INTRODUCTION

CpG islands are CpG dinucleotide-rich areas found within the promoter and 5’ exonic regions of about 60%-70% of the human genes (1, 2). Aberrant hypermethylation on promoter CpG island loci can lead to inactivation of genes that are actively expressed or increased silencing of genes that are inactive. While both genetic mutations and promoter CpG island hypermethylation changes are simultaneously found in human cancer cells, recent studies have demonstrated that the number of genes inactivated by promoter CpG island hypermethylation is four-times higher than the number of genes undergoing mutations in colorectal cancers (3, 4). Besides its importance as a gene inactivation mechanism, promoter CpG island hypermethylation is gaining attention as a potential tumor biomarker. In this respect, DNA methylation markers are being actively investigated to detect human cancers in blood, secretions, or exfoliated cytology specimens, and to predict the risk of cancer progression and development (5-9).

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of death by cancer (10, 11). The overall incidence and mortality of hepatocellular carcinoma (HCC) has been decreasing in areas with high incidence (12). However, both the incidence and mortality rates of HCC are increasing in Western countries (13). The development of HCC is the consequence of a multistep process that involves several morphologically recognizable lesions and accumulation of molecular changes at the genetic and epigenetic levels. Several studies have reported HCC-associated genetic changes, including mutations in several tumor suppressor genes (TP53, p16, PTEN, IGF2R, and RB), oncogenes (c-MYC, c-MET, PIK3CA, and CTNNB1), and other cancer-associated genes (CDH1 and CCND1) (14, 15). Not only genetic changes but also epigenetic alterations underlie the evolution of HCC. Since hypermethylation of the CDH1 promoter CpG island locus was reported in HCC (16), about 150 other genes have been found to be hypermethylated in HCC in a cancer-specific manner (17-19). However, more genes undergoing hypermethylation of promoter CpG island loci are likely to be identified in HCC. Schuebel and colleagues reported that about 400 genes actively expressed in normal colon epithelial cells are inactivated by promoter CpG island hypermethylation in colorectal cancers (3).
Of the increasingly higher number of genes being identified as undergoing promoter CpG island hypermethylation in HCC, only a few have clinical relevance and potential utility as biomarkers for prediction of recurrence or poor prognosis (20-23). The primary aim of the present study is to discover novel cancer-related DNA methylation markers for HCC. We performed a large-scale gene expression microarray analysis, which enables the simultaneous characterization of the transcription profile of tens of thousands of genes, on HCC cell lines treated with demethylating agents. Of the genes up-regulated by treatment with demethylating agents, we selected those harboring CpG island loci in their promoter sequences. We then performed methylation-specific PCR (MSP) to determine whether promoter CpG island loci of the candidate genes were hypermethylated in HCC and whether this modification was cancer-specific. We found 2 promising cancer-specific methylated genes (HIST1H2AE and EFEMP2) that are associated with poor prognosis in patients with HCC.

**MATERIALS AND METHODS**

**Cell line culture and 5-aza-2’-deoxycytidine (AZA) treatment**

A total of 8 HCC cell lines (SNU398, SNU475, SNU739, SNU761, SNU878, SNU886, HepG2, and Huh7) were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were maintained in RPMI-1640 medium with the exception of Huh7, which was maintained in Dulbecco’s modified Eagle’s medium (DMEM). HEPES buffer (25 mM), fetal bovine serum (FBS) (10%), penicillin (100 units/mL), and streptomycin (0.1 mg/mL) were added to the medium. We seeded 3 × 10⁵ cells (from each of the cell lines) per milliliter of culture medium and treated them 24 hr later with 5 μM AZA (Sigma Chemical Co., St. Louis, MO, USA) for 96 hr, during which period media and drugs were changed every 24 hr. AZA was dissolved in acetic acid and diluted to 10 mg/mL. Cells were incubated for 72 hr and then treated with trichostatin A (TSA; Sigma) at 300 nM for 24 hr. For co-treatment of cells with AZA and TSA, AZA (5 μM) was added initially for 72 hr, after which it was removed, and AZA (5 μM) and TSA (300 nM) were added for an additional 24 hr. As a negative control, cell lines were treated with acetic acid in an equal volume of medium without the drug.

**Patients and tissue specimens**

Formalin-fixed, paraffin-embedded archival tissues from 95 HCC patients (mean age 55 yr; 75 males and 20 females) were retrieved from the files of the Department of Pathology, Seoul National University Hospital (Seoul, Korea). These patients had undergone curative surgery for HCC from 2001 to 2004 at the Seoul National University Hospital. The degree of fibrosis in nonneoplastic livers was graded according to the Ishak scoring system. A liver with a fibrosis staging score of 5 or 6 was considered cirrhotic. For histological examination, tissue blocks containing tumors were selected and serially sectioned. Nonneoplastic liver tissues were obtained > 3 cm away from tumor regions and confirmed to be tumor-free by microscopic examination. We reviewed the electronic medical records of the patients to obtain clinicopathological information, including age, gender, Child-Pugh classification, and γ-glutamyltransferase (GGT) and alpha-fetoprotein (AFP) levels. Fresh-frozen tissue samples of HCC and adjacent nonneoplastic liver were obtained from HCC patients (n = 10) who underwent curative resection for HCC at the Seoul National University Hospital in 2008.

