Aberrant Promoter Methylation Profiles of Tumor Suppressor Genes in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most fatal human malignancies, but the molecular mechanisms of hepatocarcinogenesis remain unclear. Although p53 mutations are frequently observed in Asian HCC, it is not a common event in Western HCC.

Recent studies suggest that tumor suppressor genes (TSGs) can also be silenced through epigenetic disruption, such as promoter CpG island methylation, during carcinogenesis. To further understand the molecular mechanism of hepatocarcinogenesis, we have investigated the promoter methylation status of nine TSGs (SOCS-1, GSTP, APC, E-cadherin, RAR-β, p14, p15, p16, and p73) in 51 cases of HCC using methylation-specific polymerase chain reaction. We found that 82% of HCCs had methylation of at least one TSG promoter. The most frequently methylated TSGs in HCC were: SOCS-1 (65%), GSTP (54%), APC (53%), E-cadherin (49%), and p15 (49%). Methylation of SOCS-1, GSTP, APC, E-cadherin, and p15 was significantly more frequent in HCC than non-tumor liver (P < 0.05). Methylation of SOCS-1, GSTP, and p15 was also significantly more frequent in HCC than cirrhotic liver (P < 0.05). Although methylation of one or two genes could be seen in both non-tumor and cirrhotic livers, 53% of the HCC cases had three or more TSG promoters methylated, in comparison to 0% in non-tumor liver and 13% in cirrhosis (P = 0.001). Methylation of SOCS-1, APC, and p15 was more frequently seen in hepatitis C virus-positive HCC than hepatitis C virus/hepatitis B virus-negative HCC. Our data suggest that promoter hypermethylation of TSGs is a common event in HCC and may play an important role in hepatocarcinogenesis. (Am J Pathol 2003, 163:1101–1107)

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and among the most fatal of human neoplasms, but the molecular mechanisms of hepatocarcinogenesis are primarily unknown. Recent genetic studies have indicated that both the p53 and pRb pathways may be involved in hepatocarcinogenesis. It has been shown that point mutations of the p53 tumor suppressor gene (TSG) were frequently seen in HCCs in Chinese and African populations.1–3 However, p53 mutation is not a frequent event in American and European HCCs.4–6 This discrepancy may result from different ethnic backgrounds and/or different etiologies, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infections or toxin exposure. Both HBV and HCV infections are thought to be involved in hepatocarcinogenesis because chronic hepatitis and cirrhosis associated with either HBV or HCV infection precede most HCCs. It has been shown that HBV encodes a X-gene product, HBx, that not only can activate the JAK/STAT signaling pathway,7 but can also interact with p53 and impair the function of wild-type p53.8 The core protein of HCV has also been shown to modulate gene transcription, cell proliferation, and cell death.9 However, the exact mechanisms underlying virus-associated hepatocarcinogenesis are still unclear.

It has recently been proposed that aberrant methylation of CpG islands, which are CpG dinucleotide rich areas located mainly in the promoter regions of many genes, serves as an alternative mechanism for inactivation of TSGs in cancer.10–14 It has been demonstrated that many TSGs can be functionally silenced through such aberrant promoter methylation. Recent studies have revealed that aberrant methylation of TSG promoters is frequently found in several common malignancies, such as colon, lung, breast, and prostate cancer.15–17 Frequent promoter methylation of p16, p15, and GSTP genes has also been observed in the majority of HCCs in Chinese and Japanese populations.18–20 However, the frequency of TSG methylation in Western HCC has not been studied as extensively. To elucidate the molecular mechanisms of hepatocarcinogenesis in this population, we have studied methylation of nine TSGs involved in multiple cellular signaling transduction pathways in 51 cases of HCC using a multiplex methylation-specific polymerase chain reaction (MSP) method, and have compared methylation of these genes in HCC to nonmalignant liver and cirrhotic liver. Our study indicates that TSG promoter methylation is a frequent event in Western HCC.
isolated by digestion with 100 μg/ml of proteinase K and followed by conventional phenol/chloroform (1:1) extraction, consecutive 10-μm sections were cut from paraffin-embedded tissue blocks and mounted for histopathological evaluation using conventional hematoxylin and eosin (H&E) staining. H&E-stained sections also served as a guide for tissue selection for DNA analysis. For DNA extraction, consecutive 10-μm sections were directly collected into sterile Eppendorf tubes. Genomic DNA was stored at −70°C until used. DNA sequences containing promoter regions of SOCS-1, APC, E-cadherin, GSTP, p15, p16, RAR-β, p14, and p73 genes were first amplified in a single PCR run with 30 cycles using flanking primer sets as described previously.22 DNA methylation of CpG islands was then determined by PCR using specific primers for both methylated and unmethylated DNA.22 Two sets of primers were used to amplify each region of interest: one pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 1% polyacrylamide gels. Detection of DNA methylation of all TSGs, except SOCS-1, was informative in all 51 cases of HCC. Because of the large size (210 bp) of the flanking PCR products of the SOCS-1 gene, the informative rate of SOCS-1 was relatively low in formalin-fixed surgical resection specimens of HCC, but 100% in alcohol-fixed cytological specimens. Therefore, only data of promoter methylation of SOCS-1 in 26 HCC cases of cytological specimens was reported in the Results section.

