**American Medical Journal 4 (1): 21-35, 2013**
ISSN 1949-0070

doi:10.3844/amjsp.2013.21.35 Published Online 4 (1) 2013 (http://www.thescipub.com/amj.toc)

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

**[1]Qinghua Feng, [2]Sajida Hassan, [3]Trina Das, [4]Jianfei Huang, [5]Zhenqing Feng and [2]David Gretch**

[1]Department of Pathology,
[2]Department of Lab Medicine,
[3]Department of Surgery,
School of Medicine, University of Washington, Seattle, 98109, Washington
[4]Department of Pathology,
Affiliated Hospital of University of Nantong, 20 Xisi Road, Nantong, Jiangsu, 226001, China
[5]Department of Pathology, Nanjing Medical University, Nanjing, Jiangsu, 210029, China

Received 2012-09-18, Revised 2013-01-16; Accepted 2013-03-12

## 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;

**Corresponding Author:** Qinghua Feng, Department of Pathology, School of Medicine, University of Washington, Seattle, 98109, Washington Tel: 206-897-1583 Fax: 206-897-1334

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.

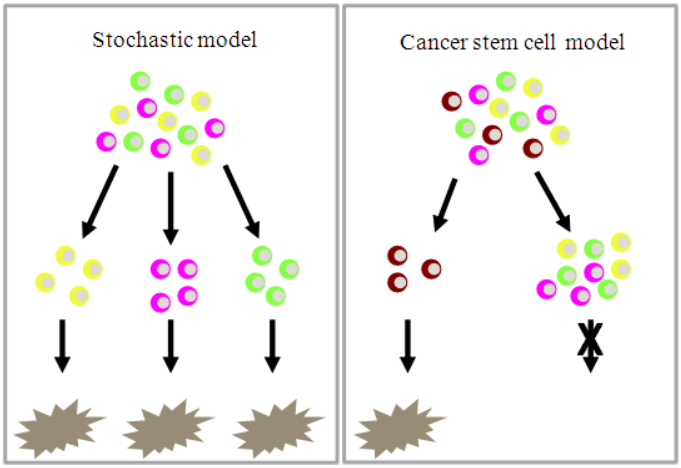
## 1.2. Cancer Stem Cells

### 1.2.1. Potential Cancer Stem Cells

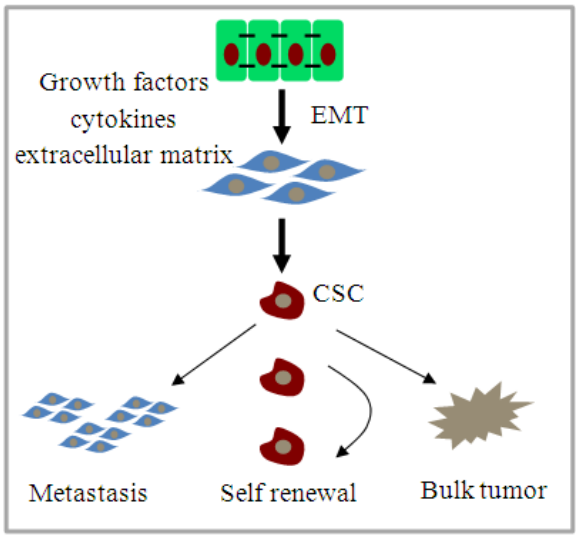
Recent studies support the hypothesis that cancer is derived from a small proportion of tumorigenic cells, called Cancer Stem Cells (CSCs), which are responsible for cancer initiation, proliferation, heterogeneity, as well as invasion and metastasis (Vlerken *et al*., 2012) (**Fig. 1A**). Similar to 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 periportal 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 cytokeratin 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.




(a)




(a)

