td>
<td>12.0</td>
<td>ISL LIM homeobox 1</td>
</tr>
<tr>
<td>KLF2</td>
<td>-14.7</td>
<td>Kruppel-like factor 2 (lung)</td>
</tr>
<tr>
<td>KLF4</td>
<td>-4.7</td>
<td>Kruppel-like factor 4 (gut)</td>
</tr>
<tr>
<td>PAX6</td>
<td>-2.9</td>
<td>paired box 6</td>
</tr>
<tr>
<td>Pou5F1</td>
<td>1.9</td>
<td>POU class 5 homeobox 1 (Oct3)</td>
</tr>
<tr>
<td>RUNX1</td>
<td>-20.0</td>
<td>runt-related transcription factor 1</td>
</tr>
<tr>
<td>Sox2</td>
<td>15.9</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>STAT3</td>
<td>-7.7</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SUZ12</td>
<td>2.3</td>
<td>suppressor of zeste 12 homolog (Drosophila)</td>
</tr>
<tr>
<td>VDR</td>
<td>9.7</td>
<td>vitamin D (1,25-dihydroxyvitamin D3) receptor</td>
</tr>
<tr>
<td><strong>Genes involved in EMT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMK2N1</td>
<td>2.8</td>
<td>calcium/calmodulin-dependent protein kinase II inhibitor 1</td>
</tr>
<tr>
<td>CDH1</td>
<td>-1.5</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>KRT19</td>
<td>-11.1</td>
<td>keratin 19</td>
</tr>
<tr>
<td>MAP1B</td>
<td>10.9</td>
<td>microtubule-associated protein 1B</td>
</tr>
<tr>
<td>MST1R</td>
<td>-1.9</td>
<td>macrophage stimulating 1 receptor</td>
</tr>
<tr>
<td>SPP1</td>
<td>-50.1</td>
<td>secreted phosphoprotein 1</td>
</tr>
<tr>
<td>TFP12</td>
<td>32.3</td>
<td>tissue factor pathway inhibitor 2</td>
</tr>
<tr>
<td>TGFbeta3</td>
<td>-11.7</td>
<td>transforming growth factor, beta 3</td>
</tr>
<tr>
<td>TIMP1</td>
<td>-100.0</td>
<td>TIMP metalloproteinase inhibitor 1</td>
</tr>
<tr>
<td>Vim</td>
<td>1.6</td>
<td>Vimentin</td>
</tr>
<tr>
<td>VPS13A</td>
<td>2.3</td>
<td>vacuolar protein sorting 13 homolog A (S. cerevisiae)</td>
</tr>
</tbody>
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In the context of cancer stem cells, virtually all known CSC markers are epigenetically regulated, either by DNA methylation or histone modification. Thus, tumor cells have the ability to switch cancer stem cell markers on and off, in order to generate tumor heterogeneity. This nature applies to both tumorigenic cancer stem cells and the bulk of non-tumorigenic cancer cells. It has been proposed that aberrant epigenetic changes might explain fundamental differences between normal stem cells and cancer stem cells. Proliferation of normal stem cells is tightly regulated, while cancer stem cells have higher proliferation rate. This in part might be explained by the different epigenetic regulation of Polycomb protein complex targets. In normal stem cells, developmentally regulated genes are repressed by PcG protein complexes through chromatin modification, which is transient and reversible. In neoplastic cells, these PcG target genes become hypermethylated, which is stable and irreversible, thus ensuring higher proliferation of CSCs. In addition, PcG proteins, including BMI1 and EZH2, are frequently over expressed in cancer, further locking CSCs in the proliferation mode. It has been hypothesized that the associated epigenetic differences are responsible for the phenotypic differences between tumorigenic CSCs and non-tumorigenic bulk cancer cells and that epigenetic reprogramming is used by CSCs to promote greater tumor heterogeneity.