**Microarray**

Illumina’s whole-genome expression arrays (Sentrix Human-Ref-8 Expression BeadChip, Illumina, Inc., San Diego, CA, USA) were used to analyze the RNA expression profiles of 5 HCC cell lines (SNU 398, SNU 761, SNU 878, HepG2, and Huh7) before and after treatment with AZA (5 μM) or TSA (300 nM).

**Methylation-specific PCR (MSP)**

For methylation analysis, 2 μg of genomic DNA obtained from HCC cell lines and tissue samples was modified using the EZ DNA Methylation™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s protocol. The specific primers for bisulfite modified DNA were designed using MSPPrimer (http://www.mspprimer.org). For genes whose primer sequences could not be obtained by using the MSPPrimer software, MSP primers were designed using MethPrimer (http://www.urogene.org/methprimer). MSP primer sequences are available on request. MSP was performed as previously described (24).

**Reverse transcription (RT)-PCR**

We isolated total RNA from 8 HCC cell lines by using the RNasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. A total of 5 μg of RNA was reverse-transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA). Semi-quantitative real-time PCR was performed with 10 ng of cDNA in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by using the SYBR® Green PCR Master Mix (Applied Biosystems). Gene expression levels were normalized to the expression of GAPDH. Oligonucleotide sequences of primers and PCR conditions are available on request.

**Statistics**

Associations between clinicopathological features and hypermethylated gene markers were analyzed using SPSS 13.0 for Windows. P values were based on Fisher’s exact test or Pearson’s chi-squared test. Survival was measured from the surgical resection day until death or the last clinical review before August 31, 2007. Overall survival was estimated by the method of the Kaplan-Meier log-rank test, and Cox proportional hazard analysis
was used to estimate multivariate relationships between several clinicopathological and hypermethylated gene markers. P < 0.05 was considered significant.

Ethics statement
This study was approved by the institutional review board of the Seoul National University Hospital (Approval No., C-1007-209-325). Written informed consent was exempted considering the retrospective nature of the study and minimal harm to the patients.

RESULTS
Pharmacological unmasking and microarray analysis in HCC cell lines
The expression profiles of 5 HCC cell lines (SNU398, SNU761, SNU878, HepG2, and Huh7) were obtained before and after treatment with either AZA or TSA by using a microarray platform (Fig. 1). To identify genes undergoing hypomethylation-dependent expression changes, we determined the expression fold changes of individual genes between mock-treated and TSA- or AZA-treated cells, and plotted TSA- and AZA-related fold changes on the x- and y-axis, respectively (Fig. 2). The DNA demethylating agent (AZA) induces reexpression of densely hypermethylated and transcriptionally inactive genes, whereas the class I and II histone deacetylase inhibitor (TSA) does not induce reexpression (25, 26). Of the genes that did not show an
increase in expression with TSA treatment (< 1.4-fold), subsets of genes displayed a peak of AZA-induced gene expression (> 2-fold). We considered the genes showing both < 1.4-fold expression with TSA treatment and > 2-fold expression with AZA treatment as candidate genes that might be inactivated by hypermethylation. In at least one of 5 cell lines, 793 genes were found to meet the selection criteria. Of these, genes that have no CpG islands in their promoters and proximal transcriptional start sites (TSS) as well as those genes whose methylation status in HCC had already been reported, were excluded from subsequent analysis (Fig. 1).

DNA methylation status of candidate genes in HCC cell lines
To identify whether the candidate genes (n = 443) are methylated in their promoter CpG island loci, we tried to design MSP primers by using the MSPrimer or MethPrimer software programs and successfully designed optimal primers for 380 genes. We analyzed the methylation status of these 380 genes in 8 HCC cell lines (SNU398, SNU475, SNU739, SNU761, SNU878, SNU886, HepG2, and Huh7) using MSP and found that 239 of 380 genes were methylated in one or more cell lines (Fig. 3), and that 167 genes were methylated in at least four cell lines. To identify the factors that might influence in their predisposition to DNA methylation, we compared the occupancy rate of Polycomb proteins (EED2 and SUZ12) and the frequency of the H3K27me3 modification between methylated genes and unmethylated genes in HCC cell lines by using the occupancy maps published for embryonic stem cells (27). Genes methylated in HCC cell lines had a higher frequency of SUZ12, EED, and H3K27me3 targets compared to genes not methylated in HCC cell lines (Fig. 4). Next, we counted the frequency of LINE-1 and ALU in upstream and downstream sequences around the transcription start site of the 380 genes and then compared the number of LINE-1 or ALU repeats between methylated and unmethylated genes in HCC cell lines. The number of ALU repeats was significantly higher in unmethylated genes than in methylated genes, whereas the number of LINE-1 was similar (Fig. 5).