**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

| Gene Symbol | Fold change | Entrez gene name |
|---|---|---|
| **Genes involved in stem cells** | | |
| BMI1 | -1.9 | BMI1 polycomb ring finger oncogene |
| CDKN2A | -1.8 | cyclin-dependent kinase inhibitor 2A (p16) |
| CDX2 | 2.9 | caudal type homeobox 2 |
| DLX1 | 5.5 | distal-less homeobox 1 |
| DNMT3B | 1.7 | DNA (cytosine-5-)-methyltransferase 3 beta |
| DNMT3L | 2.5 | DNA (cytosine-5-)-methyltransferase 3-like |
| ISL1 | 12.0 | ISL LIM homeobox 1 |
| KLF2 | -14.7 | Kruppel-like factor 2 (lung) |
| KLF4 | -4.7 | Kruppel-like factor 4 (gut) |
| PAX6 | -2.9 | paired box 6 |
| POU5F1 | 1.9 | POU class 5 homeobox 1 (Oct3) |
| RUNX1 | -20.0 | runt-related transcription factor 1 |
| Sox2 | 15.9 | SRY (sex determining region Y)-box 2 |
| STAT3 | -7.7 | signal transducer and activator of transcription 3 (acute-phase response factor) |
| SUZ12 | 2.3 | suppressor of zeste 12 homolog (Drosophila) |
| VDR | 9.7 | vitamin D (1,25- dihydroxyvitamin D3) receptor |
| **Genes involved in EMT** | | |
| CAMK2N1 | 2.8 | calcium/calmodulin-dependent protein kinase II inhibitor 1 |
| CDH1 | -1.5 | E-cadherin |
| KRT19 | -11.1 | keratin 19 |
| MAP1B | 10.9 | microtubule-associated protein 1B |
| MST1R | -1.9 | macrophage stimulating 1 receptor (c-met-related tyrosine kinase) |
| SPP1 | -50.1 | secreted phosphoprotein 1 |
| TFPI2 | 32.3 | tissue factor pathway inhibitor 2 |
| TGFB3 | -11.7 | transforming growth factor, beta 3 |
| TIMP1 | -100.0 | TIMP metallopeptidase inhibitor 1 |
| Vim | 1.6 | Vimentin |
| VPS13A | 2.3 | vacuolar protein sorting 13 homolog A (S. cerevisiae) |

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 PcGprotein 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 BIM1 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 refer 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 CpGdinucleotides, plays an important role in normal development and in gene expression regulation. The majority of CpGdinucleotides are methylated in the genome, except when they are clustered as a CpGisland 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 havetried 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 has 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).

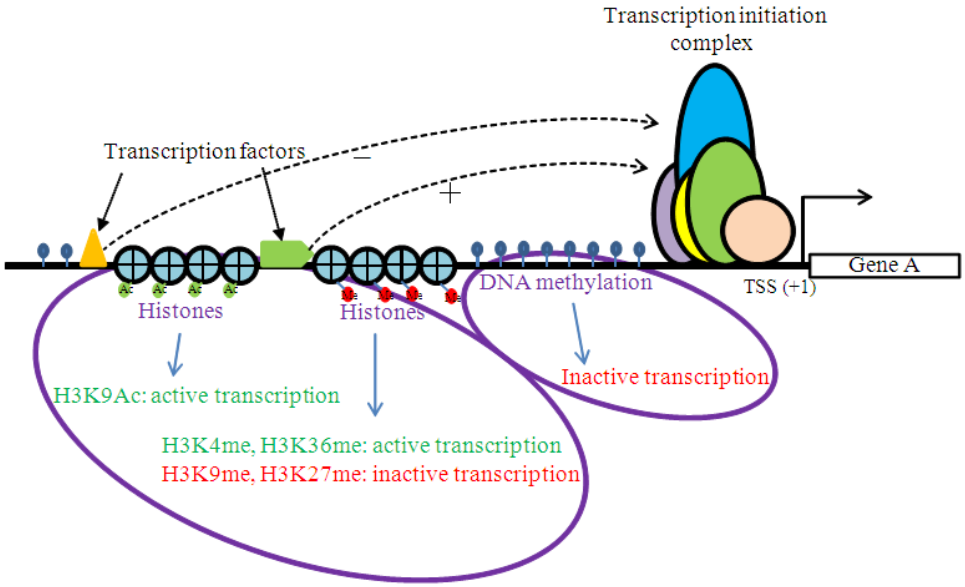



**Fig. 2.** Multiple epigenetic mechanisms influence gene expression. DNA methylation of the gene promoter usually leads to transcription inactivation, while histone modifications are either associated with active (H3K9Ac, H3K4me, KeK36me) or inactive (H3K9me, H3K27me) transcription

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 hepatomasubline 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 patternshave 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 up regulation of DNMT1 and DNMT3b in HepG2 cells (Park *et al*., 2011), while HCV core protein induces CDH1 methylation thus reducing its expression via up regulation 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.

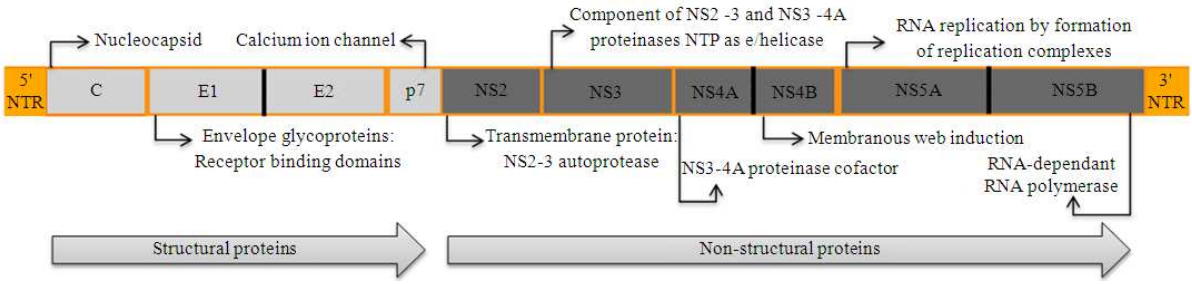



**Fig. 3.** Genome organization of HCV. HCV encodes 10 viral genes, encoding 4 structural (Core, E1, E2, p7) and 6 non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins

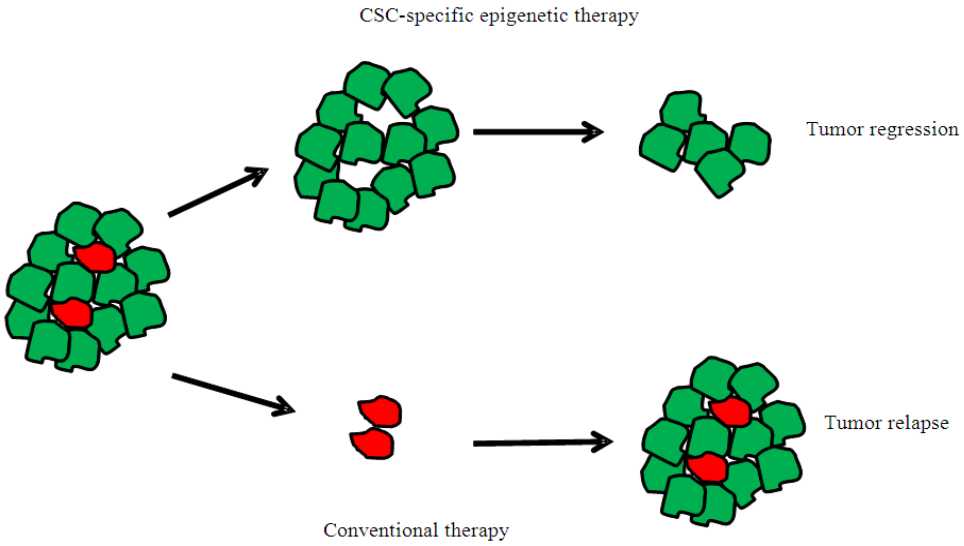



**Fig. 4.** Development of stem cell specific therapy. Based on the cancer stem cell model, the conventional cancer therapy will eradicate the bulk of tumor cells but not CSCs, which will repopulate and lead to tumor relapse. The CSC-specific epigenetic therapy specifically targets CSCs and the remaining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

## 3. REFERENCES


Ali, N., H. Allam, R. May, S.M. Sureban and Bronze *et al*., 2011. Hepatitis C virus-induced cancer stem cell-like signatures in cell culture and murine tumor xenografts. J. Virol., 85: 12292-12303. DOI: 10.1128/JVI.05920-11

Ammerpohl, O., J. Pratschke, C. Schafmayer, A. Haake and W. Faber *et al*., 2012. Distinct DNA methylation patterns in cirrhotic liver and hepatocellular carcinoma. Int. J. Cancer, 130: 1319-1328. DOI: 10.1002/ijc.26136

Archer, K.J., V.R. Mas, D.G. Maluf and R.A. Fisher, 2010. High-throughput assessment of CpG site methylation for distinguishing between HCV-cirrhosis and HCV-associated hepatocellular carcinoma. MGG, 283: 341-349. PMID: 20165882

Banerjee, A., R.B. Ray and R. Ray, 2010. Oncogenic potential of hepatitis C virus proteins. Viruses, 2: 2108-2133. DOI: 10.3390/v2092108

Battaglia, S., N. Benzoubir, S. Nobilet, P. Charneau and D. Samuel *et al*., 2009. Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. PLOS one, 4: e4355-e4355. DOI: 10.1371/journal.pone.0004355

Baylin, S.B. and P.A. Jones, 2011. A decade of exploring the cancer epigenome-biological and translational implications. Nat. Rev. Cancer, 11: 726-734. DOI: 10.1038/nrc3130

Belinsky, S.A., K.J. Nikula, W.A. Palmisan, R. Michels and G. Saccomanno *et al*., 1998. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc. Natl. Acad. Sci. USA., 95: 11891-11896. DOI: 10.1073/pnas.95.20.11891

Blight, K.J., J.A. McKeating and C.M. Rice, 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol., 76: 13001-13014. DOI: 10.1128/JVI.76.24.13001-13014.2002

