Correlative Analysis of DNA Methyltransferase Expression and Promoter Hypermethylation of Tumor Suppressor Genes in Hepatocellular Carcinoma

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Abstract. *Background: Promoter hypermethylation of tumor suppressor genes (TSGs) is a common phenomenon in liver carcinogenesis, although the controlling mechanism remains unclear. Materials and Methods: The mRNA expression of DNA methyltransferases (DNMT1, 2, 3a, 3b and splice variants 3b3 and 3b4) and methyl-CpG binding protein (MBD2) were quantitated in 51 liver specimens (41 hepatocellular carcinoma (HCC), 1 cholangiocarcinoma, 1 macroregenerative nodule and 8 HCC cell lines) and the expression levels were correlated with the promoter methylation status of 14 TSG, including APC, RASSF1A, SOCS-1, GSTP1, E-cadherin, p14, p15, p16, DAP-kinase, HIC1, MGMT, TIMP-3, hMLH1 and HLTF. Results: Up-regulations of DNMT1, DNMT2, DNMT3a, DNMT3b4 and MBD2 were suggested in more than 40% of the cases. In particular, the overexpression of DNMT3b and the splice variant DNMT3b3 were identified in as many as 91% and 97.8% of cases, respectively. Using methylation-specific PCR, the most frequently methylated TSGs were APC (90.2%), RASSF1A (86.3%), SOC-1 (74.5%), GSTP1 (72.5%), E-cadherin (64.7%) and p16 (58%). Statistical correlations did not suggest the DNMTs and MBD2 expressions in association with cumulative methylated index in individual cases, but increased expression levels of DNMT2 and DNMT3a showed significant association with the hypermethylation of GSTP1 ($p=0.014$) and DAP-kinase ($p=0.006$), respectively. Furthermore, the analysis with clinicopathological data indicated aberrant DAP-kinase methylation was significantly associated with advanced stage T3/T4 HCC tumors ($p=0.032$) and that p16 hypermethylation was distinct more prevalent in tumors arising from a cirrhotic background ($p=0.005$). Conclusion: Our study indicated that DNMT deregulations are common in liver cancers and the existence of a relationship between DNMT2 and DNMT3a overexpression and promoter hypermethylation of candidate tumor suppressor genes in HCC.*

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Key Words: Hepatocellular carcinoma, promoter hypermethylation, DNA methyltransferases, tumor suppressor genes.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is a malignancy associated with a high incidence of mortality and morbidity (1, 2). Epidemiological studies have indicated viral hepatitis infections as the main etiologic risk factor, where viral hepatitis B-induced chronic hepatitis and liver cirrhosis are considered strong predisposing factors towards tumor development (3). To date, information on the molecular pathways underlining hepatocarcinogenesis is limited, although recent advances in molecular biology have led to a rapid progress in understanding the molecular events involved. The inactivation of defined tumor suppressor genes (TSGs) is well recognized as a causative molecular defect in the development of neoplastic cells. In HCC, growing evidence has indicated that a number of critical TSGs undergo CpG island hypermethylation, which can lead to the transcriptional inactivation and loss of gene function. This, in turn, suggests CpG island hypermethylation as an important molecular mechanism in liver carcinogenesis. Aberrant promoter methylation has been further postulated to be due to increased expression and activity of DNA methyltransferases (DNMTs), which catalyze the transfer of methyl groups from S-adenosylmethionine to cytosines in CpG dinucleotides. Indeed, elevated expressions of the maintenance DNA methyltransferase ware demonstrated in a number of human carcinomas including colon, lung and liver (4-6).