Confirmation of methylation status in HCC tumor tissues
In order to confirm whether CpG island loci methylated in cell lines are also methylated in primary tumor tissues, we analyzed 10 paired HCC and adjacent nonneoplastic liver tissues for their methylation status in 239 genes using MSP (Fig. 6). We subdivided all the genes (n = 360) into 6 groups (named 0-5) based on the comparison of methylation frequencies of individual genes between HCC and nonneoplastic liver tissues: 1) higher methylation frequency in HCC than in nonneoplastic liver (group 1, n = 56), 2) fully methylated in both HCC and non-neoplastic liver (group 2, n = 96), 3) not fully methylated but identical meth-
ylation frequencies in HCC and non-neoplastic liver (group 3, n = 17), 4) genes not methylated in HCC or non-neoplastic liver (group 4, n = 18), 5) genes less frequently methylated in HCC than in non-neoplastic livers (group 5, n = 52), and 6) unmethylated genes in HCC cell lines (group 0, n = 141) (Fig. 6). To identify key biological functions associated with a list of genes within each group, we performed functional annotation analysis using the DAVID tool (http://david.abcc.ncifcrf.gov/) (Table 1). Group 1 genes showed a significant association with the neuropeptide signaling pathway, positive regulation of amine transport, and acid secretion, whereas group 5 genes exhibited a significant association with nucleosome assembly, chromatin assembly, protein-DNA complex assembly, chromatin assembly or disassembly, and tissue development.

**Correlation of promoter CpG island hypermethylation with down-regulation of gene expression**

To ensure that the microarray data represent real changes in ex-
Fig. 7. Effect of 5-aza-2’-deoxycytidine (AZA) and trichostatin A (TSA) on gene expression. RNA was isolated from hepatocellular carcinoma (HCC) cell lines left untreated or treated with AZA, TSA, or a combination of AZA and TSA. mRNA was reverse-transcribed, and gene expression was quantitated by real-time PCR and normalized to GAPDH expression.

Fig. 8. Methylation frequencies of 33 DNA methylation markers in HCC cell lines (n = 8) and tissue samples (10 paired HCC and non-neoplastic liver tissue samples). Methylation frequencies of these DNA methylation markers in HCC tissue samples were higher than those of non-neoplastic liver tissue samples: the differences were 20% or more.

Fig. 9. Methylation frequencies of 33 DNA methylation markers in hepatocellular carcinoma (HCC) tissue samples (n = 95). DNA methylation markers were distributed along the x-axis according to the decreasing order of methylation frequency.
Table 2. Correlation of promoter hypermethylated genes with clinical parameters

| Parameter                      | Value | p value |
|--------------------------------|-------|---------|
| Age (yr)                       |       |         |
| < 55 (n = 47)                  |       |         |
| ≥ 55 (n = 48)                  |       |         |
| p value                        | 0.20  | 0.03    |
| Gender                         |       |         |
| M (n = 75)                     |       |         |
| F (n = 20)                     |       |         |
| p value                        | 1.0   | 0.73    |
| Tumor size                     |       |         |
| < 6 cm (n = 45)                |       |         |
| ≥ 6 cm (n = 48)                |       |         |
| p value                        | 0.06  | 0.31    |
| AFP                            |       |         |
| < 100 (n = 48)                 |       |         |
| ≥ 100 (n = 43)                 |       |         |
| p value                        | 1.0   | 0.41    |
| GGT                            |       |         |
| < 56 (n = 48)                  |       |         |
| ≥ 56 (n = 47)                  |       |         |
| p value                        | 0.68  | 0.35    |
| Child-Pugh classification      |       |         |
| A (n = 86)                     |       |         |
| B (n = 9)                      |       |         |
| p value                        | 0.02  | 0.45    |
| Background liver               |       |         |
| Cirrhotic (n = 62)             |       |         |
| Noncirrhotic (n = 33)          |       |         |
| p value                        | 0.34  | 0.53    |
| Histologic grade               |       |         |
| 1.2 (n = 52)                   |       |         |
| 3.4 (n = 23)                   |       |         |
| p value                        | 0.25  | 1.00    |
| Microscopic vascular invasion  |       |         |
| Absent (n = 38)                |       |         |
| Present (n = 55)               |       |         |
| p value                        | 0.65  | 0.54    |
| Frequency (%)                  | 95    | 86      |

AFP, alpha-fetoprotein; GGT, serum γ-glutamyltransferase.
expression, we selected 7 genes (CRABP2, EFEMP2, HIST1H2AE, INA, NETO2, RASGRP2, and TPS3113) and analyzed their expression changes in HCC cell lines treated with AZA, TSA, or a combination of AZA and TSA using semi-quantitative real-time PCR (Fig. 7). Treatment with AZA alone resulted in induction of mRNA expression of these 7 genes. In some cases, the effect of combined AZA and TSA treatment was stronger than that of AZA or TSA alone. These data implicate that histone deacetylation may also be involved in the inhibition of gene transcription by DNA methylation.

