Aberrant Promoter Methylation Profiles of Tumor Suppressor Genes in Hepatocellular Carcinoma

Bin Yang,* Mingzhou Guo,[†] James G. Herman,[†] and Douglas P. Clark*

From the Department of Pathology* and the Sidney Kimmel Comprehensive Cancer Center,[†] The Johns Hopkins Medical Institutions, Baltimore, Maryland

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 more frequent in HCC than in nontumor 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 nontumor and cirrhotic livers, 53% of the HCC cases had three or more TSG promoters methylated, in comparison to 0% in nontumor 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 cancers.^{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.

B. Y. and M. G. contributed equally to this study.

Accepted for publication May 9, 2003.

Address reprint requests to Douglas P. Clark, M.D., Department of Pathology, Pathology Building, Rm 406, Johns Hopkins Medical Institutions, 600 North Wolfe St., Baltimore, MD 21287. E-mail: dclark@jhmi.edu.

Table 1. Clinicopathological Data of HCC Cases

Clinicopathologic characteristics	Number of cases
Total case number	51
FNA specimens	26 (51%)
Resection specimens	25 (49%)
Median age (range)	58 (27–81)
Sex	
Male	30 (59%)
Female	21 (41%)
Association with cirrhosis	
With cirrhosis	36 (71%)
Without cirrhosis	15 (29%)
Viral status	
HBV-positive	9 (18%)
HCV-positive	18 (36%)
Non-HBV, non-HCV	24 (46%)
Tumor differentiation	
Well	25 (49%)
Moderately	18 (35%)
Poorly	8 (16%)

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 through the pathology laboratory database. This study was reviewed by the Johns Hopkins Institutional Review Board and found to be exempt. No chemotherapy or radiation therapy was instituted before tumor excision or FNA procedure. The case distribution in different clinicopathological categories is summarized in Table 1. The tissue was fixed in buffered formalin for surgical specimens or in ethanol-based fixative for FNA specimens. Consecutive 4- μ m 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- μ m sections were directly collected into sterile Eppendorff tubes. Genomic DNA was isolated by digestion with 100 μ g/ml of proteinase K and followed by conventional phenol/chloroform (1:1) extraction and ethanol precipitation.

Bisulfite Modification and MSP

Bisulfite modification and MSP were conducted based on the principle that bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified. Thus, after bisulfite conversion, methylated and unmethylated DNA sequences can be distinguished by sequence-spe-

cific primers. DNA was treated with sodium bisulfite as described previously.²¹ Briefly, 1 μ g of genomic DNA was denatured by incubation with 0.2 mol/L of NaOH for 10 minutes at 37°C. Aliquots of 10 mmol/L of hydroquinone (30 μ l; Sigma Chemical Co., St. Louis, MO) and 3 mol/L of sodium bisulfite (pH 5.0, 520 μ l; Sigma Chemical Co.) were added and the solution was incubated at 50°C for 16 hours. Treated DNA was purified by use of the Wizard DNA purification system (Promega Corp., Madison, WI), desulfonated with 0.3 mol/L of NaOH, precipitated with ethanol, and resuspended in water. Modified 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%),

Table 2. Frequency of TSG Promoter Methylation in HCC and Control Tissues

Gene	HCC (n = 51) % (n)	Cirrhosis (n = 15) % (n)	Normal (n = 14) % (n)
SOCS-1	65 (17)*	7 (1)	7 (1)
GSTP	54 (28)	13 (2)	7 (1)
APC	53 (27)	27 (4)	14 (2)
E-cadherin	49 (25)	20 (3)	7 (1)
p15	47 (24)	0 (0)	0 (0)
p16	16 (8)	13 (2)	7 (1)
RAR-beta	12 (6)	13 (2)	7 (1)
p14	6 (3)	0 (0)	0 (0)
p73	6 (3)	0 (0)	0 (0)

*Total number of cases for SOCS-1 analysis is 26.

APC (53%), *E-cadherin* (49%), and *p15* (47%). Each of the genes, except *APC*, has been previously reported to be methylated frequently in HCC.^{18–20,23} 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 frequently seen 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 HCCs 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 (54%), 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

CASE #	APC	E-Cad	SOCS-1	p15	GSTP	p16	RAR-b	p14	p73
N1									
N2									
N3									
N4									
N5									
N6									
N7									
N8									
N9									
N10									
N11									
N12									
N13									
N14									
C1									
C2									
C3									
C4									
C5									
C6									
C7									
C8									
C9									
C10									
C11									
C12									
C13									
C14									
C15									
HC1			x						
HC2									
HC3									
HC4			x						
HC5									
HC6									
HC7									
HC8									
HC9									
HC10									
HC11			x						
HC12			x						
HC13			x						
HC14			x						
HC15			x						
HC16			x						
HC17			x						
HC18									
HC19									
HC20									
HC21			x						
HC22			x						
HC23			x						
HC24			x						
HC25			x						
HC26			x						
HC27			x						
HC28			x						
HC29			x						
HC30			x						
HC31			x						
HC32			x						
HC33									
HC34			x						
HC35									
HC36			x						
HC37									
HC38									
HC39									
HC40									
HC41									
HC42			x						
HC43			x						
HC44									
HC45									
HC46									
HC47									
HC48									
HC49									
HC50									
HC51									

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; **x** indicates a noninformative specimen.