At least some data suggest that HCV infection might directly participate in the generation of liver cancer stem cells. BMI1 is the key regulatory component of the Polycomb Regulatory Complex 1 (PRC1) (Cao et al., 2005). It is essential for self-renewal of both hematopoietic stem cells as well as neural stem cells through Ink4a/Arf locus (Bruggeman et al., 2005; Oguro et al., 2006). Similarly, BMI1 enhances self-renewal of hepatic stem cells through repression of Ink4a/Arf locus (Chiba et al., 2010). We have previously shown that CDKN2A is preferentially methylated in HCV caused HCC (Feng et al., 2010) and HCV infection directly down regulates CDKN2A. This suggests that
persistent HCV infection might be actively involved in maintaining pluripotency of hepatic cancer stem cells. Future studies of epigenetic profiling of HCV infected hepatocytes and potential hepatic progenitor cells are needed to elucidate the epigenetic mechanisms involved in HCV infection, hepatic cancer stem cells and hepatocarcinogenesis.

With respect to tumor heterogeneity, it is interesting to consider the extremely marked heterogeneity of HCV and HCV is clearly capable of broad regulation of cellular genes. Thus, we hypothesize that evolution and adaptation of HCV to tumor cellular conditions may serve to generate a major growth advantage for successful HCCs. HCV also exerts profound negative effects on cellular innate immunity, including anticancer regulatory pathways. This diversification potential may provide a powerful alternative mechanism for HCV as an oncogenic virus.

1.4. Epigenetic Alterations in HCC

Cancer risk is determined by the interaction between individual genetic variations and environmental exposure and environmental exposure is manifested as epigenetic alterations (Brait and Sidransky, 2011). Epigenetics refers to heritable changes of gene expression that are not mediated by alterations in the primary nucleotide gene sequence. Among these stable but reversible changes, DNA methylation and specific post-translational modifications on NH2-terminal histone tails, are the key mechanisms that control chromatin condensation and gene expression (Fig. 2). In general, acetylation of histone H3 and H4 is mostly associated with gene expression, while DNA methylation, di- and trimethylation of H3 lysine 9 (H3K9) and trimethylation of H3K27, cause or contribute to the condensation of chromatin, recruitment of Heterochromatin Protein 1 (HP1) and PcGprotein complexes. These latter events ultimately lead to gene silencing.

DNA methylation, the addition of a methyl group to the cytosine in the CpG dinucleotides, plays an important role in normal development and in gene expression regulation. The majority of CpG dinucleotides are methylated in the genome, except when they are clustered as a CpG island located in the promoter regions of many housekeeping genes. In normal cells, such promoter-associated CpG islands are usually not methylated, regardless of whether the gene is transcribed or not. In tumor cells, CpG islands in the promoter region of many tumor suppressor genes become methylated and are associated with transcriptional silencing of these genes. Consequently, methylation of a CpG island associated with a tumor suppressor gene is as potent as genetic mutations with respect to gene inactivation. Both global hypomethylation and gene-specific hypermethylation, have been reported in virtually every tumor type tested and are early events in tumorigenesis, occurring in precursor lesions of many cancer types (Belinsky et al., 1998; Esteller et al., 2000; Evron et al., 2001; Tsuda et al., 2000; Umbricht et al., 2001). Unlike genetic mutations, DNA methylation usually occurs at a fixed location in the promoter region of the gene, facilitating the development of clinical suitable assays. In addition, DNA methylation changes can be detected noninvasively in blood or other bodily fluids, making them ideal biomarkers. Finally, in contrast to genetic alterations, epigenetic changes are potentially reversible, thus are attractive therapeutic targets. In fact, 5-azacytidine, a DNA methyltransferase inhibitor, has been FDA approved for the treatment of myelodysplastic syndrome and Suberoylanilidehydroxamic Acid (SAHA), a histone deacetylase inhibitor, has been approved for the treatment of T cell cutaneous lymphoma. More inhibitors are currently being developed and tested in clinical trials for both hematological and solid tumors (Gal-Yam et al., 2008).