Statistical Analysis
Chi-square or Fisher exact tests, depending on the absolute numbers included in the analysis, were used to analyze the association of concurrent TSG methylation in HCC, cirrhosis, and nontumor liver. Chi-square test or Fisher exact test were also applied to the correlation between TSG methylation profiles and clinicopathological data, such as viral status and gender. A logistic regression analysis was used to analyze the correlation between TSG methylation profiles and the degree of tumor differentiation.

Results
Frequency of Individual TSG Methylation in HCC, Cirrhosis, and Nontumor Liver
The frequency of promoter methylation of nine genes in 51 cases of HCC, 15 cirrhotic livers, and 14 nontumor liver tissues was determined using MSP and is shown in Table 2. Forty-two (82%) cases of HCC had methylation of at least one TSG. The most frequently methylated TSG promoters in HCC were: SOCS-1 (65%), GSTP (54%),

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Table 1. Clinicopathological Data of HCC Cases

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<tr>
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<tr>
<td>Total case number</td>
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<tr>
<td>Resection specimens</td>
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<td>Median age (range)</td>
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<td>Association with cirrhosis</td>
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Materials and Methods

Tumor Samples
Fifty-one cases of HCC, including 25 cases of radical or partial hepatectomy and 26 cases of fine needle aspiration biopsy (FNA), were collected from The Johns Hopkins Hospital pathology archives. Fifteen cases of cirrhotic liver tissues remote from HCC lesions and 14 cases of nontumor liver tissues adjacent to either a hepatic adenoma or a focal nodular hyperplasia were also obtained from surgical resection specimens. The HBV and HCV infection status was tested serologically by enzyme immunoassay before this study and was documented in the Johns Hopkins Hospital pathology archives. Fifteen cases of cirrhotic liver tissues were fixed in buffered formalin for surgical specimens or in ethanol-based fixative for FNA specimens. Therefore, only data of promoter methylation of SOCS-1 in 26 HCC cases of cytological specimens was reported in the Results section.

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Chi-square or Fisher exact tests, depending on the absolute numbers included in the analysis, were used to analyze the association of concurrent TSG methylation in HCC, cirrhosis, and nontumor liver. Chi-square test or Fisher exact test were also applied to the correlation between TSG methylation profiles and clinicopathological data, such as viral status and gender. A logistic regression analysis was used to analyze the correlation between TSG methylation profiles and the degree of tumor differentiation.

