Abstract

**Purpose:** This study aims to determine the relationship between CpG island DNA hypermethylation and global genomic DNA hypomethylation and their prognostic implications in hepatocellular carcinoma. The association of DNA methylation changes with clinicopathologic factors and the chronological ordering of DNA methylation changes along multistep hepatocarcinogenesis were also assessed.

**Experimental Design:** Hepatocellular carcinoma (n = 20) and nonneoplastic liver samples (n = 72) were analyzed for their methylation status at 41 CpG island loci and 3 repetitive DNA elements (LINE-1, ALU, and SAT2) using MethyLight or combined bisulfite restriction analysis. After selection of 19 CpG island loci showing cancer-specific DNA methylation, another set of 99 hepatocellular carcinoma samples was analyzed for these loci.

**Results:** The number of methylated genes in hepatocellular carcinoma was significantly higher in hepatocellular carcinoma patients with a cirrhotic liver than in hepatocellular carcinoma patients with a noncirrhotic liver (9.9 versus 7.0, P = 0.001). Hepatocellular carcinoma from female patients showed a higher number of methylated genes than hepatocellular carcinoma from male patients (11.2 versus 8.4, P = 0.006). The genes CRABP1 and SYK showed significant association between CpG island hypermethylation and patients’ poor survival. SAT2 hypomethylation occurred earlier than LINE-1 or ALU hypomethylation along the multistep hepatocarcinogenesis. Depending on the type of CpG island locus, a direct, inverse, or no relationship between CpG island hypermethylation and repetitive DNA hypomethylation was observed in hepatocellular carcinomas.

**Conclusion:** The varying relationships between the hypermethylation of individual CpG island loci and the hypomethylation of repetitive elements suggests that they are not mechanically linked. SYK and CRABP1 hypermethylation may serve as useful tumor markers for prognostication of hepatocellular carcinoma patients.

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Overall, genomic DNA has relatively few CpG dinucleotides, whereas there are foci of high CpG dinucleotide frequency. These CpG-rich sequences, called CpG islands, are found in approximately 70% of human gene promoters and 5’ exon sequences (1, 2). The majority of CpG sites in repetitive DNA sequences, which cover about 45% of human genomic DNA, are methylated, but CpG islands in promoters are usually protected from aberrant DNA hypermethylation. However, these normal methylation patterns are lost in cancer cells: CpG sites of promoter CpG islands that are supposed to be methylation-free become methylated, whereas CpG sites in repetitive DNA elements that are normally methylated become demethylated (3, 4). Promoter CpG island hypermethylation contributes to carcinogenesis by shutting off expression of tumor suppressor or tumor-related genes, and genomic DNA hypomethylation is implicated in carcinogenesis by inducing chromosomal instability, as evidenced by a recent study in which knocking out DNA methyltransferase genes brought about genomic hypomethylation and accompanying structural alterations and/or changes in chromosome copy number (5). Although both focal CpG island hypermethylation and overall genomic hypomethylation are found in the same cancer, the relationship between them varies by tissue types of human cancers.

Hepatocellular carcinoma is the fifth most common malignancy among men worldwide: in countries of East Asia, hepatocellular carcinoma is among the leading causes of death (6).
Translational Relevance

Promoter CpG island hypermethylation is now recognized as a potential mechanism for inactivation of tumor suppressor genes or tumor-related genes, and acts as an alternative to gene mutations. In addition to its role in gene inactivation, promoter CpG island hypermethylation is now gaining attention as a molecular marker of tumor detection and prediction of clinical outcome. Hepatocellular carcinoma is the fifth most common malignancy worldwide and a leading cause of death in many parts of Africa and Asia. Although several therapeutic modalities are available, the long-term prognosis of hepatocellular carcinoma patients is still poor. In the present study, we have analyzed 99 hepatocellular carcinoma tissue samples for their methylation status in 19 cancer-specific CpG island loci and found that SYK and CRABPI hypermethylation was an independent prognostic factor heralding poor prognosis for hepatocellular carcinoma patients. Compared with other protein or RNA biomarkers with prognostic significance in hepatocellular carcinoma, DNA methylation marker is more stable and can be analyzed in formalin-fixed, paraffin-embedded tissue samples. The ability of SYK and CRABPI hypermethylation to predict a poor prognosis may help to guide optimal application of therapeutic modalities to hepatocellular carcinoma patients according to individual risk.

