Hepatocellular carcinoma (HCC) usually develops on the basis of chronic hepatitis and liver cirrhosis, where inactivation of several tumor suppressor genes (TSGs) takes place via methylation of the promoter. Interestingly, these methylation events are more prevalent in a background liver at high risk of HCC than one at low risk. Abnormal methylation is also observed in precancerous nodules such as dysplastic nodules and adenomas, suggesting that epigenetic alteration is an early event for HCC carcinogenesis. It is possible that infection with the hepatitis virus induces alteration of methylation at promoters of TSGs. Some studies suggested that viral proteins interfere with DNA methyltransferase in chronic hepatitis B. Induction of epigenetic alteration in chronic hepatitis C might, however, be a consequence of oxidative stress. In addition, we proposed age should be taken into consideration for HCC development via epigenetic pathways. Further investigations are required to understand the mechanism of inducing epigenetic instability during hepatocarcinogenesis.

Key words: Hepatocellular carcinoma, DNA methylation, Hepatitis virus, Aging, Tumor suppressor gene

Introduction

Accumulation of several genetic and epigenetic alterations take place in hepatocellular carcinoma (HCC), and these are considered to play a critical role in the activation of oncogenes and inactivation of tumor suppressor genes (TSGs) during hepatocarcinogenesis (Nishida et al., 1992, 1994, 2003; Thorgeirsson and Grisham, 2002). In human HCC, an alteration of DNA methylation status is commonly detected, suggesting the importance of epigenetic instability in the development of this type of cancer. Generally, in normal cells, cytosines of CpG in non-promoter regions are methylated, whereas those in active gene promoters remain unmethylated. In HCC, however, the methylation pattern found in normal cells is disturbed, and this leads to inactivation of TSGs, chromosomal fragility, and activation of transposable elements, all of which will play a role in cancer development. In this review, we focus on the alteration of DNA methylation and its role in hepatocarcinogenesis with special attention to hepatitis virus infection, which is a major cause of chronic hepatitis and HCC worldwide.

Alteration of methylation status of DNA in HCC and background liver

Alteration of methylation in several TSGs has been reported in HCC. As epigenetic change of TSGs is commonly detected when compared to genetic changes such as point mutations in HCC tissue (Nishida et al., 1993, 2003), this type of alteration is considered to be a key mechanism for TSG inactivation during hepatocarcinogenesis (Table 1). Some methylation events, such as those at the CHFR (checkpoint with forkhead associated and ring finger) and SKY (spleen tyrosine kinase), were reportedly detected specifically in advanced stages of HCC (Sakai et al., 2005; Yuan et al., 2006), but most methylation in these cancer-related genes is observed even in the early stages of a tumor as well as in precancerous lesions (Lehmann et al., 2005; Zhu et al., 2007; Nishida et al., 2008). This evidence
suggests that epigenetic alteration could play a role at an early stage of HCC formation.

Abnormal methylation at promoters of TSGs has been reported in both HCC and noncancerous background liver, although the level of methylation in noncancerous tissue is low compared to that of HCC (Lee et al., 2003; Nishida et al., 2008). In addition, some of these methylation events can also be detected in normal liver, with the level increasing with age (Fig. 1) (Li et al., 2004; Ko et al., 2008; Nishida et al., 2008). Interestingly, abnormal methylation tends to be more prevalent in background liver at high risk of HCC, such as cirrhotic noncancerous livers of HCC patients, than in that at low risk, such as mild hepatitis without HCC (Lee et al., 2003; Harder et al., 2008). Furthermore, frequent methylation of the p16 tumor suppressor gene is reportedly detected in liver with hepatitis B virus (HBV) or hepatitis C virus (HCV); however, no methylation of p16 was observed in livers with autoimmune hepatitis or primary biliary cirrhosis, or in fatty livers where the risk of HCC is low (Kaneto et al., 2001). This evidence suggests that the presence of chronic infection from the hepatitis virus or differences in the manner of inflammation may reflect the frequencies of methylation events, which finally related to the increasing risk of liver cancer.