1.5. DNA Methylation Changes Associated with Specific Etiologies

Several studies have tried to identify specific DNA methylation patterns associated with various risk factors in hepatocarcinogenesis, such as viral infections, aflatoxin exposure and alcohol consumption. p16 methylation was present in early stages of HBV-associated hepatocarcinogenesis, not only in high frequency in HCCs, but also was in Cirrhotic Nodules (CNs) and Dysplastic Nodules (DNs), known precursor lesions of HCCs (Shim et al., 2003). Further, p16 methylation had been shown to preferentially occur in liver tissues with HBV infections compared to liver tissues without HBV infection (Jicai et al., 2006). Although a few studies did not detect differences of p16 methylation between HBV-HCCs and HCV-HCCs (Fukai et al., 2005; Kaneto et al., 2001), several studies consistently observed higher frequency of p16 methylation in HCV-HCCs than HBV-HCCs (Katoh et al., 2006; Li et al., 2004; Narimatsu et al., 2004). However, whether p16 methylation occurs in HCCs without hepatitis virus infection is inconclusive, with some studies reporting no methylation (Fukai et al., 2005; Li et al., 2004), while others reported 31-50% methylation (Katoh et al., 2006; Narimatsu et al., 2004).
Specific gene methylations were also reported to be associated with viral infections. Methylation of four genes (MINT31, p16, GSTP1 and RASSF1A) was more frequent in HCC associated with viral infections (Katoh et al., 2006). Methylation of CIITA was significantly associated with chronic but not acute HBV infection (He et al., 2006). Methylation of CTGF, RARB, E-cadherin and p73 was more frequent in HBV-associated HCCs than in HCV-associated HCCs (Chiba et al., 2005; Yang et al., 2003), while RUNX3, APC, SOCS-1 and p14 were preferentially methylated in HCV-HCC (Mori et al., 2005; Yang et al., 2003). However, it is not known whether the observed difference of gene specific hypermethylation was statistically or clinically significant. Several recent studies also linked environmental exposures to specific DNA methylation patterns. High frequencies of p16, GSTP1, MGMT and RASSF1 methylation were significantly associated with high level of AFB1-DNA adducts in HCC tumors (Zhang et al., 2002; 2003; 2005; 2006). Although these studies suggest that different gene specific hypermethylation might be associated with HCCs of different etiologies, systematic studies on a large number of HCC cases are needed to confirm these observations.

We previously conducted a retrospective study to identify tumor suppressor genes differentially methylated in HCV-associated HCC (Feng et al., 2010). DNA methylation status of 10 genes (APC, CCND2, CDKN2A, GSTP1, HOXA9, RARB, RASSF1, RUNX, SFRP1 and TWIST1) was determined using MethyLight assays on 65 archived liver tissue blocks (25 normal, 12 HBV-HCC and 28 HCV-HCC). Five genes (APC, CDKN2A, HOXA9, RASSF1 and RUNX) were significantly more frequently methylated in malignant liver tissues than normal liver tissues. Among HCC cases, HOXA9, RASSF1 and SFRP1 were methylated more frequently in HBV positive HCC cases while RARB and CDKN2A were methylated only in HCV positive HCC cases. (p16) was significantly more frequently methylated in HCV positive HCC cases. Subsequently, immunohistochemistry analysis of CDKN2A (p16) protein expression on 26 HCC cases demonstrated the inverse correlation between the protein expression of CDKN2A (p16) and DNA methylation of CDKN2A (p16) (unpublished data). Interestingly, expression of both CDKN2A (p16) and RARB was reduced in HCV replicon cells (Huh7.5 hepatoma subline with genotype 1a strain H77 replicon (FL-Neo replicon)) by gene expression microarray analysis (Blight et al., 2002), while de novo DNA methyltransferases 3L and 3B were up regulated 2.5 and 1.7 fold respectively. Finally, CDKN2A (p16) promoter is associated with decreased histone 3 lysine 27 trimethylation (H3K27m3) in HCV replicon cells (unpublished data).
1.6. DNA Methylation Changes Associated with Disease Progression