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APC (53%), E-cadherin (49%), and p15 (47%). Each of the genes, except APC, has been previously reported to be methylated frequently in HCC. The four other genes studied demonstrated much less frequent methylation: p14 (6%), p73 (6%), RAR-β (12%), and p16 (16%). The frequency of methylation of E-cadherin, GSTP, APC, p15, SOCS-1, p16, and RAR-β promoters was much lower in both cirrhotic and nontumor livers (Table 2). There was no methylation of p14, p15, or p73 in either nontumor or cirrhotic livers. Methylation of SOCS-1, GSTP, APC, E-cadherin, and p15 was significantly more frequent in HCC than in nontumor livers (P = 0.04, P = 0.002, P = 0.01, P = 0.005, and P = 0.001, respectively). Methylation of SOCS-1, GSTP, and p15 was also more frequent in HCC than cirrhosis (P = 0.03, P = 0.01, and P = 0.001, respectively). Methylation was also more frequent in HCC than cirrhosis for APC (53% versus 26%) and E-cadherin (49% versus 20%), but these changes were not statistically significant (P = 0.09 and P = 0.07, respectively). There was no significant difference in the methylation frequency of any tested TSGs between nontumor liver and cirrhosis (P > 0.05).

Cumulative Methylation Patterns in HCC, Cirrhosis, and Nontumor Liver

Methylation status of eight TSGs (GSTP, APC, p15, p14, p73, p16, and RAR-β) was included in the analysis of cumulative methylation patterns. Methylation of SOCS-1 was not included because of the low informative rate in formalin-fixed surgical specimens (see Material and Methods). Methylation of multiple TSGs was commonly seen in HCC (Figure 1). Fifty-three percent of the HCC cases had three or more TSG promoters methylated in comparison to 0% in nontumor livers and 13% in cirrhotic livers (P = 0.001). Methylation of four or more TSG promoters was only observed in HCC cases (Figure 1 and Table 3). Methylation of two or three TSGs was not specifically associated with either HCC or cirrhosis. Single TSG methylation was more frequently seen in nontumor liver (36%) than in HCC (5%) (P = 0.04). Eighteen percent of HCC cases showed no methylation of any TSGs tested, in comparison to 47% in cirrhosis and 57% in nontumor liver.

Subgrouping HCC Based on TSG Methylation Profiles

Based on the methylation profiles shown in Figure 1, our 51 cases of HCC can be divided into three groups: nonmethylation group, scattered methylation group, and dense methylation group. Nine cases (18%) of HCC showed no methylation of any nine TSGs tested; the scattered methylation group contained 24 cases (45%), which showed methylation of one to three TSG promoters; the dense methylation group included 14 cases (28%), which had methylation of more than four TSG promoters. The most frequently methylated TSGs in the dense methylation group were APC, E-cadherin, SOCS-1, p15, and GSTP. Forty-two cases (82%) of HCC showed TSG methylation of either APC or E-cadherin or both. Interestingly, 76% of the methylated HCC cases exhibited a mutually exclusive methylation pattern between APC and E-cadherin.

Correlation between TSG Methylation Profile and Clinicopathological Data

Correlation of HCC methylation profiles to clinicopathological data revealed several interesting associations. Overall, TSG methylation was more common among HBV-positive or HCV-positive HCC than HBV/HCV-negative HCC (Figure 2). Methylation of SOCS-1, APC, and p15 was associated with HCV-positive HCC relative to HBV/HCV-negative HCC (P = 0.004, P = 0.006, and P = 0.03, respectively). Although not statistically significant, there seems to be a trend toward methylation of p16, p73, and RAR-β in HBV-positive HCC (Figure 2). There was no significant difference between HCV-positive and HBV-positive HCC in the frequency of methylation of any tested TSGs (P > 0.05). There was no association between the frequency of TSG promoter methylation and gender or the histopathological differentiation of the tumor (P > 0.05).

Discussion

HCC, the major type of primary liver cancer, is one of the most common cancers worldwide and a leading cause of...
Figure 1. Methylation profiles of nine TSGs in HCC, cirrhosis, and nontumor livers. Cases were labeled as N1 to N14 for nontumor liver; C1 to C15 for cirrhosis; and HC1 to HC51 for HCC. A filled box indicates that promoter methylation was detected by MSP; an open box indicates that no methylation was detected; • indicates a noninformative specimen.
death in many countries. Although many of the major viral
and environmental risk factors for HCC development
have been unraveled, the genetic and epigenetic
pathways leading to malignant transformation of liver cells
have remained obscure. The heterogeneous geographi-
cal distribution of HCC further complicates efforts to iden-
tify the common genetic and epigenetic events respon-
sible for hepatocarcinogenesis. One recent study
indicated that expression of two crucial DNA methyltrans-
ferases, DNMT1 and DNMT3a, was significantly higher in
HCC compared to nontumor liver,23 suggesting that epi-
genetic mechanisms may be important. It will be impor-
tant to identify the genes silenced through promoter CpG
island hypermethylation during hepatocarcinogenesis.