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

TSG methylation	HCC (n = 51) % (n)	Cirrhosis (n = 15) % (n)	Nontumor (n = 14) % (n)
0 gene	18 (9)	47 (7)	57 (8)
1 gene	6 (3)	27 (4)	36 (5)
2 genes	23 (12)	13 (2)	7 (1)
3 genes	25 (13)	13 (2)	0 (0)
≥4 genes	28 (14)	0 (0)	0 (0)

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 geographical distribution of HCC further complicates efforts to identify the common genetic and epigenetic events responsible for hepatocarcinogenesis. One recent study indicated that expression of two crucial DNA methyltransferases, DNMT1 and DNMT3a, was significantly higher in HCC compared to nontumor liver,²⁴ suggesting that epigenetic mechanisms may be important. It will be important 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 methylation profiling in diagnostic pathology, it is important to study hepatic tissue surrounding lesions. Our data suggest 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 harbored three or more methylated TSGs. In fact, methylation 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 single gene. Also, this suggests that disruption of multiple signal transduction pathways is biologically required during hepatocarcinogenesis.

Previous studies have suggested that the Wnt signal transduction pathway may be important in hepatocarcinogenesis. In this pathway, the oncoprotein β -catenin normally interacts with E-cadherin at the plasma membrane. 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 accumulation 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 proliferation. This dysregulation may result from mutations in *APC*, *E-cadherin*, *Axin*, or β -catenin genes. Immunohistochemical 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 methylation of *APC*, *E-cadherin*, or both in 82% of HCCs. Interestingly, ~76% of methylated HCC cases showed mutually exclusive *APC* and *E-cadherin* methylation, suggesting 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 cytokine signaling (*SOCS-1*, also known as JAB and SSI-1) is a protein that suppresses the JAK/STAT pathway by rendering cells unresponsive to cytokine stimulation. One recent study has indicated that the *SOCS-1* gene was

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.

methyated in the majority of Chinese HCC cases.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.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.^{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.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,^{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%).^{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-dependent kinase inhibitor, is aberrantly methylated in several human neoplasms, especially hematopoietic malignancies.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.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.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.

References

1. Bressac B, Kew M, Wands J, Ozturk M: Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 1991, 350:429–431
2. Hollstein MC, Wild CP, Bleicher F, Chutimataewin S, Harris CC, Srivatanakul P, Montesano R: p53 mutations and aflatoxin B1 exposure in hepatocellular carcinoma patients from Thailand. *Int J Cancer* 1993, 53:51–55
3. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC: Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 1991, 350:427–428
4. Boix-Ferrero J, Pellin A, Blesa R, Adrados M, Llombart-Bosch A: Absence of p53 gene mutations in hepatocarcinomas from a Mediterranean area of Spain. A study of 129 archival tumour samples. *Virchows Arch* 1999, 434:497–501
5. Challen C, Lunec J, Warren W, Collier J, Bassendine MF: Analysis of the p53 tumor-suppressor gene in hepatocellular carcinomas from Britain. *Hepatology* 1992, 16:1362–1366
6. Kazachkov Y, Khaoustov V, Yoffe B, Solomon H, Klintmalm GB, Tabor E: p53 abnormalities in hepatocellular carcinoma from United States patients: analysis of all 11 exons. *Carcinogenesis* 1996, 17:2207–2212
7. Lee YH, Yun Y: HBx protein of hepatitis B virus activates Jak1-STAT signaling. *J Biol Chem* 1998, 273:25510–25515
8. Kew MC: Increasing evidence that hepatitis B virus X gene protein and p53 protein may interact in the pathogenesis of hepatocellular carcinoma. *Hepatology* 1997, 25:1037–1038
9. Forrester K, Lupold SE, Ott VL, Chay CH, Band V, Wang XW, Harris CC: Effects of p53 mutants on wild-type p53-mediated transactivation are cell type dependent. *Oncogene* 1995, 10:2103–2111
10. Herman JG, Baylin SB: Promoter-region hypermethylation and gene silencing in human cancer. *Curr Top Microbiol Immunol* 2000, 249: 35–54
11. Herman JG, Jen J, Merlo A, Baylin SB: Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res* 1996, 56:722–727
12. Baylin SB, Belinsky SA, Herman JG: Aberrant methylation of gene promoters in cancer—concepts, misconceptions, and promise. *J Natl Cancer Inst* 2000, 92:1460–1461
13. Baylin SB, Herman JG: DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 2000, 16:168–174
14. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP: Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998, 72:141–196
15. Esteller M, Corn PG, Baylin SB, Herman JG: A gene hypermethylation profile of human cancer. *Cancer Res* 2001, 61:3225–3229
16. Herman JG: p16(INK4): involvement early and often in gastrointestinal malignancies. *Gastroenterology* 1999, 116:483–485
17. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB: Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. *Cancer Res* 1997, 57:837–841
18. Wong IH, Lo YM, Yeo W, Lau WY, Johnson PJ: Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. *Clin Cancer Res* 2000, 6:3516–3521
19. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC, Herman JG: SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* 2001, 28:29–35
20. Tchou JC, Lin X, Freije D, Isaacs WB, Brooks JD, Rashid A, De Marzo AM, Kanai Y, Hirohashi S, Nelson WG: GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. *Int J Oncol* 2000, 16:663–676
21. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, 93:9821–9826
22. House M, Guo M, Iacobuzio-Donahue C, Herman J: Molecular progression of promoter methylation in intraductal papillary mucinous neoplasms (IPMN) of the pancreas. *Carcinogenesis* 2003, 24:193–198
23. Matsumura T, Makino R, Mitamura K: Frequent down-regulation of E-cadherin by genetic and epigenetic changes in the malignant