To clarify the role of DNMTs in the aberrant promoter hypermethylation of TSGs in HCC, the expression levels of a number of DNMTs in primary HCC tumors and cell lines

Table I. *Primer sequences and PCR conditions for MSP analysis.*

Gene		Primer sequence (Sense)	Primer sequence (Antisense)	Annealing temp. (˚C)
APC	M	5'-TATTGCGGAGTGCGGGTC-3'	5'-TCGACGAACTCCCGACGA-3'	60
	U	5'-GTGTTTTATTGTGGAGTGTGGGTT-3'	5'-CCAATCAACAAACTCCCAACAA-3'	60
RASSF1A	M	5'-GGGTTTTGCGAGAGCGCG-3'	5'-GCTAACAAACGCGAACCG-3'	60
	U	5'-GGTTTTGTGAGAGTGTGTTTAG-3'	5'-CACTAACAAACACAAACCAAAC-3'	60
SOCS-1	M	5'-TTCGCGTGTATTTTTAGGTCGGTC-3'	5'-CGACACAACTCCTACAACGACCG-3'	60
	U	5'-TTATGAGTATTTGTGTGTATTTTTAGGTGGGTT-3'	5'-CACTAACAACACAACTCCTACAACAACCA-3'	60
GSTP1	M	5'-TTCGGGGTGTAGCGGTCGTC-3'	5'-GCCCCAATACTAAATCACGACG-3'	55
	U	5'-GATGTTTGGGGTGTAGTGGTTGTT-3'	5'-CCACCCCAATACTAAATCACAACA-3'	55
E-cadherin	M	5'-TTAGGTTAGAGGGTTATCGCGT-3'	5'-TAACTAAAAATTCACCTACCGAC-3'	57
	U	5'-TAATTTTAGGTTAGAGGGTTATTGT-3'	5'-CACAACCAATCAACAACACA-3'	53
p16	M	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	5'-GACCCCGAACCGCGACCGTAA-3'	65
	U	5'-TTATTAGAGGGTGGGGTGGATTGT-3'	5'-CAACCCCAAACCACAACCATAA-3'	60
DAP-kinase	M	5'-GGATAGTCGGATCGAGTTAACGTC-3'	5'-CCCTCCCAAACGCCGA-3'	60
	U	5'-GGAGGATAGTTGGATTGAGTTAATGTT-3'	5'-CAAATCCCTCCCAAACACCAA-3'	60
HIC1	M	5'-TTCGGGTTAGGGTCGTAGTC-3'	5'-CTAACCGAAAACTATCAACCCTCG-3'	60
p15	M	5'-GCGTTCGTATTTTGCGGTT-3'	5'-CGTACAATAACCGAACGACCGA-3'	60
	U	5'-TGTGATGTGTTTGTATTTTGTGGTT-3'	5'-CCATACAATAACCAAACAACCAA-3'	60
MGMT	M	5'-TTTCGACGTTCGTAGGTTTTCGC-3'	5'-GCACTCTTCCGAAAACGAAACG-3'	66
	U	5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3'	5'-AACTCCACACTCTTCCAAAAACAAAACA-3'	66
TIMP-3	M	5'-CGTTTCGTTATTTTTTGTTTTCGGTTTC-3'	5'-CCGAAAACCCCGCCTCG-3'	59
	U	5'-TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT-3'	5'-CCCCCAAAAACCCCACCTCA-3'	59
hMLH1	M	5'-ACGTAGACGTTTTATTAGGGTCGC-3'	5'-CCTCATCGTAACTACCCGCG-3'	59
	U	5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3'	5'-ACCACCTCATCATAACTACCCACA-3'	59
p14	M	5'-GTGTTAAAGGGCGGCGTAGC-3'	5'-AAAACCCTCACTCGCGACGA-3'	64
	U	5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3'	5'-CACAAAAACCCTCACTCACAACAA-3'	64
HLTF	M	5'-TGGGGTTTCGTGGTTTTTTCGCGC-3'	5'-CCGCGAATCCAATCAAACGTCGACG-3'	66
	U	5'-ATTTTTGGGGTTTTGTGGTTTTTTTGTGT-3'	5'-ATCACCACAAATCCAATCAAACATCAACA-3'	66

M, methylated primers; U, unmethylated primers.

were examined and were correlated with the findings of the promoter methylation status of 14 TSGs commonly involved in cellular regulatory pathways. The expressions of the DNMTs, DNMT1, DNMT2, DNMT3a, DNMT3b and the splice variants DNMT3b3 and DNMT3b4, as well as of the methyl-CpG binding protein MBD2 were examined, since these enzymes have a reported role in the maintenance of the genome methylation status integrity and have been implicated in the transcriptional regulatory changes of human cancer. Moreover, our clinicopathological correlative analysis was extended to include HCC tumors arising from liver cirrhosis and the non-cirrhotic background of chronic hepatitis. Our findings suggest a relationship between deregulated DNMT expressions and the induction of DNA hypermethylation of CpG island-containing TSGs in HCC.