Bosch, F.X., J. Ribes, R. Cleries and M. Diaz, 2005. Epidemiology of hepatocellular carcinoma. Clin. Liver Dis., 9: 191-211. DOI: 10.1016/j.cld.2004.12.009

Brait, M. and D. Sidransky, 2011. Cancer epigenetics: Above and beyond. Toxicol. Mech. Methods, 21: 275-288. DOI: 10.3109/15376516.2011.562671

Bruggeman, S.W., M.E. Valk-Lingbeek, P.P.V.D. Stoop, J.J. Jacobs and K. Kieboom *et al*., 2005. Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. Genes Dev., 19: 1438-1443. DOI: 10.1101/gad.1299305

Cai, M.Y., J.H. Hou, H.L. Rao, R.Z. Luo and M. Li *et al*., 2011. High expression of H3K27me3 in human hepatocellular carcinomas correlates closely with vascular invasion and predicts worse prognosis in patients. Mol. Med., 17: 12-20. DOI: 10.2119/molmed.2011.00054

Cao, R., Y. Tsukada and Y. Zhang, 2005. Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol. Cell, 20: 845-854. DOI: 10.1016/j.molcel.2005.12.002

Chiba, T., A. Seki, R. Aoki, H. Ichikawa and M. Negishi *et al*., 2010. Bmi1 promotes hepatic stem cell expansion and tumorigenicity in both Ink4a/Arf-dependent and -independent manners in mice. Hepatology, 52: 1111-1123. DOI: 10.1002/hep.23793

Chiba, T., O. Yokosuka, K. Fukai, Y. Hirasawa and M. Tada *et al*., 2005. Identification and investigation of methylated genes in hepatoma. Eur. J. Cancer, 41: 1185-1194. DOI: 10.1016/j.ejca.2005.02.014

Chiba, T., O. Yokosuka, M. Arai, M. Tada and K. Fukai *et al*., 2004. Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. J. Hepatol., 41: 436-445. DOI: 10.1016/j.jhep.2004.05.018.

Clouston, A.D., E.E. Powell, M.J. Walsh, M.M. Richardson and A.J. Demetris *et al*., 2005. Fibrosis correlates with a ductular reaction in hepatitis C: Roles of impaired replication, progenitor cells and steatosis. Hepatology, 41: 809-818. DOI: 10.1002/hep.20650

Crea, F., R. Danesi and W.L. Farrar, 2009. Cancer stem cell epigenetics and chemoresistance. Epigenomics, 1: 63-79. DOI: 10.2217/epi.09.4

Deng, Y.B., G. Nagae, Y. Midorikawa, K. Yagi and S. Tsutsumi *et al*., 2010. Identification of genes preferentially methylated in hepatitis C virus-related hepatocellular carcinoma. Cancer Sci., 101: 1501-1510. DOI: 10.1111/j.1349-7006.2010.01549.x

Duong, F.H., V. Christen, S. Lin and M.H. Heim, 2010. Hepatitis C virus-induced up-regulation of protein phosphatase 2A inhibits histone modification and DNA damage repair. Hepatology, 51: 741-751. PMID: 20043320.

El-Serag, H.B. and K.L. Rudolph, 2007. Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. Gastroenterology, 132: 2557-2576. DOI: 10.1053/j.gastro.2007.04.061

El-Serag, H.B., 2002. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology, 36: S74-83. DOI: 10.1053/jhep.2002.36807

Esteller, M., A. Sparks, M. Toyota, M. Sanchez-Cespedes and G. Capella *et al*., 2000. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res., 60: 4366-4371. PMID: 10969779

Evron, E., W.C. Dooley, C.B. Umbricht, D. Rosenthal and N. Sacchi *et al*., 2001. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet, 357: 1335-1336. DOI: 10.1016/S0140-6736(00)04501-3

Feng, Q., J.E. Stern, S.E. Hawes, H. Lu and M. Jiang *et al*., 2010. DNA methylation changes in normal liver tissues and hepatocellular carcinoma with different viral infection. Exp. Mol. Pathol., 88: 287-292. DOI: 10.1016/j.yexmp.2010.01.002

Fujinaga, H., T. Tsutsumi, H. Yotsuyanagi, K. Moriya and K. Koike, 2011. Hepatocarcinogenesis in hepatitis C: HCV shrewdly exacerbates oxidative stress by modulating both production and scavenging of reactive oxygen species. Oncology, 81: 11-17. DOI: 10.1159/000333253

Fukai, K., O. Yokosuka, F. Imazeki, M. Tada and R. Mikata *et al*., 2005. Methylation status of p14ARF, p15INK4b and p16INK4a genes in human hepatocellular carcinoma. Liver Int., 25: 1209-1216. DOI: 10.1111/j.1478-3231.2005.01162.x