On the other hand, overexpression of DNA methyltransferase (DNMT), which is considered a key enzyme for maintaining methylation status or inducing de novo DNA methylation, is also reported in both HCC tissue and noncancerous liver (Saito et al., 2001). The abnormal expression of DNMT could be responsible for the alteration of methylation status, and infection by the

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**Table 1.** Tumor suppressor genes inactivated by promoter methylation in human hepatocellular carcinoma.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of the gene product</th>
<th>Freq.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3σ</td>
<td>Regulation of G2/M checkpoint</td>
<td>89%</td>
<td>Iwata et al., 2000</td>
</tr>
<tr>
<td>APC</td>
<td>Regulation of WNT/β-catenin signaling</td>
<td>53%</td>
<td>Yang et al., 2003</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>T type Ca channel signaling</td>
<td>21%</td>
<td>Shen et al., 2002</td>
</tr>
<tr>
<td>CASP8</td>
<td>Apoptosis</td>
<td>72%</td>
<td>Yu et al., 2002</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin: Cell adhesion</td>
<td>67%</td>
<td>Kanai et al., 1997</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator: drug resistance.</td>
<td>77%</td>
<td>Ding et al., 2004</td>
</tr>
<tr>
<td>CHFR</td>
<td>Checkpoint with forkhead-associated domain and RING finger domain: Regulation of G2/M checkpoint</td>
<td>35%</td>
<td>Sakai et al., 2005</td>
</tr>
<tr>
<td>COX2</td>
<td>Prostaglandin synthase</td>
<td>18%</td>
<td>Shen et al., 2002</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
<td>62%</td>
<td>Shen et al., 2002</td>
</tr>
<tr>
<td>GADD45α</td>
<td>DNA repair and cell death</td>
<td><em>N.R.</em></td>
<td>Qi et al., 2004</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione transferase: Detoxification and metabolism</td>
<td>65%</td>
<td>Zhong et al., 2002</td>
</tr>
<tr>
<td>HDPR1</td>
<td>Human homologue of Dapper: Regulation of WNT/β-catenin signaling</td>
<td>51%</td>
<td>Yau et al., 2005</td>
</tr>
<tr>
<td>HIC1</td>
<td>Regulation of p53 signaling</td>
<td>85%</td>
<td>Park et al., 2006</td>
</tr>
<tr>
<td>NORE1B</td>
<td>Regulation of Ras signaling</td>
<td>62%</td>
<td>Macheiner et al., 2006</td>
</tr>
<tr>
<td>NQO1</td>
<td>Quinone oxidoreductase 1: Metabolism</td>
<td>50%</td>
<td>Tada et al., 2005</td>
</tr>
<tr>
<td>p16</td>
<td>Inhibitor of cyclin-dependent kinase: Regulation of G1/S checkpoint</td>
<td>73%</td>
<td>Wong et al., 1999</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Regulation of Ras signaling</td>
<td>85%</td>
<td>Zhang et al., 2002</td>
</tr>
<tr>
<td>RIZ1</td>
<td>Regulation of Ras signaling</td>
<td>29%</td>
<td>Lehmann et al., 2005</td>
</tr>
<tr>
<td>RUNX3</td>
<td>Regulation of TGF-β signaling</td>
<td>48%</td>
<td>Xiao and Liu, 2004</td>
</tr>
<tr>
<td>SEMA3B</td>
<td>SEMAPHORIN 3B. A member of the semaphorin family important in axonal guidance</td>
<td>83%</td>
<td>Tischkoff et al., 2005</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled-related protein 1: Regulation of WNT/β-catenin signaling</td>
<td>48%</td>
<td>Shih et al., 2006</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1: Regulation of JAK/STAT signaling</td>
<td>65%</td>
<td>Yoshikawa et al., 2001</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Regulation of JAK/STAT signaling</td>
<td>33%</td>
<td>Niwa et al., 2005</td>
</tr>
<tr>
<td>SYK</td>
<td>The spleen tyrosine kinase: Suppressor of tumor formation and metastasis</td>
<td>27%</td>
<td>Yuan et al., 2006</td>
</tr>
<tr>
<td>WT1</td>
<td>A transcription factor that functions as a tumour suppressor</td>
<td>46%</td>
<td>Zhang et al., 2007</td>
</tr>
</tbody>
</table>

*N.R: Percentage was not reported.*
hepatitis virus and chronic inflammation might play a role in the induction of DNMT in hepatocytes.