Materials and Methods

Patients and cell lines. Tumorous liver tissues were collected from 43 patients (aged 24-76; 72% male) who underwent curative surgery at the Prince of Wales Hospital, Hong Kong SAR. Histological examination confirmed the diagnosis of hepatocellular carcinoma in 41 cases (H1-H41) and one case each of cholangiocarcinoma (CC1) and macroregenerative nodule (MN1). Identifiable cirrhosis in the non-tumorous liver was also indicated in 29 cases (67.4%), while histological signs of chronic hepatitis were suggested in the remaining 14 cases. Serological analysis indicated patients to be the predominantly chronic carriers of viral hepatitis B (HBV) (95.3%). The disease staging of HCC tumors according to the American Joint Committee on Cancer (AJCC) criteria classified 26 cases as T1 or T2 (also considered as early stages herein) and 15 cases as T3 or T4 (advanced stages).

Eight HCC cell lines were used in this study, including five (HKCI-1, 2, 4, 6 and C3) established from local tissue specimens (7, 8) and three (PLC/PRF/5, Hep3B and HepG2) purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in a humidified chamber in a 5% CO_2 atmosphere at 37˚C and propagated as previously reported (7) or as recommended by the ATCC.

Bisulfite modification and methylation-specific PCR (MSP). DNA was extracted from frozen tissues and cultured cells by the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Purified DNA samples were chemically modified by sodium bisulfite as described previously (9). Chemical modification, converting all unmethylated cytosines to uracils and leaving methylcytosines unaltered, was performed by treating 1 mg DNA with the CpGenome DNA modification kit (Chemicon, Temecula, CA, USA). The bisulfite-modified DNA

Table II. *Primer and probe sequences for quantitative RT-PCR of DNMTs and MBD2.*

Gene		Primer and probe	Product size
DNMT1	sense:	5'-GGTTCTTCCTCCTGGAGAATGTC-3'	141 bp
	antisense:	5'-GGGCCACGCCGTACTG-3'	
	probe:	5'-6-FAM-CCTTCAAGCGCTCCATGGTCCTGAA-TAMRA-3'	
DNMT2	sense:	5'-AAGCTGTAAGCCAGCCCATATAC-3'	148 bp
	antisense:	5'-TCAGCAGTGAACAGAACCTACATG-3'	
	probe:	5'-6-FAM-ACATTTTTCATATGTGTTCAGACAGAGCCTATTACAAGTC-TAMRA-3'	
DNMT3a	sense:	5'-CAATGACCTCTCCATCGTCAAC-3'	89 bp
	antisense:	5'-CATGCAGGAGGCGGTAGAA-3'	
	probe:	5'-6-FAM-AGCCGGCCAGTGCCCTCGTAG-TAMRA-3'	
DNMT3b	sense:	5'-CCATGAAGGTTGGCGACAA-3'	69 bp
	antisense:	5'-TGGCATCAATCATCACTGGATT-3'	
	probe:	5'-6-FAM-CACTCCAGGAACCGTGAGATGTCCCT-TAMRA-3'	
DNMT3b3	sense:	5'-GATGAACAGGATCTTTGGCTT-3'	163 bp
	antisense:	5'-GCCTGGCTGGAACTATTCACA-3'	
	probe:	5'CGTGGTGCCCGCCAGAAGCT 3'	
DNMT3b4	sense:	5'-CGGGATGAACAGTTAAAGAAAGTA-3'	140 bp
	antisense:	5'-CCAAAGATCCTTTCGAGCTC-3'	
	probe:	5'CCAAGTCGAACTCGATCAAACAGGG 3'	
MBD2	sense:	5'-GAATGAACAGCCACGTCAGCTT-3'	127 bp
	antisense:	5'-GCTACCTGGACCAACTCCTTGA-3'	
	probe:	5'-6-FAM-TCTGGGAGAAGAGGCTACAAGGACTTAGTGCA-TAMRA-3'	
β-actin	sense:	5'-TGAGCGCGGCTACAGCTT-3'	58 bp
	antisense:	5'-CCTTAATGTCACACACGATT-3'	
	probe:	5'-6-FAM-ACCACCACGGCCGAGCGG-TAMRA-3'	

resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) was then amplified using primer pairs targeting specifically either the methylated or unmethylated sequences of candidate genes. A total of 14 tumor suppressor genes, including *APC, RASSF1A, SOCS-1, GSTP1, E-cadherin, p14, p15, p16, DAP-kinase, HIC1, MGMT, TIMP-3, hMLH1* and *HLTF* were examined. The primer sequences are listed in Table I. PCR was performed in a 25-μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM each of deoxynucleotide triphosphates, 1 μM of each primer and 1 unit of AmpliTaq Gold DNA polymerase (PE Biosystem, Foster City, CA, USA). The condition for amplification was 12 min at 95˚C, followed by 38 cycles of denaturation at 95˚C for 30 sec, annealing at temperature as specified in Table I for 40 sec and extension at 72˚C for 45 sec. Universal methylated DNA (Chemicon) was used as positive control, whereas distilled water was included as negative control during amplification. PCR products obtained were separated on 10% polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

Quantitative RT-PCR analysis for DNMTs and MBD2 expression levels. Total RNA was isolated from frozen tissues and cultured cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg of total cellular RNA. cDNA synthesis was performed in a 100-μl reaction volume containing TaqMan RT buffer, 5.5 mM $MgCl_2$, 0.5 mM each of deoxynucleotide triphosphates, 2.5 μM of random hexamers, 40 units of RNase inhibitor and 125 units of MultiScribe Reverse Transcriptase (PE Biosystems). The condition for RT reaction was 10 min at 25˚C, 30 min at 48˚C; followed by enzyme inactivation at 95˚C for 5 min. cDNA derived from 3 different normal livers was used as control (Ambion, Austin, TX; Clontech Laboratory Inc., Palo Alto, CA; and Strategene, La Jolla, California, USA). To prevent the re-amplification of carryover PCR products, cDNA was treated with 0.25 unit of AmpErase uracil-N-glycosylase at 50˚C for 2 min (PE Biosystems). PCR was performed in a 25-μl reaction volume containing 1 x TaqMan buffer, 3.5 mM of $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphates, 300 nM of primer, 200 nM of probe and 0.625 unit of AmpliTaq Gold DNA polymerase (PE Biosystems). The condition was 10 min at 95˚C, followed by 45 cycles of denaturation at 95˚C for 30 sec and annealing/extension at 60˚C for 1 min. Real-time detection of the fluorescence emission was performed using iCycler (BioRad). The sequences of primer pairs and probes used for quantitative RT-PCR are shown in Table II. PCR for each sample-primer set was done in triplicate and in parallel with b-actin amplification. The expression levels of DNMTs and MBD2 were normalized to that of b-actin and were expressed as a ratio relative to the average value derived from normal livers.

Statistical analysis. The association among promoter methylation status of tumor suppressor genes, expression levels of DNMTs and MBD2, and clinicopathological data were analyzed by Spearman's correlation test (2-tailed). All statistical analyses were performed using SPSS software and a p-value <0.05 was considered significant.