Hepatocellular carcinoma is a human cancer in which aberrant DNA methylation frequently occurs (7). To date, approximately 94 genes have been shown to be hypermethylated in hepatocellular carcinoma (8–12); most of these genes have been identified from methylation-specific PCR studies. Although methylation-specific PCR is a very sensitive assay to assess DNA hypermethylation, the results are dependent on the number of PCR cycles, the amount of input DNA, and the PCR reaction mixture conditions. Thus, the reported methylation frequencies of identical genes in hepatocellular carcinoma have varied among studies (8–10, 12). Real-time PCR-based methylation-specific PCR (MethyLight) can overcome issues related to PCR cycling and provide reliable quantitative information about the methylation status of the target CpG island loci. MethyLight assay has been evaluated and validated for its precision and performance characteristics (13, 14).

Promoter CpG island hypermethylation is an early event in chronic liver diseases (9, 10, 15, 16), whereas genome-wide hypomethylation has been reported to occur primarily later at the hepatocellular carcinoma stage, and not in chronic liver diseases (17). Although several studies have analyzed DNA hypermethylation or hypomethylation in hepatocellular carcinoma, studies analyzing both types of methylation changes in hepatocellular carcinoma are very limited (12, 18), and the relationship between DNA hypermethylation and hypomethylation in hepatocellular carcinoma remains unclear. Furthermore, no study in the literature has investigated DNA hypermethylation and hypomethylation together in patients with chronic liver diseases without hepatocellular carcinoma.

In the present study, we analyzed hepatocellular carcinoma (n = 20) and nonneoplastic livers (n = 72) with regard to methylation status in single-copy genes (n = 41) and repetitive DNA sequences (LINE-1, ALU, and SAT2) using MethyLight or combined bisulfite restriction analysis (COBRA). Through comparison of the methylation status of 41 CpG island loci between hepatocellular carcinoma and nonneoplastic livers, 19 CpG island loci were found to show cancer-specific hypermethylation. These 19 loci were further examined in another set of hepatocellular carcinoma samples (n = 99) to determine the relationship between hypermethylation of the cancer-specific loci and clinicopathologic features of hepatocellular carcinoma. Additionally, the relationship between hypermethylation and hypomethylation was also explored in hepatocellular carcinoma and chronic liver diseases.

Materials and Methods

Patients and tissue specimens. Formalin-fixed, paraffin-embedded archival tissues from 129 hepatitis B virus–positive hepatocellular carcinoma patients (mean age, 52.3 y; 104 males and 25 females), 25 hepatitis B virus–positive liver cirrhosis patients (52.4 y; 17 males and 8 females), 32 chronic hepatitis B patients (44.9 y; 23 males and 9 females), and 25 healthy liver donors (31.2 y; 17 males and 8 females), were retrieved from the files of the Department of Pathology, Seoul National University Hospital (Seoul, Korea). The hepatocellular carcinoma patients underwent curative surgery from 1999 to 2004 at Seoul National University Hospital, and the hepatocellular carcinoma tumor stages were classified according to the tumor-node-metastasis (TNM) criteria. The degree of fibrosis in nonneoplastic livers was graded based on the Ishak scoring system (19), and a liver with a fibrosis staging score of 5 or 6 was considered cirrhotic. Through microscopic examination of histologic slides, tissue blocks containing tumors only were selected and serially sectioned. Cirrhotic portions in matched hepatocellular carcinoma were obtained from the cirrhotic liver >3 cm away from tumors and were confirmed to be tumor-free by microscopic examination. We reviewed the medical records of the hepatocellular carcinoma patients and obtained clinicopathologic information including age, gender, Child-Pugh classification, bilirubin, and γ-glutamyltransferase and α-fetoprotein levels. Informed consent was obtained from each participant or participant’s guardian. This study was approved by the Institutional Review Board of Seoul National University College of Medicine.