- progression of hepatocellular carcinomas. *Clin Cancer Res* 2001, 7:594–599
24. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S: Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. *Hepatology* 2001, 33:561–568
25. Matsuda Y, Ichida T, Matsuzawa J, Sugimura K, Asakura H: p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology* 1999, 116:394–400
26. Roncalli M, Bianchi P, Bruni B, Laghi L, Destro A, Di Gioia S, Gennari L, Tommasini M, Malesci A, Coggi G: Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. *Hepatology* 2002, 36:427–432
27. Barth AI, Nathke IS, Nelson WJ: Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. *Curr Opin Cell Biol* 1997, 9:683–690
28. Hajra KM, Fearon ER: Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer* 2002, 34:255–268
29. Ilyas M, Tomlinson IP: The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. *J Pathol* 1997, 182:128–137
30. Novak A, Dedhar S: Signaling through beta-catenin and Lef/Tcf. *Cell Mol Life Sci* 1999, 56:523–537
31. Behrens J: Control of beta-catenin signaling in tumor development. *Ann NY Acad Sci* 2000, 910:21–33
32. Nhieu JT, Renard CA, Wei Y, Cherqui D, Zafrani ES, Buendia MA: Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am J Pathol* 1999, 155:703–710
33. Suzuki T, Yano H, Nakashima Y, Nakashima O, Kojiro M: Beta-catenin expression in hepatocellular carcinoma: a possible participation of beta-catenin in the dedifferentiation process. *J Gastroenterol Hepatol* 2002, 17:994–1000
34. Wong CM, Fan ST, Ng IO: Beta-catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. *Cancer* 2001, 92:136–145
35. Chen TC, Hsieh LL, Ng KF, Jeng LB, Chen MF: Absence of APC gene mutation in the mutation cluster region in hepatocellular carcinoma. *Cancer Lett* 1998, 134:23–28
36. de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C: Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* 1998, 95:8847–8851
37. Huang H, Fujii H, Sankila A, Mahler-Araujo BM, Matsuda M, Cathomas G, Ohgaki H: Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. *Am J Pathol* 1999, 155:1795–1801
38. Legoix P, Bluteau O, Bayer J, Perret C, Balabaud C, Belghiti J, Franco D, Thomas G, Laurent-Puig P, Zucman-Rossi J: Beta-catenin mutations in hepatocellular carcinoma correlate with a low rate of loss of heterozygosity. *Oncogene* 1999, 18:4044–4046
39. Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY: Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. *Am J Pathol* 2000, 157:763–770
40. Cui J, Zhou X, Liu Y, Tang Z: Mutation and overexpression of the beta-catenin gene may play an important role in primary hepatocellular carcinoma among Chinese people. *J Cancer Res Clin Oncol* 2001, 127:577–581
41. Fujie H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, Makuuchi M, Kimura S, Koike K: Frequent beta-catenin aberration in human hepatocellular carcinoma. *Hepatol Res* 2001, 20:39–51
42. Clevers H: Axin and hepatocellular carcinomas. *Nat Genet* 2000, 24:206–208
43. Jin M, Piao Z, Kim NG, Park C, Shin EC, Park JH, Jung HJ, Kim CG, Kim H: p16 is a major inactivation target in hepatocellular carcinoma. *Cancer* 2000, 89:60–68
44. Gong Y, Deng S, Zhang M, Wang G, Minuk GY, Burczynski F: A cyclin-dependent kinase inhibitor (p21(WAF1/CIP1)) affects thymidine incorporation in human liver cancer cells. *Br J Cancer* 2002, 86:625–629
45. Lin YW, Chen CH, Huang GT, Lee PH, Wang JT, Chen DS, Lu FJ, Sheu JC: Infrequent mutations and no methylation of CDKN2A (P16/MTS1) and CDKN2B (p15/MTS2) in hepatocellular carcinoma in Taiwan. *Eur J Cancer* 1998, 34:1789–1795