Results

Promoter hypermethylation status of TSGs. The promoter methylation status of 14 TSGs in 51 liver samples, including 41 HCC, 1 CC, MN and 8 HCC cell lines was determined

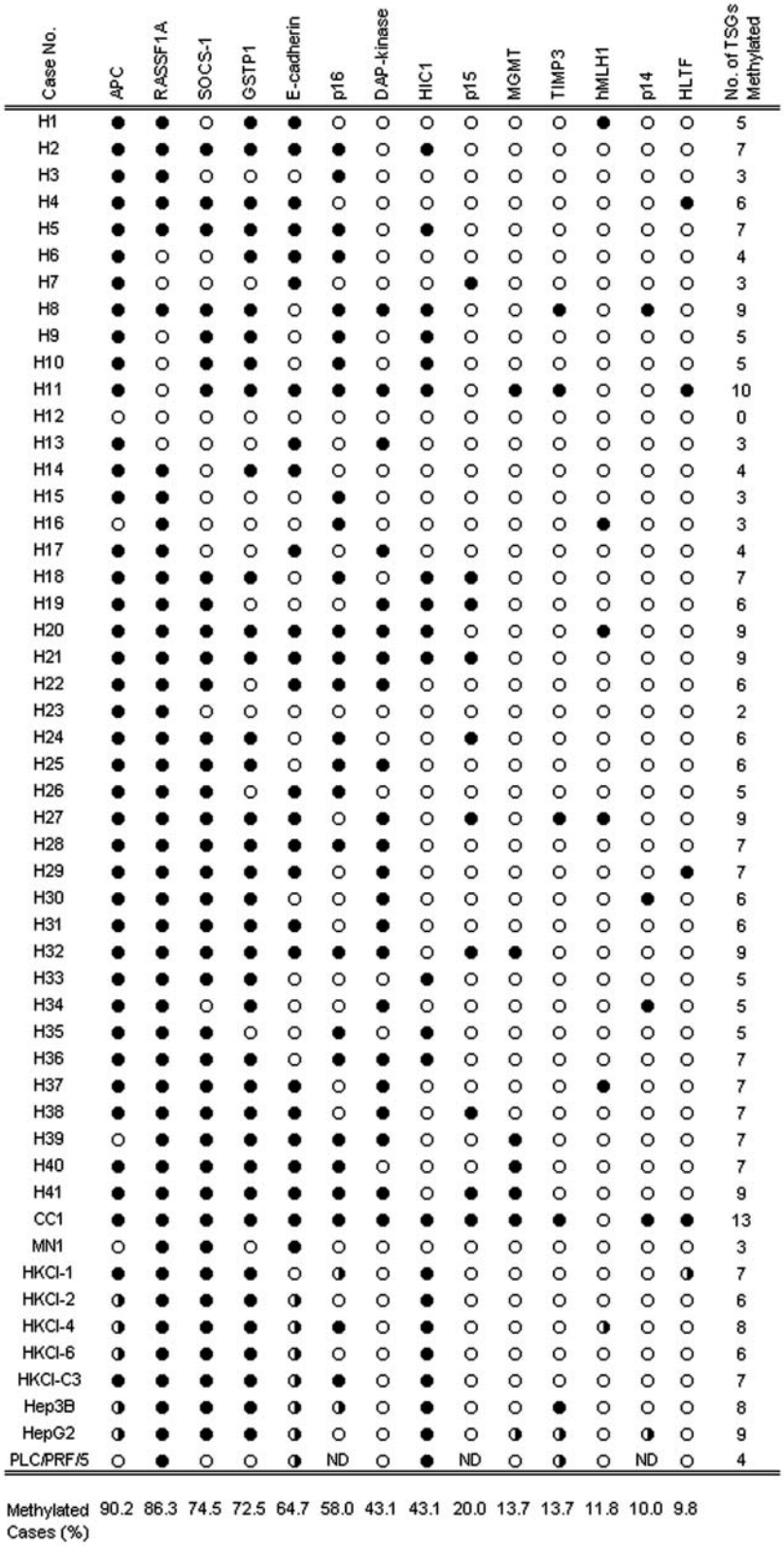

Figure 1. *Summary of methylation analysis on 14 candidate tumor suppressor genes. The promoter methylation status of TSGs was determined by MSP. Open, solid and half-filled circles represent non-methylated, methylated and partially methylated genes, respectively. "ND" indicates an non-informative case. The percentage of cases methylated for each gene and the number of TSGs methylated for each sample are shown.*