MethyLight analysis. We extracted genomic DNA from 15 to 20 paraffin-embedded tissue sections (10-μm thick). The paraffin was removed from the tissue by rinsing in xylene, and genomic DNA was isolated using a QIAamp tissue kit (Qiagen). Sodium bisulfite conversion of genomic DNA was done as described (20). DNA methylation was analyzed using MethyLight technology (13). From 177 DNA methylation markers that had been tested in previous studies for gastric cancer and colorectal cancer (21, 22), we selected 52 CpG DNA methylation markers that were found to be hypermethylated in gastric cancer or colorectal cancer tissues at higher frequencies or higher levels than those of their normal counterpart mucosas. Initially, we examined a training set of hepatocellular carcinoma (n = 10) and healthy donor liver tissues (n = 10) for the selected 52 DNA methylation markers using MethyLight, and excluded 11 CpG island loci because they were methylated in all the normal liver tissue samples or not methylated in the neoplastic and nonneoplastic liver tissue samples. The remaining 41 CpG island loci were selected for subsequent DNA methylation analysis. The DNA sequences of the MethyLight reaction primers and probes for these 41 CpG island loci and SAT2 are shown in Supplemental Table S1. MethyLight PCR was done as described (13). Briefly, two sets of primers and probes, designed specifically to bind to bisulfite-converted DNA, were used in the reaction: one set of primers and probe for every methylated target to be analyzed (methylated reaction) and one set of primers and probe for the reference locus, ALU (normalization control reaction). The
normalization control reaction refers to the methylation-independent measurement control to control for DNA amplification and normalize for input DNA. M.SssI-treated genomic DNA was used as a constant reference sample to determine the percentage of methylated reference (PMR) at a particular locus. PMR was defined as 100 \times \frac{\text{(methylated reaction/control reaction)}_{\text{sample}}}{\text{(methylated reaction/control reaction)}_{\text{M.SssI-Reference}}\text{. Although there was no standard cutoff value for classifying methylated and unmethylated loci using DNA methylation analyses, a CpG island locus was considered methylated if PMR > 4. The PMR cutoff value of 4 has been validated by previous studies based on the distributions of PMR values in the tested CpG island loci and correlation of loss of protein expression with methylation for CDKN2A, MLH1, and MGMT, where a PMR cutoff of 4 was used (14, 23).

**LINE-1 and ALU COBRA.** COBRA for LINE-1 and ALU was done as described (20). In short, bisulfite-modified DNA was amplified with oligonucleotide primers specific for bisulfite-modified LINE-1 or ALU DNA sequences. For LINE-1, the amplified PCR products were digested with TaqI and TaqI, which cut the unmethylated DNA allele and methylated allele, respectively. For ALU, digestion of the amplified PCR products with TaqI digests the methylated ALU allele into two fragments but does not cut the unmethylated ALU allele. The digested PCR products were electrophoresed on 10% polyacrylamide gels and then stained with ethidium bromide. The intensities of digested bands were measured with ImageJ software, and LINE-1 and ALU methylation levels were determined using the following formula: (intensities of methylated bands)/(intensities of methylated bands + intensities of unmethylated bands) \times 100.

**Statistics.** Associations between clinicopathologic variables and epigenetic alterations were analyzed using Fisher’s exact test or Pearson’s \( \chi^2 \) test. The two-tailed Student’s \( t \)-test was used to evaluate the significance of the differences observed between two means. One-way ANOVA was used when comparing similarity for three or more groups. The two-tailed Student’s \( t \)-test was used to test the association between DNA hypomethylation and DNA hypermethylation. Survival was measured from the date of resection of hepatocellular carcinoma to the date of death or the last clinical review before August 31, 2007. Overall survival rates were assessed using the Kaplan-Meier log-rank test, and Cox proportional hazard analysis was used to examine multivariate relationships between several clinicopathologic and epigenetic variables. A value of \( P < 0.05 \) was considered significant. Statistical analyses were carried out with SPSS software (version 12.0).