The progression of hypomethylation in non-promoter CpG is also important for carcinogenesis because it may induce chromosome instability and activation of oncogenes or transposons. The methylation level of satellite 2 (SAT2) in pericentromeric satellite regions reportedly decreases along with the progression of chronic hepatitis and HCC (Lee et al., 2009). It is reported that expression of DNMT3b4, which may lack DNA methyltransferase activity and compete with DNMT3b3 for targeting to pericentromeric satellite regions, results in DNA hypomethylation on SAT2 and plays a critical role for inducing chromosomal instability (Saito et al., 2002). As mentioned above, the demethylation at non-promoter CpG is also found at an early stage of HCC carcinogenesis, and progresses along with the tumor (Kim et al., 2009; Lee et al., 2009).

Role of hepatitis virus in DNA methylation

Virus infection and chronic inflammation could be important factors in epigenetic instability. Previous reports suggest that the presence of simian virus 40, Epstein-Barr virus, and HBV are associated with abnormal DNA methylation (Li et al., 2005).

After infection, DNA viruses and retroviruses can be integrated into the host genome, whereas DNA methylation is known to act as a protection mechanism against a disturbance of the host genome. Integration of viral DNA might, therefore, lead to induction of DNA methylation that acts as a defense for keeping genomic integrity. On the other hand, it also induces deregulation of gene expression through epigenetic alteration during persistent hepatitis viral infection. In addition, some reports suggest that viral proteins, such as hepatitis B virus X protein (HBx), interact with DNMT and interfere with the function of this enzyme (Zheng et al., 2009). The effect of inflammation for induction of epigenetic instability should be taken into consideration, as chronic viral infection is usually accompanied by inflammation. Although the association between epigenetic instability and chronic viral hepatitis still remains to be explored, the possible mechanisms for induction of abnormal methylation in chronic hepatitis are discussed here.

HBV and DNA methylation

As described above, the promoters of several TSGs showed abnormal methylation in HCC as well as background liver of chronic hepatitis and liver cirrhosis. Among them, methylation of the GSTP1, RASSF1A, E-cadherin, and p16 genes are reportedly more frequent in HBV-positive HCC than HBV-negative tumors (Zhong et al., 2002, 2003; Shim et al., 2003; Jicai et al., 2006; Su et al., 2007). Several reports suggested that induction of these methylation events might be affected by the presence of HBx. For example, promoter methylation of the p16 gene is shown to be closely correlated with higher HBx expression in precancerous lesions (Zhu et al., 2007). In addition, it is shown that HBx induces DNMT1 expression, which leads to the hypermethylation of the p16 and may play an important role in the early stage of HBV-associated hepatocarcinogenesis (Zhu et al., 2007). HBx also reportedly represses E-cadherin expression at the transcription level by inducing methylation-mediated promoter inactivation (Lee et al., 2005).

Other reports also suggested the role of HBx for induction of DNMTs. Junk et al. (2007) showed the possibility that HBx might activate DNMT1 expression via a regulatory circuit involving the p16/retinoblastoma protein (Rb)/E2F1 pathway (Jung et al., 2007). Inhibition of DNMT1 activity not only abolished the methylation-mediated p16 repression but also suppressed DNMT1 expression itself, suggesting a cross-talk between DNMT1 and p16 (Jung et al., 2007).