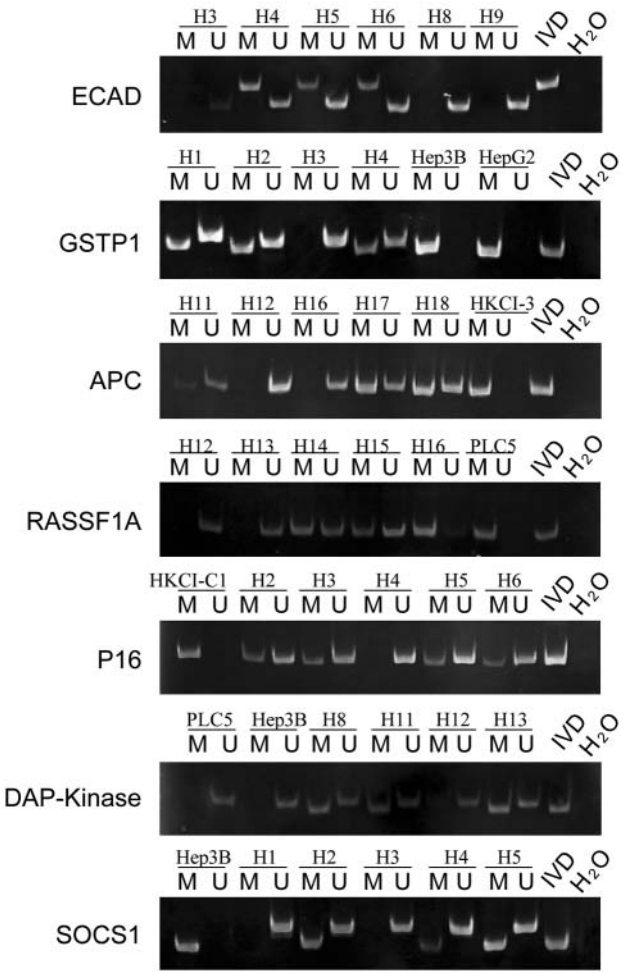

Figure 2. *Methylation-specific PCR analysis of selected genes in liver cancer. MSP was done using primers specific for methylated and non-methylated DNA for the indicated genes. Each reaction included a water control (H_2O) and a positive control for methylation using in vitro methylated DNA (IVM). PCR reactions were analyzed on a 10% acrylamide gel and the DNA bands were visualized after staining with ethidium bromide under UV light. The seven most highly methylated genes are shown. Representative examples from primary HCC tumors (H1-H18) and cell lines (Hep3B, HepG2, PLC5, HKCI-3, HKCI-C3) are presented.*

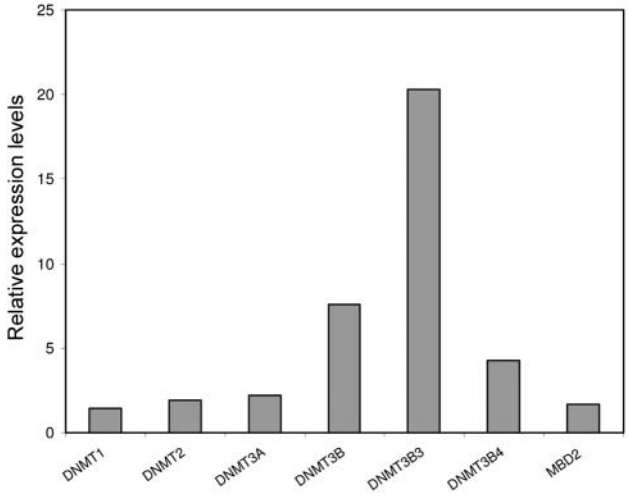

Figure 3. *Relative expression levels of DNMTs and MBD2. *mRNA levels determined from quantitative RT-PCR analysis in primary tumors and cell lines were normalized against β-actin. The median expression levels are presented.*