**Results**

**Quality of formalin-fixed, paraffin-embedded tissues and its suitability for MethyLight assay.** The liver tissue samples used for this study, including a training set of 10 hepatocellular carcinoma tissues and 10 healthy donor liver tissues, the first tester set of 20 hepatocellular carcinoma tissues and 72 noncancerous liver tissues, and the second tester set of 99 hepatocellular carcinoma tissues, were formalin-fixed paraffin-embedded tissues with storage time ranging from three to eight years. The concern was regarding the suitability of formalin-fixed paraffin-embedded tissues for methylation analysis. After bisulfite modification of the same amount of DNA, ALU-based MethyLight control reaction was done to quantify the number of input target DNA and the threshold cycle values were found to be comparable among DNA samples from varying storage time. For five samples (five hepatocellular carcinoma tissue specimens), snap-frozen and formalin-fixed paraffin-embedded tissues were available in parallel. MethyLight assays were repeated five times for five pairs of specimens and eight CpG island loci. All of the measurements showed nearly identical methylation levels (Supplemental Table S2).

**CpG island hypermethylation analysis of hepatocellular carcinoma and nonneoplastic liver tissues.** We did MethyLight analysis of 41 CpG island loci on a total of 92 liver samples, including hepatocellular carcinoma (\( n = 20 \)) and noncancerous liver tissues (\( n = 72 \)), and determined PMR values for each sample and CpG island locus (Supplemental Fig. S1). Twenty-three CpG island loci were significantly methylated in hepatocellular carcinoma tissue samples at levels higher than those of noncancerous liver tissues (\( P < 0.05 \); Student’s \( t \)-test); TMEFF2, HOXA1, p16, DLEC1, APC, PTGS2, RASSF1A, CDKN1C, GSP1, RUNX3, SOCS3, CRABP1, CCN2D, GRIN2B, PCS1, NEUROG1, SYK, CACNA1G, BCL2, SFRP1, CALCA, TERT, and SCGB3A1 (in descending order of statistical significance; Fig. 1A). When we calculated DNA methylation frequencies for each CpG island locus (PMR > 4) for hepatocellular carcinoma and noncancerous liver tissues, hepatocellular carcinoma tissues showed hypermethylation at 20 CpG island loci at frequencies higher than those of noncancerous liver tissues (\( P < 0.05 \); \( \chi^2 \) test), which included HOXA1, CDKN1C, CRABP1, DLEC1, p16, CCN2D, CACNA1G, RUNX3, PTGS2, BCL2, GRIN2B, NEUROG1, GSP1, SYK, SFRP1, CALCA, SOCS3, APC, TERT, and TNFRSF10C (in descending order of statistical significance; Fig. 1B). All these 19 loci except for TNFRSF10C exhibited significantly higher methylation levels in hepatocellular carcinoma than in noncancerous liver tissues and thus were considered cancer-specific CpG island loci. For the 41 tested CpG island loci, the mean number of methylated loci (PMR > 4) in hepatocellular carcinoma was 16.8, much higher than in premalignant stages (Fig. 2), whereas the mean number of methylated loci in liver cirrhosis was 10.1, significantly higher than that in chronic hepatitis (10.1 versus 8.5; \( P = 0.014 \); Student’s \( t \)-test). When comparison analysis was confined to the 19 cancer-specific CpG island loci, the mean number of methylated CpG island loci in hepatocellular carcinoma was 9.4, much higher than those of noncancerous liver tissues (liver cirrhosis, 2.2; chronic hepatitis, 1.8; normal livers, 1.5; ANOVA, \( P < 0.001 \)).

**Methylation levels of LINE-1, ALU and SAT2 in hepatocellular carcinoma and nonneoplastic liver tissues.** We did COBRA assays for LINE-1 and ALU and MethyLight analysis of the satellite DNA region SAT2 on hepatocellular carcinoma (\( n = 20 \)) and nonneoplastic tissue samples (\( n = 72 \)). Figure 3 shows the methylation levels of LINE-1, ALU, and SAT2 for the hepatocellular carcinoma and nonneoplastic liver tissues. Methylation levels of LINE-1 and ALU did not change in the premalignant stages from normal liver to chronic liver disease but sharply dropped in hepatocellular carcinoma tissues. The methylation level of SAT2 declined in chronic hepatitis liver samples and further declined in hepatocellular carcinoma tissues.