Gal-Yam, E.N., Y. Saito, G. Egger and P.A. Jones, 2008. Cancer epigenetics: Modifications, screening and therapy. Ann. Rev. Med., 59: 267-280. DOI: 10.1146/annurev.med.59.061606.095816

Gao, W., Y. Kondo, L. Shen, Y. Shimizu and T. Sano *et al*., 2008. Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. Carcinogenesis, 29: 1901-1910. DOI: 10.1093/carcin/bgn170

Ghosh, A.K., R. Steele, K. Meyer, R. Ray and R.B. Ray, 1999. Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. J. Gen. Virol., 80: 1179-1183. PMID: 10355764

Grebely, J. and G.J. Dore, 2011. What is killing people with hepatitis C virus infection? Seminars Liver Dise., 31: 331-339. DOI: 10.1055/s-0031-1297922

Gryder, B.E., Q.H. Sodji and A.K. Oyelere, 2012. Targeted cancer therapy: Giving histone deacetylase inhibitors all they need to succeed. Future Med. Chem., 4: 505-524. DOI: 10.4155/fmc.12.3

Guo, N., R. Chen, Z. Li, Y. Liu and D. Cheng *et al*., 2011. Hepatitis C virus core upregulates the methylation status of the RASSF1A promoter through regulation of SMYD3 in hilar cholangiocarcinoma cells. Acta Bioch. Biophys. Sinica, 43: 354-361. DOI: 10.1093/abbs/gmr021

Hamamoto, R., Y. Furukawa, M. Morita, Y. Iimura and Y. Silva *et al*., 2004. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat. Cell. Biol., 6: 731-740. DOI: 10.1038/ncb1151

Harris, P.J., G. Speranza and C. Dansky Ullmann, 2012. Targeting embryonic signaling pathways in cancer therapy. Expert Opin. Therapeutic Targets, 16: 131-145. DOI: 10.1517/14728222.2011.645808

He, Y., Y. Zhao, S. Zhang, W. Chen and S. Lin *et al*., 2006. Not polymorphism but methylation of class II transactivator gene promoter IV associated with persistent HBV infection. J. Clin. Virol., 37: 282-286. PMID: 16996793

Hernandez-Vargas, H., M.P. Lambert, F.L. Calvez-Kelm, G. Gouysse and S. McKay-Chopin *et al*., 2010. Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. PloS One, 5: e9749-e9749. DOI: 10.1371/journal.pone.0009749

Jiang, G., L. Liu, I.M. Buyse, D. Simon and S. Huang, 1999. Decreased RIZ1 expression but not RIZ2 in hepatoma and suppression of hepatoma tumorigenicity by RIZ1. Int. J. Cancer, 83: 541-546. DOI: 10.1002/(SICI)1097-0215(19991112)83:4<541::AID-IJC17>3.0.CO;2-F.

Jicai, Z., Y. Zongtao, L. Jun, L. Haiping and W. Jianmin *et al*., 2006. Persistent infection of hepatitis B virus is involved in high rate of p16 methylation in hepatocellular carcinoma. Mol. Carcinog., 45: 530-536. DOI: 10.1002/mc.20188

Jones, P.A. and S.B. Baylin, 2002. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet., 3: 415-428. PMID: 12042769

Jones, P.A. and S.B. Baylin, 2007. The epigenomics of cancer. Cell, 128: 683-692. DOI: 10.1016/j.cell.2007.01.029

Jordan, N.V., G.L. Johnson and A.N. Abell, 2011. Tracking the intermediate stages of epithelial-mesenchymal transition in epithelial stem cells and cancer. Cell Cycle, 10: 2865-2873. DOI: 10.4161/cc.10.17.17188

Kane, M.A., 2003. Global control of primary hepatocellular carcinoma with hepatitis B vaccine: The contributions of research in Taiwan. Cancer Epidemiol. Biomarkers Prev., 12: 2-3. PMID: 12540496

Kaneto, H., S. Sasaki, H. Yamamoto, F. Itoh and M. Toyota *et al*., 2001. Detection of hypermethylation of the p16(INK4A) gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. Gut, 48: 372-377. DOI: 10.1136/gut.48.3.372

Katoh, H., T. Shibata, A. Kokubu, H. Ojima and M. Fukayama *et al*., 2006. Epigenetic instability and chromosomal instability in hepatocellular carcinoma. Am. J. Pathol., 168: 1375-1384. DOI: 10.2353/ajpath.2006.050989

Kondo, Y., L. Shen, S. Suzuki, T. Kurokawa and K. Masuko *et al*., 2007. Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatol. Res., 37: 974-983. PMID: 17584191

Kouzarides, T., 2007. Chromatin modifications and their function. Cell, 128: 693-705. DOI: 10.1016/j.cell.2007.02.005