We have studied methylation profiles of nine TSGs
involved in several signal transduction pathways in 51
cases of Western HCC. We found that methylation of TSG
promoters was a frequent event in HCC, as 82% cases of
HCC had at least one TSG promoter methylated. Among
nine TSGs tested, the most frequently methylated were
SOCS-1, GSTP, APC, E-cadherin, and p15. In comparison
to nontumor liver tissues, methylation of these five TSGs
was specifically associated with HCC. Methylation of
SOCS-1, GSTP, and p15 was also significantly more
frequent in HCC than in cirrhotic liver. Other recent studies
have also shown frequent methylation of SOCS-1, p15,
and E-cadherin genes in Chinese and Japanese
HCC.18,19,25 It seems that silencing of TSGs through
promoter CpG island methylation may play an important role
during hepatocarcinogenesis.

It should be noted that our comparison tissues were
not normal liver, but rather cirrhotic liver from patients
with HCC and nontumor liver from patients with benign
liver abnormalities, such as adenomas. Previous studies
have found no methylation in normal liver from patients
without disease.19 However, to assess the utility of meth-
ylation profiling in diagnostic pathology, it is important to
study hepatic tissue surrounding lesions. Our data sug-
gest that gene methylation profiling may be clinically
useful in distinguishing HCC from nonmalignant liver.

The most striking methylation pattern in HCC is the
concurrent methylation of multiple TSGs. Although single
or double TSG methylation can be seen in nontumor and
cirrhotic liver tissue, the majority of our HCC cases har-
bored three or more methylated TSGs. In fact, methyl-
ation of four or more TSG promoters was only seen in
HCC. Such multiple TSG methylation patterns were also
observed in HCC by others using a set of genes involved
in cell-cycle regulation.26 These results emphasize the
importance of analyzing multiple TSGs rather than a sin-
gle gene. Also, this suggests that disruption of multiple
signal transduction pathways is biologically required dur-
ing hepatocarcinogenesis.

Previous studies have suggested that the Wnt signal
transduction pathway may be important in hepatocarcin-
genesis. In this pathway, the oncoprotein β-catenin
normally interacts with E-cadherin at the plasma mem-
brane. Its turnover is mediated by phosphorylation and
ubiquitin-mediated degradation via the APC-Axin,GSK3b
complex.27–31 In many human neoplasms, this normal
regulation is disrupted, resulting in the cytoplasmic and
nuclear accumulation of β-catenin. Such an accumula-
tion of β-catenin subsequently turns on the transcription
factor TCF-1, which in turn enhances the expression of a
set of genes involved in cell-cycle control and cell prolif-
eration. This disregulation may result from mutations in
APC, E-cadherin, Axin, or β-catenin genes. Immunohisto-
chemical analyses of HCCs indicate abnormal nuclear
accumulation of β-catenin in many cases.32–34 However,
mutations in the APC, β-catenin, or Axin genes appear to
be uncommon in HCC,35–42 suggesting that other gene-
silencing mechanisms may be important. We found meth-
ylation of APC, E-cadherin, or both in 82% of HCCs.
Interestingly, ~76% of methylated HCC cases showed
mutually exclusive APC and E-cadherin methylation, sug-
gestting that inactivation of either APC or E-cadherin may
lead to the accumulation of β-catenin in HCC.