HBx not only up-regulates DNMT1, DNMT3a1, and DNMT3a2 and selectively promotes regional hypermethylation of specific TSGs, but also induces global hypomethylation of SAT2 repeat sequences by down-regulating DNMT3b (Park et al., 2007). Interestingly, the prevalence of these specific methylation abnormalities was significantly correlated with HBx expression in HBV-positive?HCC (Park et al., 2007). HBx therefore seems to be an important player for epigenetic modulation during HBV-related hepatocarcinogenesis.

HCV and DNA methylation

Methylation of some TSGs, such as the SOCS-1, APC, and p15 genes, is reportedly observed at higher prevalence in HCV-positive HCC than in HCV-negative tumors (Yang et al., 2003; Li et al., 2004; Ko et al., 2008). However, HCV itself, which is known as an RNA virus, does not integrate into and directly disturb the host genome. On the contrary, this virus causes long-term inflammation of the liver, usually over the course of a decade, and spontaneous improvement of HCV-related hepatitis is unusual (Mindikoglu and Miller, 2009). In this setting, longstanding exposure to reactive oxygen species (ROS) may promote DNA damage (Loft and Poulsen, 1996; Matsumoto et al., 2003), and so the relationship between ROS and epigenetic alterations in hepatocyte requires clarification.

Lim et al. (2008) demonstrated that ROS induces methylation at the E-cadherin promoter by inducing Snail expression, a transcription factor that down-regulates E-cadherin, through the PI3K/Akt/GSK3ß pathway (Lim et al., 2008). It was shown that Snail induced DNA methylation by recruiting histone deacetylase 1 and DNMT1 at the E-cadherin promoter. The correlation among ROS induction, expression of Snail, methylation, and down-regulation of E-cadherin is
also observed (Lim et al., 2008). Therefore it could be possible that chronic inflammation induced regional hypermethylation at promoters of certain TSGs. Meanwhile Valinluck and Sowers have shown that inflammation-mediated cytosine damage products can mimic 5-methylcytosine in directing enzymic DNA methylation and induce inappropriate methylation within a CpG sequence (Valinluck and Sowers, 2007a,b), which might also lead to silencing of TSGs critical for HCC development.

Furthermore, the deposition of iron, which also induces oxidative stress, is commonly observed in the liver of HCV-positive chronic hepatitis (Barton et al., 1995). It is well known that its depletion by phlebotomy is effective for reducing HCC risk in chronic hepatitis C, suggesting that iron, which may induce ROS in hepatocytes, plays an important role in HCV-related hepatocarcinogenesis (Kato et al., 2001). Interestingly hemochromatosis liver, which carries an iron overload in hepatocytes and a risk for developing HCC, also shows several methylation events of TSGs, such as the RASSF1A, cyclinD2, p16, GSTP1, SOCS-1, and APC.

![Fig. 2. Comparison of mean percentage methylation levels between HCV-positive noncancerous liver and normal liver. Among 21 loci examined, the low-level methylation was detected at 12 loci in noncancerous liver and normal liver, and the levels were compared at these loci between HCV-positive noncancerous liver and normal liver. 6 of 12 loci showed significantly higher levels of methylation in HCV-positive tissue than normal liver (*Statistically significant by the Wilcoxon rank sum test). On the centrally, only 1 locus showed significant difference between HBV-positive noncancerous liver and normal liver, and none of the loci showed a significantly higher methylation level in virus-negative noncancerous liver and normal liver (data not shown).](image)

![Fig. 3. Correlation between methylation levels in normal liver and age. A. Relationship between methylation levels and age at 4 representative promoters of TSGs (HIC-1, APC, CASP8 and p16). The r values of Pearson correlation test and ρ value of Spearman correlation tests are shown. We also found correlation between methylation levels and age at 4 additional loci (GSTP1, SOCS1, RASSF1A and SFRP2: Data not shown). B. Comparison of distribution of methylation level at above-mentioned 8 loci and age in normal liver. For normalization of methylation levels at different loci, we calculated Z-scores. In normal liver, methylation levels was significantly higher in patients ≥ 65 years old compared with those younger than 65 (*P value by the Wilcoxon rank sum test).](image)
with downregulation of these genes (Lehmann et al., 2007). This evidence clearly supports the idea that exposure to iron can be a risk of abnormal methylation and emergence of HCC. Oxidative stress due to longstanding inflammation as well as iron deposition could, therefore, play a role in inducing epigenetic instability in hepatocytes, but the mechanism still remains to be explored in detail.