Ksiazkiewicz, M., A. Markiewicz and A.J. Zaczek, 2012. Epithelial-mesenchymal transition: A hallmark in metastasis formation linking circulating tumor cells and cancer stem cells. Pathobiology, 79: 195-208. PMID: 22488297

Kwon, G.Y., B.C. Yoo, K.C. Koh, J.W. Cho and W.S. Park *et al*., 2005. Promoter methylation of E-cadherin in hepatocellular carcinomas and dysplastic nodules. J. Korean Med. Sci., 20: 242-247. DOI: 10.3346/jkms.2005.20.2.242

Lachenmayer, A., S. Toffanin, L. Cabellos, C. Alsinet and Y. Hoshida *et al*., 2012. Combination therapy for hepatocellular carcinoma: Additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib. J. Hepatol., 56: 1343-1350. DOI: 10.1016/j.jhep.2012.01.009

Laird, P.W., 2003. The power and the promise of DNA methylation markers. Nat. Rev. Cancer, 3: 253-266. DOI: 10.1038/nrc1045

Lee, S., H.J. Lee, J.H. Kim, H.S. Lee and J.J. Jang *et al*., 2003. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. Am. J. Pathol., 163: 1371-1378. DOI: 10.1016/S0002-9440(10)63495-5

Levrero, M., 2006. Viral hepatitis and liver cancer: The case of hepatitis C. Oncogene, 25: 3834-3847. DOI: 10.1038/sj.onc.1209562

Li, L. and W.B. Neaves, 2006. Normal stem cells and cancer stem cells: The niche matters. Cancer Res., 66: 4553-4557. DOI: 10.1158/0008-5472.CAN-05-3986

Li, X., A.M. Hui, L. Sun, K. Hasegawa and G. Torzilli *et al*., 2004. p16INK4A hypermethylation is associated with hepatitis virus infection, age and gender in hepatocellular carcinoma. Clin. Cancer Res., 10: 7484-7489. DOI: 10.1158/1078-0432.CCR-04-1715

Machida, K., C.L. Chen, J.C. Liu, C. Kashiwabara and D. Feldman *et al*., 2012. Cancer stem cells generated by alcohol, diabetes and hepatitis C virus. J. Gastroenterol. Hepatol., 27: 19-22. DOI: 10.1111/j.1440-1746.2011.07010.x

Machida, K., H. Tsukamoto, H. Mkrtchyan, L. Duan and A. Dynnyk *et al*., 2009. Toll-like receptor 4 mediates synergism between alcohol and HCV in hepatic oncogenesis involving stem cell marker Nanog. Proc. Natl. Acad. Sci. USA., 106: 1548-1553. DOI: 10.1073/pnas.0807390106

Magerl, C., J. Ellinger, T. Braunschweig, E. Kremmer and L.K. Koch *et al*., 2010. H3K4 dimethylation in hepatocellular carcinoma is rare compared with other hepatobiliary and gastrointestinal carcinomas and correlates with expression of the methylase Ash2 and the demethylase LSD1. Hum. Pathol., 41: 181-189. DOI: 10.1016/j.humpath.2009.08.007

Mani, S.A., W. Guo, M.J. Liao, E.N. Eaton and A. Ayyanan *et al*., 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell, 133: 704-715. DOI: 10.1016/j.cell.2008.03.027

Micallef, J.M., J.M. Kaldor and G.J. Dore, 2006. Spontaneous viral clearance following acute hepatitis C infection: A systematic review of longitudinal studies. J. Viral Hepatitis, 13: 34-41. DOI: 10.1111/j.1365-2893.2005.00651.x

Mori, T., S. Nomoto, K. Koshikawa, T. Fujii and M. Sakai *et al*., 2005. Decreased expression and frequent allelic inactivation of the RUNX3 gene at 1p36 in human hepatocellular carcinoma. Liver Int., 25: 380-388. PMID: 15780064

Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi and T. Tsutsumi *et al*., 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med., 4: 1065-1067. DOI: 10.1038/2053

Munakata, T., M. Nakamura, Y. Liang, K. Li and S.M. Lemon, 2005. Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C virus NS5B RNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. USA., 102: 18159-18164. DOI: 10.1073/pnas.0505605102

Narimatsu, T., A. Tamori, N. Koh, S. Kubo and K. Hirohashi *et al*., 2004. p16 promoter hypermethylation in human hepatocellular carcinoma with or without hepatitis virus infection. Intervirology, 47: 26-31. DOI: 10.1159/000076639

Nishida, N., 2010. Impact of hepatitis virus and aging on DNA methylation in human hepatocarcinogenesis. Histol. Histopathol., 25: 647-654. PMID: 20238302