by methylation-specific PCR (Figure 1). The most frequent methylated TSG promoters were *APC* (90.2%), *RASSF1A* (86.3%), *SOCS-1* (74.5%), *GSTP1* (72.5%), *E-cadherin* (64.7%) and *p16* (58.0%). *DAP-kinase* and *HIC1* were methylated at the frequency of 43.1%. The remaining six genes studied including *p15* (20.0%), *MGMT* and *TIMP-3* (13.7% each), *hMLH1* (11.8%), *p14* (10.0%) and *HLTF* (9.8%), exhibited a much lower incidence of promoter hypermethylation. Concurrent methylation of multiple TSGs was common in our series, with a mean number of 6.1 methylated genes detected per case. In 51 samples, 49 cases (96.1%) exhibited three or more methylated TSG promoters, whereas 22 (43.1%) showed hypermethylation of seven or more TSG promoters. One case (H12) did not demonstrate methylation changes of any TSG investigated. The frequency of promoter methylation of each individual gene and the cumulative methylation percentage in each case are shown in Figure 2.

TSG methylation status and clinicopathological correlations. The correlative analysis of individual TSG methylation status with clinicopathological data on 43 primary tumors revealed two interesting associations. First, tumor specimens derived from cirrhosis showed a significantly higher incidence of *p16* hypermethylation ($p=0.005$, r=0.416). Twenty-one out of 29 HCC cases (72.4%) with underlying liver cirrhosis had a methylated *p16,* whereas only 4 out of the 14 cases (28.6%) that arose from a non-cirrhotic liver displayed *p16* promoter hypermethylation. Secondly, the more advanced stages T3/T4 HCCs exhibited more frequent methylated *DAP-kinase* (11/15 cases; 73.3%) than the early stage T1/T2 tumors (10/26 cases; 38.5%) ($p=0.032$, r=0.336).

mRNA expression levels of DNMTs and MBD2. The mRNA expression levels of DNMT1, 2, 3a, 3b and of the splice variants 3b3 and 3b4, and MBD2 in primary tumors and cell lines were determined by quantitative RT-PCR and expressed as a ratio relative to normal livers. The up-regulation of DNMT1, 2, 3a, 3b, 3b3, 3b4 and MBD2 by 2-fold or more was suggested in 40.0%, 48.6%, 56.8%,

91.1%, 97.8%, 65.9% and 45.9% of cases examined, respectively. The highest expressions were those of DNMT3b, 3b3 and 3b4 at median increased levels of 7.6-, 20.3- and 4.3-fold, respectively (Figure 3). The possible relationships between DNMTs and MBD2 expression levels clinicopathological characteristics of the cases analyzed was explored further. No significant association between expression levels of any of the DNMT genes and MBD2 with clinicopathological categories, including the presence of cirrhosis, disease stage of tumors, gender or age, was found ($p>0.05$).

Relationship between TSG hypermethylation and levels of DNMT and MBD2 expression. To examine the effect of DNMTs and MBD2 deregulations on promoter hypermethylation of TSGs, the correlation between these two factors in primary tumors and cell lines, of which both DNA methylation and expression studies were simultaneously performed, was analyzed. Hypermethylation of *DAP-kinase* showed significant association with the overexpression of DNMT3a ($p=0.006$, $r=0.441$), where the median expression level of DNMT3a in hypermethylated DAP-kinase cases was 4.75 and 1.75 in unmethylated cases. In addition, the overexpression of DNMT2 was found to be associated with the hypermethylation of *GSTP1* ($p=0.014$, $r=0.399$). The median expression level of DNMT2 in methylated *GSTP1* cases was 2.91 and in non-methylated cases was 0.41.