Activation of the JAK/STAT pathway has also been
implicated in hepatocarcinogenesis. Suppressor of cyto-
kine signaling (SOCS-1, also known as JAB and SSI-1) is
a protein that suppresses the JAK/STAT pathway by ren-
dering cells unresponsive to cytokine stimulation. One
recent study has indicated that the SOCS-1 gene was

Table 3. Cumulative Methylation Patterns of Eight TSG Promoters in HCC and Control Tissues

<table>
<thead>
<tr>
<th>TSG methylation</th>
<th>HCC (n = 51)</th>
<th>Cirrhosis (n = 15)</th>
<th>Nontumor (n = 14)</th>
</tr>
</thead>
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<tr>
<td>0 gene</td>
<td>18 (9)</td>
<td>47 (7)</td>
<td>57 (8)</td>
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<tr>
<td>1 gene</td>
<td>6 (3)</td>
<td>27 (4)</td>
<td>36 (5)</td>
</tr>
<tr>
<td>2 genes</td>
<td>23 (12)</td>
<td>13 (2)</td>
<td>7 (1)</td>
</tr>
<tr>
<td>3 genes</td>
<td>25 (13)</td>
<td>13 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>≥4 genes</td>
<td>28 (14)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Figure 2. Association of TSG promoter methylation with the viral status of
HCC. The percentage of HCC cases with TSG methylation is indicated for
groups of patients that are positive for HBV, HCV, and neither HBV nor HCV.
methylated in the majority of Chinese HCC cases.\textsuperscript{19} The tumor suppressor function of SOCS-1 was further demonstrated by suppression of cell growth and colony formation after restoration of SOCS-1 into HCC cell lines.\textsuperscript{19} Using a multiplex MSP approach, we have analyzed the methylation status of SOCS-1 in 26 FNA specimens of Western HCC. Consistent with previous studies, we have found that the SOCS-1 gene is frequently methylated in Western HCC. Methylation of SOCS-1 was more frequent in HBV-positive and HCV-positive HCC than HBV/HCV-negative HCC. It has been shown that activation of the JAK/STAT pathway by phosphorylation of STAT3 was observed both in cells constitutively expressing HBx and in cells that SOCS-1 was inactivated epigenetically.\textsuperscript{7,19} Whether methylation of SOCS-1 mediates HBx-induced activation of JAK/STAT signaling pathway in HBV-positive HCC needs to be further characterized. Our study contributes to the thought that an imbalance of cytokine stimulation initiated by viral infection and subsequent silencing of SOCS-1 may play a role in hepatocarcinogenesis.

Another recent study has indicated that the p16 gene is frequently inactivated in HCC through homozygous deletion or promoter hypermethylation.\textsuperscript{43} However, the reported frequency of p16 promoter methylation varies greatly in other reports. A high frequency of p16 methylation has been reported in Asian HCCs,\textsuperscript{25,44} particularly those associated with viral infections. Recent studies from Korean and Taiwan populations showed a relatively low frequency of p16 promoter methylation (0 to 35%).\textsuperscript{43,45} In our samples, we found that only 16% of Western HCC contained p16 promoter methylation. The discrepancies between these studies on p16 methylation may be because of geographic/ethnic differences. Alternatively, it could also be because of the different conditions used for MSP analysis.

The p15 gene, which encodes another cyclin-dependant kinase inhibitor, is aberrantly methylated in several human neoplasms, especially hematopoietic malignancies.\textsuperscript{17} It has been reported that p15 promoter methylation is present in 64% of Chinese HCC and 25% of HCC patients’ plasma and serum samples.\textsuperscript{18} In our study, we found that 47% of HCC harbored p15 promoter methylation. Because methylation of p15 is not commonly seen in other solid carcinomas, except brain tumors, we examined the association of p15 methylation with viral infections in HCC. Interestingly, we found that p15 promoter methylation, along with SOCS-1 and APC, is more frequently seen in HBV-positive and HCV-positive HCC than HBV/HCV-negative HCC. Our study suggests that silencing of p15 through promoter methylation may be involved in virus-induced hepatocarcinogenesis. However, the absence of p15 promoter methylation in cirrhosis in our study argues against the hypothesis that disruption of p15 may be an early event of hepatocarcinogenesis.\textsuperscript{26}

In summary, we have shown that silencing of TSGs through promoter methylation is a frequent event in HCC. Epigenetic abnormalities in HCC not only play important roles during hepatocarcinogenesis, but also might be useful in the diagnosis of HCC.