**Low-level methylation at promoters of the TSGs is detected in aging liver, and HCV enhances the rise of methylation level**

As described above, viral hepatitis and hemochromatosis can be a background of HCC, and alteration of methylation is already found in the liver of these conditions (Kondo et al., 2000; Shen et al., 2002; Lehmann et al., 2007; Nishida et al., 2008). Because HCV is a common etiology of HCC, we analyzed a methylation level at promoters of several TSGs in HCC, corresponding to noncancerous and normal liver, and examined methylation progress in each stage of liver tissues (Nishida et al., 2008). Hierarchal clustering analysis of HCC tissues using the difference in methylation levels between cancerous and noncancerous livers of 19 CpG loci clearly classified HCC into two groups, one with extensive methylation and the other with limited methylation. Interestingly, most HCV-related HCCs came in the extensive methylation group (Nishida et al., 2008). A comparison of methylation levels between HCV-positive noncancerous livers and normal livers reveals that methylation levels are higher in HCV-positive tissue at every loci examined (Fig. 2). This evidence strongly suggests that the presence of HCV promotes an increase of methylation levels at TSGs that is important for the development of HCC.

Some of the reports made showed that the presence of methylation of the *p16* and *SOCS1* genes is associated with age (Li et al., 2004; Ko et al., 2008). We also showed that a low level of methylation was detected at the promoter of the *HIC-1, CASP8, GSTP1, SOCS1, RASSSF1A, p16* and *APC* genes in pathologically and clinically normal liver (Nishida et al., 2008). In the normal liver, the methylation levels at the promoter of these TSGs, where dense methylation was observed in HCC tissue, positively correlated with age. The methylation levels in cases aged 65 or older were significantly higher than in those aged below 65 years (Fig. 3). These data support the hypothesis that the alteration of methylation accrues as a function of age in hepatocyte.

As mentioned above, the relationship among the methylation level of TSGs, presence of HCV, and age suggests the important roles of both HCV and age for development of HCC via epigenetic pathways. Saneto et al. (2008) compared the condition of background livers in HCV-positive old and young patients who developed HCC at the first time (Saneto et al., 2008). It is well known that risk of HCC is clearly correlated with the fibrosing stage of the liver in chronic hepatitis C (Yoshida et al., 1999). However, they demonstrated that the patients in the elderly group had better liver function, and that elderly patients could even develop HCC from chronic hepatitis with mild fibrosis and lack of cirrhosis (Saneto et al., 2008). From this point of view, it could be possible that HCV infection enhances the rise of methylation level at CpG loci, where abnormal methylation emerges in an age-related manner (Nishida et al., 2008). In addition, as described above, dense methylation at these CpG loci, where the age-related methylation takes place, is considered to be unique to HCC (Nishida et al., 2007), suggesting the importance of these TSGs in human hepatocarcinogenesis via epigenetic pathways. Thus both HCV infection and aging act in concert with the accumulation of methylation events in liver tissue, and finally lead to increased risk of liver cancer (Fig. 4).

**Conclusion**

Chronic hepatitis and liver cirrhosis are the high risk fields in developing HCC, and hepatocytes that carry inactivated TSGs due to promoter methylation are already present in these settings. According to previous studies, viral proteins, such as HBx, directly affect the mechanisms that maintain the integrity of methylation status in chronic hepatitis B. In chronic hepatitis C, on the other hand, oxidative stress and age might be more important factors for epigenetic instability; however, further investigation is required. The role of iron deposition could also be important for the epigenetic alteration that is critical for hepatocarcinogenesis.
DNA methylation in human HCC

References


Nishida N., Nishimura T., Ito T., Komeda T., Fukuda Y. and Nakao K.
DNA methylation in human HCC


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