Several candidate gene approach studies have reported on the role of DNA methylation of various panels of genes during the stepwise progression of HCC. Methylation of several genes occurred not only in HCC and its precursor lesions, but also in chronic hepatitis and liver cirrhosis, suggesting that these changes are early events during HCC progression. DNA methylation of four genes (Col1A2, IGFBP2, CTGF, fibronectin (1) increased from normal liver, chronic hepatitis, liver cirrhosis to hepatoma (Chiba et al., 2005). Frequency of E-cadherin promoter methylation increased from dysplastic nodules to early stage and late stage HCCs (Kwon et al., 2005). Similarly, methylation of p16, p15 and SFRP1 was not only present in HCC, but was also present at low frequencies in chronic hepatitis and liver cirrhosis samples (Fukai et al., 2005; Shih et al., 2006). Further, methylation analysis in various liver tissues demonstrated that the number and frequency of genes methylated progressively increased in liver cirrhosis, dysplastic nodules and HCC, supporting the hypothesis that CpG island methylation of tumor-related genes is an early and frequent event and methylation changes accumulate during a multistep hepatocarcinogenesis (Lee et al., 2003).

1.7. Global DNA Methylation Profiling

Recently several genome-wide DNA methylation profiling studies have been performed on HCC tissues, using MeDIP-chip (Deng et al., 2010), CpG island Amplification Microarray (MCAM) (Gao et al., 2008; Shitani et al., 2012), IlluminaGoldenGate assay (Archer et al., 2010; Shin et al., 2010) or Illumina humanmethylation27 bead array (Ammerpohl et al., 2012; Hernandez-Vargas et al., 2010; Shen et al., 2012). However, most of these studies were not designed to investigate the direct role of HCV infection in epigenetic changes during HCC development: some had less than 5 HCV-HCC cases (Ammerpohl et al., 2012; Deng et al., 2010), lacked normal controls and only adjacent non-cancerous liver tissues were included (Hernandez-Vargas et al., 2010; Shen et al., 2012; Shin et al., 2010; Shitani et al., 2012), while others did not stratify DNA methylation changes by different etiological agents (Ammerpohl et al., 2012; Gao et al., 2008; Shin et al., 2010). At least one study indicated that certain DNA methylation changes already occurred in liver cirrhosis caused by HCV infection and persisted in HCC and specific DNA methylation patterns are associated with either cirrhosis or HCC (Ammerpohl et al., 2012). Thus far, the largest global methylation profiling study included 62 pairs of tumor/adjacent non-tumorous liver tissues and methylation of those candidate genes was detected in plasma samples from HCC patients. However, most of the HCCs included were HBV+ (Shen et al., 2012). Only three studies attempted to identify HCV-HCC specific methylation changes. Archer et al. (2010) compared DNA methylation changes using the Illumina Golden Gate array on 76 liver tissues, including 20 HCV+ HCC and adjacent non-tumorous liver tissues, 16 HCV+ cirrhotic liver tissues and 20 normal liver tissues. Both cirrhotic and HCC-specific methylation changes were identified. Deng et al. (2010) analyzed DNA methylation changes by methylated DNA immunoprecipitation-on-chip on 3 HBV-HCC, 3 HCV-HCC and 3 normal liver tissues and showed that DNA methylation preferentially occurred in HCV-related HCC cases. Hernandez-Vargas et al. (2010) compared DNA methylation changes in 30 HCC tumors with various etiologies (HBV, HCV, alcohol) and matched surrounding non-tumorous liver tissues using Illumina bead array technology and identified DNA methylation changes associated with specific etiological agents. These studies suggest HCV infection induces specific DNA methylation changes in HCC; however, the direct role of HCV viral proteins in epigenetic regulation is unclear.