Oguro, H., A. Iwama, Y. Morita, T. Kamijo and M.V. Lohuizen *et al*., 2006. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. J. Exp. Med., 203: 2247-2253. DOI: 10.1084/jem.20052477

Oliva, J., B.A. French, X. Qing and S.W. French, 2010. The identification of stem cells in human liver diseases and hepatocellular carcinoma. Exp. Mol. Pathol., 88: 331-340. DOI: 10.1016/j.yexmp.2010.01.003

Park, S.H., J.S. Lim, S.Y. Lim, I. Tiwari and K.L. Jang, 2011. Hepatitis C virus Core protein stimulates cell growth by down-regulating p16 expression via DNA methylation. Cancer Lett., 310: 61-68. PMID: 21757290

Perz, J.F., G.L. Armstrong, L.A. Farrington, Y.J. Hutin and B.P. Bell, 2006. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J. Hepatol., 45: 529-538. DOI: 10.1016/j.jhep.2006.05.013

Pogribny, I.P. and I. Rusyn, 2012. Role of epigenetic aberrations in the development and progression of human hepatocellular carcinoma. Cancer Lett. DOI: 10.1016/j.canlet.2012.01.038

Popovic, R. and J.D. Licht, 2012. Emerging epigenetic targets and therapies in cancer medicine. Cancer Discov., 2: 405-413. DOI: 10.1158/2159-8290.CD-12-0076

Ray, R.B., L.M. Lagging, K. Meyer and R. Ray, 1996. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J. Virol., 70: 4438-4443. PMID: 8676467

Ripoli, M., R. Barbano, T. Balsamo, C. Piccoli and V. Brunetti *et al*., 2011. Hypermethylated levels of E-cadherin promoter in Huh-7 cells expressing the HCV core protein. Virus Res., 160: 74-81. DOI: 10.1016/j.virusres.2011.05.014

Roskams, T., 2003. Progenitor cell involvement in cirrhotic human liver diseases: From controversy to consensus. J. Hepatol., 39: 431-434. DOI: 10.1016/S0168-8278(03)00333-7

Sakamuro, D., T. Furukawa and T. Takegami, 1995. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J. Virol., 69: 3893-3896. PMID: 7745741

Scheel, C. and R.A. Weinberg, 2012. Cancer stem cells and epithelial-mesenchymal transition: Concepts and molecular links. Semin Cancer Biol., 22: 396-403. PMID: 22554795

Schutte, K., J. Bornschein and P. Malfertheiner, 2009. Hepatocellular carcinoma--epidemiological trends and risk factors. Dig. Dis., 27: 80-92. DOI: 10.1159/000218339

Shen, J., S. Wang, Y.J. Zhang, M. Kappil and H.C. Wu *et al*., 2012. Genome-wide DNA methylation profiles in hepatocellular carcinoma. Hepatology, 55: 1799-1808. DOI: 10.1002/hep.25569

Shih, Y.L., R.Y. Shyu, C.B. Hsieh, H.C. Lai and K.Y. Liu *et al*., 2006. Promoter methylation of the secreted frizzled-related protein 1 gene SFRP1 is frequent in hepatocellular carcinoma. Cancer, 107: 579-590. DOI: 10.1002/cncr.22023

Shim, Y.H., G.S. Yoon, H.J. Choi, Y.H. Chung and E. Yu, 2003. p16 Hypermethylation in the early stage of hepatitis B virus-associated hepatocarcinogenesis. Cancer Lett., 190: 213-219. DOI: 10.1016/S0304-3835(02)00613-4

Shin, S.H., B.H. Kim, J.J. Jang, K.S. Suh and G.H. Kang, 2010. Identification of novel methylation markers in hepatocellular carcinoma using a methylation array. J. Korean Med. Sci., 25: 1152-1159. DOI: 10.3346/jkms.2010.25.8.1152

Shitani, M., S. Sasaki, N. Akutsu, H. Takagi and H. Suzuki *et al*., 2012. Genome-wide analysis of DNA methylation identifies novel cancer-related genes in hepatocellular carcinoma. Tumour Biol., 33: 1307-1317. DOI: 10.1007/s13277-012-0378-3

Sudo, T., T. Utsunomiya, K. Mimori, H. Nagahara and K. Ogawa *et al*., 2005. Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br. J. Cancer, 92: 1754-1758. DOI: 10.1038/sj.bjc.6602531

Tai, A.W. and R.T. Chung, 2009. Treatment failure in hepatitis C: mechanisms of non-response. J. Hepatol., 50: 412-420. DOI: 10.1016/j.jhep.2008.11.010

Takebe, N. and S.P. Ivy, 2010. Controversies in cancer stem cells: Targeting embryonic signaling pathways. Clin. Cancer Res., 16: 3106-3112. DOI: 10.1158/1078-0432.CCR-09-2934