**Hypermethylation versus clinicopathologic features of hepatocellular carcinoma.** Of the 41 CpG island loci, 19 CpG island loci were considered cancer-specific methylation markers because they were significantly hypermethylated in hepatocellular carcinoma compared with nonneoplastic liver tissues with respect to both the methylation level and the methylation frequency. Another set of hepatocellular carcinoma samples (\( n = 99 \)) was analyzed for the 19 loci and the 3 repetitive DNA elements using the MethyLight assay or COBRA, which were done in triplicate. The
relationships between methylation frequencies of each CpG island locus and the clinicopathologic features of hepatocellular carcinoma are summarized in Supplemental Table S3. The number of methylated genes in hepatocellular carcinoma was closely associated with the background liver condition; genomic DNA from hepatocellular carcinoma tissue associated with cirrhosis (Ishak grade 5 or 6) showed a higher number of methylated genes than genomic DNA from hepatocellular carcinoma tissue associated with noncirrhotic liver (Ishak grade, 4 or less; 9.9 versus 7.0, \( P = 0.001; n = 66 \) versus \( n = 33 \)). A gender difference was noted in the number of methylated genes: genomic DNA from hepatocellular carcinoma tissue samples of female patients showed a significantly higher number of methylated genes than genomic DNA from hepatocellular carcinoma tissues from male patients (11.2 versus 8.4, \( P = 0.006; n = 20 \) versus \( n = 79 \)). When the comparison was restricted to hepatocellular carcinoma cases with a cirrhotic liver background (because female patients with hepatocellular carcinoma were more frequently associated with cirrhotic liver than were male patients with hepatocellular carcinoma), the increased tendency of CpG island hypermethylation in female patients was still recognized: the mean number of methylated genes in male patients (\( n = 48 \)) versus female patients (\( n = 18 \)) was 9.2 versus 11.6 (\( P = 0.030 \)). No significant differences, however, were identified in the number of methylated genes between low (Edmonson-Steiner grade I, II) and high (grade III, IV) histologic grades (9.2 versus 7.6; \( P = 0.073 \)), between low and high TNM stages (9.0 versus
8.6; \( P = 0.688 \), between smaller and larger tumor sizes (8.6 versus 9.2; \( P = 0.433 \)), or between older patients and younger patients (9.4 versus 8.3; \( P = 0.182 \)).

Survival was analyzed in 94 patients, excluding 5 patients because of follow-up loss. Of the 19 CpG island loci, 2 loci, CRABP1 and SYK, exhibited a significant association between gene hypermethylation and patients' poor prognosis (Fig. 4; \( P = 0.048 \) and 0.009, respectively, Kaplan-Meier log-rank test). Table 1 summarizes the prognostic significance of clinicopathologic factors. Age, serum \( \gamma \)-glutamyltranspeptidase level, tumor size, microscopic vascular invasion, and TNM stage were significant factors, whereas the background liver state (that is, association or not with cirrhosis) was a marginally significant factor. These five clinicopathologic factors, plus the two DNA methylation markers CRABP1 and SYK found to be prognostic on univariate analysis were entered into a multivariate analysis to identify independent predictors of overall survival. Multivariate analysis using the Cox proportional hazards model revealed that CRABP1 methylation status (\( P = 0.046 \)) and SYK methylation status (\( P = 0.002 \)) were significant factors affecting overall survival of hepatocellular carcinoma patients (Table 2). When we applied different PMR cutoff values of 1, 4, 7, 10, 15, and 20, SYK and CRABP1 showed persistent association between gene hypermethylation and patients' poor prognosis (Supplemental Fig. S2).

**Hypomethylation of repetitive DNA sequences versus clinicopathologic features of hepatocellular carcinoma.** Significant association was found between the methylation level of LINE-1 and tumor stage; high-TNM-stage hepatocellular carcinoma tumors showed LINE-1 methylation levels lower than those of low-TNM-stage hepatocellular carcinoma tumors (Supplemental Table S4). ALU and SAT2 methylation levels were also somewhat lower in high-stage tumors than in low-stage tumors, but the differences were not statistically significant. No significant associations were found between methylation levels of repetitive DNA sequences and other clinicopathologic features (gender, age, associated liver condition, tumor size, and histologic grade).