1.8. Histone Modification Changes in HCC

Besides DNA methylation changes, alterations in histone modification patterns have been observed in HCC (Pogribny and Rusyn, 2012). Acetylation and methylation of histone lysine residues are the best-studied histone modifications so far. Usually, histone 3 lysine 9 acetylation (H3K9Ac) and histone 3 lysine 4 methylation (H3K4Me) are associated with active transcription, while histone 3 lysine 9 and 27 methylation (H3K9Me and H3K27Me) are associated with gene silencing (Kouzarides, 2007). Both global and gene specific histone modifications have been detected in HCC tissues. For example, compared to normal liver tissues, HCC has higher level of histone H3 lysine 27 methylation (H3K27Me) (Cai et al., 2011), lower level of global histone H4 lysine 20 methylation (H4K20Me) and undetectable levels of H3K4Me (Magerl et al., 2010). Silencing of p21 (WAF1), CTGF, CYR61 and NIPSNAP1 is associated with reduced levels of histone H3 and H4 lysine acetylation (Chiba et al., 2004), silencing p16 and RASSF1A is associated with increased level of H3K9Me, silencing PGR and ERα is associated with H3K27Me (Kondo et al., 2007; Yao et al., 2010),
while silencing of RIZ1 is associated with both H3K9Me and promoter DNA methylation (Zhang et al., 2010). Aberrant expression of histone modification enzymes have also been detected in HCC. For example, Histone Deacetylases (HDACs) are aberrantly expressed in HCC and panobinostat, a novel pan-HDAC inhibitor, has shown anti-tumor efficacy in preclinical models of HCC (Lachenmayer et al., 2012). Histone methyltransferases(SMYD3, RIZ1 and EZH2) are also aberrantly expressed in HCC (Hamamoto et al., 2004; Jiang et al., 1999; Sudo et al., 2005). Mechanistically, it has been shown that HCV infection inhibits histone H4 arginine argininemethyltransferase 1 (PRMT1) through upregulation of Protein Phosphatase 2A (PP2Ac) (Duong et al., 2010). Currently, histone modification analysis in HCC has been limited to phenotypic characterization. The lack of simple association of individual histone modifications with gene expression and disease progression suggest the existence of complex networks among various histone modifications and DNA methylations. Future studies focusing on simultaneous profiling of DNA methylation and chromatin modification should help delineate epigenetic mechanism during HCC development.

1.9. Carcinogenesis of HCV Associated HCC
1.9.1. Direct Oncogenic Role of HCV Genes

The precise molecular mechanism underlying HCV infection caused hepatocarcinogenesis is not fully understood. Because HCV is an RNA virus that does not involve a DNA intermediate, it has been proposed that HCV infection leads to hepatocarcinogenesis indirectly through viral induced inflammation and oxidative stress. Subsequently, this microenvironment sets the stage for malignant transformation of hepatocytes through accumulation of both genetic and epigenetic changes (Levrero, 2006). However, inflammation alone could not fully explain HCV induced hepatocarcinogenesis, as patients with autoimmune hepatitis rarely develop HCC despite the presence of persistent liver inflammation and cirrhosis (Fujinaga et al., 2011). More recent studies have suggested that HCV might play a more direct role in HCC carcinogenesis through interaction between viral and cellular proteins (Banerjee et al., 2010) (Fig. 3). At least four of the ten HCV viral genes (core, NS3/4A, NS5A and NS5B) can potentiate oncogenic transformation in vitro (Banerjee et al., 2010). For example, HCV core protein can transform primary Rat Embryo Fibroblast (REF) cells together with the H-ras gene (Moriya et al., 1998; Ray et al., 1996); HCV NS3/4A protein can transform NIH 3T3 cells (Sakamuro et al., 1995); HCV NS5A protein differentially modulates transcription of p21WAF1 and PCNA, thus promotingmurine fibroblast cell growth with a tumorigenic phenotype (Ghosh et al., 1999). Although NS5B fails to regulate cell cycle progression in Huh7 cells, it does stimulate proliferation and transformation in U2OS osteosarcoma cells (Munakata et al., 2005). Finally, transgenic mice expressing the HCV core protein develop HCC resembling early stage of HCC in human patients with chronic hepatitis C infection (Moriya et al., 1998). Since most of the putative transforming potentials of the HCV proteins have been defined using in vitro systems and laboratory HCV strains, it is unclear whether these observations are applicable to HCV infection in vivo. Further, the effects of HCV quasispecies in these proposed tumorigenic mechanisms need further investigation.