Teodoridis, J.M., G. Strathdee, J.A. Plumb and R. Brown, 2004. CpG-island methylation and epigenetic control of resistance to chemotherapy. Biochem. Soc. Trans., 32: 916-917. DOI: 10.1042/BST0320916

Thomas, M.B. and A.X. Zhu, 2005. Hepatocellular carcinoma: The need for progress. J. Clin. Oncol., 23: 2892-2899. DOI: 10.1200/JCO.2005.03.196

Tsai, W.L. and R.T. Chung, 2010. Viral hepatocarcinogenesis. Oncogene, 29: 2309-2324. DOI: 10.1200/JCO.2005.03.196

Tsamandas, A.C., I. Syrokosta, K. Thomopoulos, V. Zolota and D. Dimitropoulou *et al*., 2006. Potential role of hepatic progenitor cells expression in cases of chronic hepatitis C and their relation to response to therapy: A clinicopathologic study. Liver Int., 26: 817-826. PMID: 16911464

Tsuda, H., K. Yamamoto, T. Inoue, I. Uchiyama and N. Umesaki, 2000. The role of p16-cyclin d/CDK-pRb pathway in the tumorigenesis of endometrioid-type endometrial carcinoma. Br. J. Cancer, 82: 675-682. PMID: 10682682

Tysnes, B.B., 2010. Tumor-initiating and -propagating cells: Cells that we would like to identify and control. Neoplasia, 12: 506-515. PMID: 20651980

Umbricht, C.B., E. Evron, E. Gabrielson, A. Ferguson and J. Marks *et al*., 2001. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene, 20: 3348-3353. DOI: 10.1038/sj.onc.1204438

Vlerken, L.E.V., E.M. Hurt and R.E. Hollingsworth, 2012. The role of epigenetic regulation in stem cell and cancer biology. J. Mol. Med., 90: 791-801. PMID: 22660276

Wu, X., J.M. Robotham, E. Lee, S. Dalton and N.M. Kneteman *et al*., 2012. Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. PLoS Pathogens, 8: e1002617-e1002617. DOI: 10.1371/journal.ppat.1002617

Wu, X.Z., 2008. Origin of cancer stem cells: The role of self-renewal and differentiation. Ann. Surg. Oncol., 15: 407-414. DOI: 10.1245/s10434-007-9695-y

Yang, B., M. Guo, J.G. Herman and D.P. Clark, 2003. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am. J. Pathol., 163: 1101-1107. DOI: 10.1016/S0002-9440(10)63469-4

Yang, X., F. Lay, H. Han and P.A. Jones, 2010. Targeting DNA methylation for epigenetic therapy. Trends Pharmacol. Sci., 31: 536-546. DOI: 10.1016/j.tips.2010.08.001

Yao, J.Y., L. Zhang, X. Zhang, Z.Y. He and Y. Ma *et al*., 2010. H3K27 trimethylation is an early epigenetic event of p16INK4a silencing for regaining tumorigenesis in fusion reprogrammed hepatoma cells. J. Biol. Chem., 285: 18828-18837. DOI: 10.1074/jbc.M109.077974

Zhang, C., H. Li, Y. Wang, W. Liu and Q. Zhang *et al*., 2010. Epigenetic inactivation of the tumor suppressor gene RIZ1 in hepatocellular carcinoma involves both DNA methylation and histone modifications. J. Hepatol., 53: 889-895. DOI: 10.1016/j.jhep.2010.05.012

Zhang, Y.J., H. Ahsan, Y. Chen, R.M. Lunn and L.Y. Wang *et al*., 2002. High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol. Carcinog, 35: 85-92. DOI: 10.1002/mc.10076

Zhang, Y.J., P. Rossner, Jr., Y. Chen, M. Agrawal and Q. Wang *et al*., 2006. Aflatoxin B1 and polycyclic aromatic hydrocarbon adducts, p53 mutations and p16 methylation in liver tissue and plasma of hepatocellular carcinoma patients. Int. J. Cancer, 119: 985-991. DOI: 10.1002/ijc.21699

Zhang, Y.J., Y. Chen, H. Ahsan, R.M. Lunn and P.H. Lee *et al*., 2003. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int. J. Cancer, 103: 440-444. DOI: 10.1002/ijc.10852

Zhang, Y.J., Y. Chen, H. Ahsan, R.M. Lunn and S.Y. Chen *et al*., 2005. Silencing of glutathione S-transferase P1 by promoter hypermethylation and its relationship to environmental chemical carcinogens in hepatocellular carcinoma. Cancer Lett., 221: 135-143. DOI: 10.1016/j.canlet.2004.08.028