**Relationship between CpG island hypermethylation and repetitive DNA hypomethylation.** The methylation levels of repetitive DNA sequences (LINE-1, ALU, and SAT2) were compared with the methylation status of each of the 19 genes (Supplemental Fig. S3 and Supplemental Table S5). Three different groups of genes were identified based on the relationship between methylation level of the repetitive DNA...
sequences and CpG island hypermethylation of the genes. One group of genes that included CACNA1G, CDKN1C, and p16 tended to show a positive relationship between repetitive DNA hypomethylation and CpG island hypermethylation. A second group of genes tended to show an inverse relationship between repetitive DNA hypomethylation and CpG island hypermethylation, and included CCND2, CRABP1, DLEC1, GRIN2B, HOXA1, NEUROG1, PTGS2, SFRP1, SYK, and TERT. A third

| Table 1. Univariable analysis of clinicopathologic parameters with regard to survival in hepatocellular carcinoma patients |
|-----------------|----------------|-----------------|-----------------|-----------------|
| Characteristics | No. of patients | No. of deaths | Overall survival (%) |
|                 |                |                | 3y               | 5y               | P               |
| Gender          |                |                |                  |                  |                 |
| Female          | 19             | 11             | 57.0             | 43.4             | 0.536           |
| Male            | 75             | 33             | 64.9             | 52.8             |                 |
| Age (y)         |                |                |                  |                  |                 |
| ≥52             | 44             | 27             | 44.7             | 32.5             | 0.002           |
| >59             | 50             | 17             | 79.5             | 66.5             |                 |
| Child-Pugh classification | | | | | |
| A               | 83             | 37             | 63.7             | 53.3             | 0.223           |
| B               | 11             | 7              | 60.6             | 30.3             |                 |
| C               | 0              | 0              |                  |                  |                 |
| GGT <56 IU/L    | 45             | 14             | 74.0             | 61.5             | 0.007           |
| >56 IU/L        | 49             | 30             | 53.7             | 41.2             |                 |
| AFP <100 ng/mL  | 49             | 19             | 68.8             | 59.2             | 0.091           |
| >100 ng/mL      | 42             | 24             | 56.6             | 37.7             |                 |
| Bilirubin <1.5 mg/dL | 76        | 35             | 62.9             | 51.9             | 0.815           |
| >1.5 mg/dL      | 18             | 9              | 65.7             | 46.0             |                 |
| Tumor size <5.6 cm | 46         | 12             | 80.8             | 73.3             | <0.001          |
| >5.6 cm         | 48             | 32             | 46.9             | 30.2             |                 |
| Histologic grade 1, 2 | 64     | 25             | 69.4             | 55.1             | 0.475           |
| 3, 4            | 30             | 13             | 54.4             | 48.4             |                 |
| Microscopic vascular invasion Absent | 50     | 27             | 78.4             | 61.3             | 0.042           |
| Present         | 44             | 15             | 53.1             | 43.7             |                 |
| Satellite nodule Absent | 83    | 4              | 62.5             | 50.5             | 0.527           |
| Present         | 11             | 40             | 69.3             | 55.4             |                 |
| Cirrhosis Chronic hepatitis | 30    | 10             | 72.6             | 67.9             | 0.078           |
| Cirrhosis       | 64             | 34             | 58.9             | 42.8             |                 |
| TNM stage I, II | 76             | 31             | 67.9             | 56.3             | 0.005           |
| III, IV         | 18             | 13             | 44.4             | 27.7             |                 |
| CRABP1 Unmethylated | 52    | 20             | 70.4             | 59.9             | 0.048           |
| Methylated      | 42             | 24             | 54.2             | 39.8             |                 |
| SYK Unmethylated | 60     | 22             | 70.7             | 59.1             | 0.009           |
| Methylated      | 34             | 22             | 49.8             | 36.8             |                 |

Abbreviations: GGT, γ-glutamyltranspeptidase; AFP, α fetoprotein.

| Table 2. Multivariate analysis of factors associated with poor survival rate in hepatocellular carcinoma patients (Cox proportional hazard analysis) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| β               | SE             | Hazard ratio (95% confidence interval) | P               |
| CRABP1          | 0.900          | 0.368           | 2.459 (1.197-5.054) | 0.046           |
| SYK             | 0.730          | 0.342           | 2.381 (1.217-4.657) | 0.002           |
| Age             | -0.981         | 0.354           | 0.375 (0.187-0.735) | 0.006           |
| γ Glutamyl transpeptidase | 0.673       | 0.213           | 1.960 (1.292-2.973) | 0.002           |
| Tumor size      | 0.820          | 0.376           | 2.271 (1.087-4.746) | 0.029           |
group of APC, BCL2, CALCA, GSTP1, RUNX3, and SOCS3 showed no relationship between repetitive DNA hypomethylation and CpG island hypermethylation.