1.10. HCV Viral Proteins and Epigenetic Alterations in HCC

Conventionally, it has been proposed that HCV induces epigenetic changes either through the generation of Reactive Oxygen Species (ROS) or chronic inflammation (Nishida, 2010). More recent studies suggest that HCV viral genes might be directly involved in epigenetic regulation. For example, HCV core protein from HCV genotype 1b can inhibit CDKN2A (p16) expression by inducing promoter methylation via upregulation of DNMT1 and DNMT3b in HepG2 cells (Park et al., 2011), while HCV core protein induces CDH1 methylation thus reducing its expression via upregulation of SIRT1 in Huh7 cells (Ripoli et al., 2011). Similarly, it has also been shown that HCV core protein induces RASSF1A promoter methylation through upregulatingexpression of SET and MYND domain containing 3 (SMYD3), a novel histone methyltransferase, in cholangiocarcinoma cells (Guo et al., 2011). However, the presence of HCV biovariability strongly underscores the importance of using clinical HCV isolates to investigate hepatocarcinogenesis. A recent study demonstrated that only liver-cancer derived HCV core protein is capable of shifting TGF-β responses from cytostatic effects to EMT development (Battaglia et al., 2009). It is important to determine whether HCV variants isolated from clinical samples and their encoded viral proteins can induce specific HCC-specific DNA methylation changes when expressed in hepatoma cell lines. We hypothesize that DNA methylation changes directly induced by HCV infection are likely the driving force for tumor development and maintenance.
Fig. 3. Genome organization of HCV. HCV encodes 10 viral genes, encoding 4 structural (Core, E1, E2, p7) and 6 non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins.

Fig. 4. Development of stem cell specific therapy. Based on the cancer stem cell model, the conventional cancer therapy will eradicate the bulk of tumor cells but not CSCs, which will repopulate and lead to tumor relapse. The CSC-specific epigenetic therapy specifically targets CSCs and the remaining bulk tumor will spontaneously regress.

1.11. Summary and Future Directions

The development of high-throughput methods for analysis of epigenetic alterations in tumors have provided convincing evidence that epigenetic alteration is as important as genetic mutations in tumorigenesis. First, aberrant DNA methylation has been detected in virtually all types of cancer studied so far and is an early event during tumorigenesis (Jones and Baylin, 2002; 2007; Laird, 2003). Second, proteins important for epigenetic regulation are frequently mutated during tumor development (Baylin and Jones, 2011). Third, chemoresistance can be caused by epigenetic changes (Crea et al., 2009; Teodoridis et al., 2004). Fourth, both EMT and differentiation of tumor initiating cells are epigenetically regulated (Jordan et al., 2011; Scheel and Weinberg, 2012). Finally, DNA methyltransferase and histone deacetylase inhibitors have shown good efficacy in treating hematopoietic malignancies (Gryder et al., 2012; Popovic and Licht, 2012; Yang et al., 2010). However, little is known about which epigenetic changes are the cause and which one are the consequence of tumor development and maintenance. The ability to distinguish these epigenetic changes will not only provide better diagnostic markers, but also novel therapeutic and prevention targets (Fig. 4).

Characterization of epigenetic landscapes in hepatic cancer stem cells will not only identify markers for tumor detection, but also elucidate the mechanism of the origin of hepatic cancer stem cells. Current evidence suggest that cancer stem cells can either originate from normal stem cells via accumulation of aberrant
epigenetic profiles and genetic mutations, or from differentiated cells acquiring stem cell like properties. Perhaps the greatest prospect is the possibility that understanding the mechanisms driving epigenetic differences between normal and hepatic cancer stem cells will help in the discovery of novel therapies specifically and efficiently targeting hepatocellular carcinoma without altering normal tissue homeostasis.

2. CONCLUSION

HCV c Recent studies suggest that epigenetic mechanisms are not only essential for the dynamic transcriptional regulation in embryonic and somatic stem cells, but are also actively involved in tumorigensis: genes important for pluripotency are epigenetically regulated and aberrant epigenetic changes have been detected in virtually all human malignancies studied, including Hepatocellular Carcinoma (HCC). Infection with Hepatitis C Virus (HCV) is a major risk factor for the development of HCC. Despite the fact that HCV is a RNA virus without a DNA intermediate, recent studies demonstrate that HCV viral proteins may actively participate in epigenetic regulation of hepatic cancer stem cell phenotypes and induce HCC-specific epigenetic changes. Identification of host epigenetic alterations induced by HCV infection and epigenetic differences between hepatic cancer stem cells and the bulk non-tumorigenic cancer cells, may yield potential biomarkers for early detection, as well as therapeutic targets for HCV associated HCC.

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