Discussion

Methylation-mediated gene silencing contributes to tumorigenesis in many tumor types. In this study, we examined the promoter methylation status of 14 genes commonly involved in cellular pathways, including apoptosis (*DAP-kinase*), cell adherence (*E-cadherin*), cell cycle (*p14, p15 and p16*), detoxification (*GSTP1*), DNA repair (*hMLH1* and *MGMT*) and signal transduction (*APC, RASSF1A* and *SOCS-1*) in HCC, cholangiocarcinoma and a macroregenerative nodule. In keeping with other studies, our results showed that promoter hypermethylation is a frequent event in liver cancer, where the most frequent genes displaying hypermethylation were *APC, RASSF1A, SOCS-1, GSTP1, E-cadherin* and *p16* (>55% of cases), followed by *DAP-kinase* and *HIC1*. Concurrent methylation of multiple TSGs was also common, where 3 or more hypermethylated promoters per case could be detected in as many as 96% of cases. Furthermore, on average the hypermethylation of 6.1 genes were identified per specimen. Such high incidences of aberrant methylation inevitably suggested that epigenetic inactivation of multiple regulatory genes might have a contributory role to the accelerated cell proliferation and/or transformation mechanism in HCC developments.

Most HCCs arise in the liver, with underlying diseases induced by persistent hepatitis virus infection, including chronic hepatitis and cirrhosis. We found that tissue specimens derived from cirrhosis showed significant correlation with hypermethylation of *p16* compared to those from chronic hepatitis ($p=0.005$). The *p16* gene encodes a critical negative regulator of cell cycle progression and is one of the most frequently inactivated tumor suppressor genes detected in human cancer cells (10). While it is not clear how the inactivation of *p16 via* epigenetic mechanism plays a role in hepatocarcinogenesis through the development of cirrhosis, our finding is consistent with another study. In the report described by Yu *et al.*, methylation of p16 in Chinese patients was detected in 75% of cirrhotic HCC but in only 33% of the non-cirrhotic group (11). In addition, we found methylation of the promoter region of *DAP-kinase* to be associated with advanced stage HCC tumors, which are often characterized by vascular invasion and intrahepatic metastasis ($p=0.032$). *DAP-kinase* is a pro-apoptotic enzyme with multiple functions in programmed cell death and tumor metastasis (12). Similar to our finding, it has been shown in non-small-cell lung cancer that methylation of the *DAP-kinase* promoter was associated with an increase in tumor size, lymph node involvement and the progression of early tumors to late stage disease (13).

Though it is now well established that global hypomethylation accompanied by region-specific hypermethylation events are frequent in tumor cells, the underlying mechanism remains largely unclear. It is not fully understood why certain CpG islands appear to be more susceptible to aberrant methylation. DNMTs are key enzymes responsible for establishing and maintaining genomic methylation patterns, while methyl-CpG binding proteins have been implicated in subsequent transcriptional repression. Recent studies showed a significant association between overexpression of DNMT1 and methylation on C-type CpG islands in colorectal and stomach cancer (14, 15) and that over-expression of DNMT3b4 may well induce the hypomethylation status of peri-centromeric satellite regions in HCC (7). These findings prompted our present investigation for the relationship between DNMT and MBD2 expressions and aberrant TSG hypermethylation in liver cancer. On the whole, a substantial increase in the expression of DNMTs and MBD2 were found in tumors compared to normal livers. In particular, up-regulations of DNMT2 and DNMT3a correlated significantly with hypermethylation of *GSTP1* ($p=0.014$) and *DAP-kinase* ($p=0.006$), respectively. Though it may not be likely that deregulation of DNMT2 or DNMT3a alone would have a direct impact on the genomic changes, it is conceivable that these DNMTs in cooperation with other local factors, such as the histones, may have specific gene targets or chromosomal regions. Our findings

also suggest that the epigenetic silencing of *GSTP1* and *DAP-kinase* from the DNMT2 and DNMT3a deregulations may have predisposed cells to a condition favoring HCC development, by increasing the promutagenic oxidative stresses and repressing the pro-apoptotic signals, respectively.

In conclusion, promoter hypermethylation of TSGs is a frequent event in HCC and may be associated with DNMTs over expression. Methylation findings reported here might have further clinical implications, since a panel of 6 to 8 highly methylated genes has been identified and may represent useful biomarkers for further developments in the early diagnosis and disease monitoring of patients with HCC.

Acknowledgements

This work was supported by a Central Allocation Grant from the Hong Kong Research Grants Council (Ref. No.: CUHK2/02C), China.

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Received March 7, 2006
Accepted April 25, 2006