Discussion

In the present study, we analyzed methylation of 41 promoter CpG island loci in a total of 92 tissue samples of hepatocellular carcinoma and nonneoplastic livers, and found that 19 CpG island loci were hypermethylated in a cancer-specific manner. We further analyzed methylation of these 19 cancer-specific CpG island loci in another set of 99 hepatocellular carcinoma liver tissue samples to correlate the methylation status of each locus with clinicopathologic features and to determine whether these DNA methylation markers can predict clinical outcomes of hepatocellular carcinoma. The methylation status of CRABP1 and SYK was found to be a significant prognostic factor upon both univariate analysis and multivariate analysis. Hypermethylation of SYK, a protein tyrosine kinase involved in B cell and T cell development and activation (24, 25), has been implicated in an adverse outcome for hepatocellular carcinoma patients (26), whereas CRABP1 has not been studied for its CpG island hypermethylation in hepatocellular carcinoma. CRABP1, which is expressed in almost all tissues, encodes a high-affinity cellular retinoic binding protein, and belongs to a family of small cytosolic lipid binding proteins. Although the function of Crabp1 is not completely understood, Crabp1 mainly regulates the availability of retinoic acid to its nuclear receptors by facilitating catabolism and/or sequestering retinoic acid (27, 28). A recent study has suggested a tumor suppressor function for Crabp1 in esophageal squamous cell carcinoma cells lacking the protein reduces cell growth, whereas RNA interference-mediated knockdown of CRABP1 in cells expressing the gene promotes cell growth (29). CRABP1 hypermethylation and coupled loss of expression were first reported in papillary thyroid cancer tissues and a colon cancer cell line and then in esophageal squamous cell carcinoma tissues (29, 30). The CRABP1 CpG island locus is one of the markers on a panel used to determine the CpG island methylator phenotype in colorectal cancers (23) and CpG island methylator phenotype-positive colorectal cancers are known to lead to an adverse clinical outcome (31). Our present study is the first to report that CRABP1 is frequently hypermethylated in hepatocellular carcinoma and that CRABP1 hypermethylation has a prognostic implication in hepatocellular carcinoma.

In the present study, CpG island hypermethylation in hepatocellular carcinoma was closely associated with background liver condition and gender. Hepatocellular carcinoma tumors arising in a cirrhotic background showed a higher number of methylated CpG island loci than tumors arising in noncirrhotic liver. Female hepatocellular carcinoma patients showed a higher number of methylated CpG island loci than male hepatocellular carcinoma patients. A close link between CpG island hypermethylation and cirrhosis of hepatocellular carcinoma patients was also noted in another study (8), in which p16 hypermethylation was found more frequently in hepatocellular carcinomas in cirrhotic than in noncirrhotic livers. Although the underlying mechanism for such a close link between CpG island hypermethylation and background liver condition is unclear at present, such information suggests that epigenetic modulations of hepatocellular carcinoma may be influenced by the disease state of the background liver. A gender dependence in the predisposition of hepatocellular carcinoma tumors toward CpG island hypermethylation has also been found in a study by Katoh et al. (32), in which GSTP1 hypermethylation was more frequently found in hepatocellular carcinoma tumors of female patients than in those of male patients. Animal studies have shown sex hormone–induced DNA methylation changes at specific gene regulatory regions, e.g., estrogen treatment in rat increases DNA methylation of the prolactin gene in liver tissue (33), and DNA methylation changes of the estrogen receptor gene and aromatase gene have been induced in Japanese medaka fish by estrogen treatment (34). However, gender-dependent differences in the number of methylated loci of hepatocellular carcinoma tumors cannot be explained by the presence of sex hormone alone because a gender dependence in the extent of methylation was not noted in nonneoplastic liver tissues (data not shown).

Approximately 45% of the human genome is interspersed repetitive DNA sequences, of which LINE-1 and ALU are the majority (35, 36). Although LINE-1 and ALU are dispersed mainly throughout the euchromatic regions of the genome, ALU repeats are preferentially located in the GC-rich and gene-rich genomic regions, in contrast to the preferential location of LINE-1 in AT-rich genomic regions (37). SAT2, a satellite DNA sequence, is found in pericentromeric heterochromatin of possibly all chromosomes. These repetitive DNA elements are normally heavily methylated, and a recent study showed a correlation between PCR measures of LINE-1, ALU, and SAT2 methylation and 5-methylcytosine content in the human genome (38), indicating the usefulness of these methylation analyses as a surrogate measure of genomic methylation levels. In the present study, these repetitive DNA elements showed discordance in timing of hypomethylation along the multistep hepatocarcinogenesis from normal liver to hepatocellular carcinoma. SAT2 hypomethylation occurred in the chronic hepatitis stage, whereas LINE-1 and ALU hypomethylation occurred in the hepatocellular carcinoma stage. These findings are consistent with previous results (17, 18, 39, 40). Studies from the Hirohashi group have shown that DNA hypomethylation of chromosomal loci on 16q and 17p occurs only in hepatocellular carcinoma and not in chronic liver diseases, and that DNA hypomethylation of SAT2 and SAT3 occurs more often in liver tissues of chronic liver diseases from hepatocellular carcinoma patients compared with histologically normal liver tissues from hepatocellular carcinoma patients or patients with liver metastasis of primary colon cancer (18, 39, 40). In the study of Lin et al. (17), genome-wide 5-methylcytosine content determined by an in vitro methyl acceptor assay with M.SssI did not differ among normal, noncirrhotic, and cirrhotic liver tissues but was reduced significantly in hepatocellular carcinoma tissues. Considering the fact that satellite DNA sequences, of which LINE-1, ALU, and SAT2, are consistent with previous results (17, 18, 39, 40), Studies from the Hirohashi group have shown that DNA hypomethylation of chromosomal loci on 16q and 17p occurs only in hepatocellular carcinoma and not in chronic liver diseases, and that DNA hypomethylation of SAT2 and SAT3 occurs more often in liver tissues of chronic liver diseases from hepatocellular carcinoma patients compared with histologically normal liver tissues from hepatocellular carcinoma patients or patients with liver metastasis of primary colon cancer (18, 39, 40).
process of hepatocarcinogenesis. However, because of the limitation that COBRA assays analyze single-CpG sites on DNA sequence that restriction enzymes recognize, more comprehensive assays (e.g., pyrosequencing methylation analysis of repetitive DNA elements) are needed to support such a conclusion.

Opposing DNA methylation changes of focal CpG island hypermethylation and diffuse genomic hypomethylation are common findings in human cancers, regardless of tissue type, and have been shown in the same tumors (12, 41–44), which has led to the suspicion that these two changes are mechanically linked. In the present study, genomic hypomethylation, represented by the methylation levels of repetitive DNA elements as surrogate markers, and CpG island hypermethylation were prominent in hepatocellular carcinoma tissues compared with precancerous liver tissues. Despite the presence of both types of aberrant DNA methylation changes in hepatocellular carcinoma, the relationship between CpG island hypermethylation and genomic DNA hypomethylation varied among CpG islands. A positive relationship between CpG island hypermethylation and genomic DNA hypomethylation was found for three CpG island loci, 11 loci showed an inverse relationship between CpG island hypermethylation and genomic hypomethylation, and the remainder exhibited no relationship between the methylation status at CpG islands and the overall genome. The variability between individual CpG island hypermethylation and repetitive DNA hypomethylation status indicated that there is no mechanical link between focal CpG island hypermethylation and generalized genomic DNA hypomethylation in liver cancer cells.

In summary, we found that CpG island hypermethylation of hepatocellular carcinoma tumors was closely associated with the background liver condition and gender. The relationship between CpG island hypermethylation and repetitive DNA hypomethylation varied depending on the type of CpG island locus and was not mechanically linked in hepatocellular carcinomas. SYK and CRABP1 hypermethylation was closely associated with poor prognosis for hepatocellular carcinoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Prognostic Implications of and Relationship Between CpG Island Hypermethylation and Repetitive DNA Hypomethylation in Hepatocellular Carcinoma

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