**Correlation of DNA methylation markers with clinicopathological features of HCC**

Genes with methylation frequencies higher in HCC than in non-neoplastic liver tissues are more likely to play an important role in hepatocarcinogenesis. To identify a possible correlation of gene hypermethylation with clinicopathological features of HCC patients, we selected 33 genes showing a methylation difference higher than 19% between HCC and nonneoplastic liver samples (Fig. 8) and analyzed their methylation statuses in another set of HCC tissue samples (n = 95) by MSP. Hypermethylation was detected in 1 or more genes in all HCC samples. RNASE4 showed the highest methylation frequency (90%), followed by DUOX1 (82%), CRABP2 (66%), and NETO2 (61%) (Fig. 9). The methylation status of the DNA methylation markers was correlated with clinicopathological features such as age, gender, tumor size, AFP and GGT levels, microscopic vascular invasion, and clinical outcome. The results of these association studies are summarized in Table 2. The number of methylated genes was significantly higher in older (≥ 55 yr) than in younger patients (11.7 vs 8.8, P = 0.030 by Student's t-test). NETO2, DHDH, MAP6D1, C1orf59, CYB5R2, ULBP1, SPINT1, and ZNF342 were more frequently hypermethylated in tumors from patients older than 55 yr than in tumors from patients younger than 55 yr (P < 0.05 for all genes). HSPA12B was more frequently methylated in tumors from female patients than in tumors from male patients (P = 0.008). RNASE4 was more frequently methylated in tumors smaller than 6 cm than in HCC tumors bigger than 6 cm (P = 0.018). The methylation frequencies of NETO2, DHDH, and SPINT1 were significantly higher in HCC cases with AFP production smaller than 100 ng/mL than in HCC cases with AFP production higher than 100 ng/mL, whereas the methylation frequency of RASGRP2 and HSPA12B was significantly lower in HCCs with low AFP production (< 100 ng/mL). INA, PPP1R14A, and ECEL1 were more frequently methylated in HCC patients with low serum GGT levels (< 56 ng/mL) than in HCC patients with high serum GGT levels (≥ 56 ng/mL) (P < 0.05 for all genes). The methylation frequencies of C1orf59, OXTR, and CYB5R2 were significantly lower in HCC tumors with microscopic vein invasion than in HCCs without microscopic vein invasion (P < 0.05 for all genes).

**Correlation of DNA methylation markers with clinical outcome**

Correlation of DNA methylation markers with patient survival was analyzed in 90 patients. Five patients were excluded because of follow-up loss. Of the 33 CpG loci, HIST1H2AE and EFEMP2 exhibited an association between gene hypermethylation and poor prognosis (P = 0.022 and P = 0.081, respectively, by Kaplan-Meier log-rank test) (Fig. 10, Table 3). Among several other clinicopathological factors, tumor size, serum GGT levels, and microscopic vascular invasion showed prognostic significance. Taking into account these 3 clinicopathological factors, HIST1H2AE and EFEMP2 were included into a multivariate analysis.

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**Fig. 10.** Kaplan-Meier survival curves of 90 hepatocellular carcinoma (HCC) patients. Correlation of (A) HIST1H2AE, and (B) EFEMP2 methylation status with overall survival.
to identify independent predictors of overall survival (Table 4). Multivariate analysis by using the COX proportional hazards model revealed that HIST1H2AE (P = 0.010) and EFEMP2 (P = 0.020) methylation status are significant variables affecting the overall survival of HCC patients. The methylation status of these two genes suggested about 2.6-fold increased risk as compared to patients who had these genes unmethylated.

**DISCUSSION**

In this study, we conducted a genome-wide microarray expression screening in 5 HCC cell lines treated with AZA or TSA and characterized a set of methylation-regulated genes. We were able to identify 221 novel DNA methylation markers in HCC. At the same time, we observed that a significant number of genes up-regulated after AZA treatment, were actually not methylated in HCC cell lines and primary HCC tissue samples. While the mechanism of how AZA reactivates expression of unmethylated genes has not been elucidated yet, several possibilities exist. First, the up-regulation of unmethylated genes might be secondary to AZA-induced expression of upstream genes that encode transcription factors and are inactivated by promoter CpG island hypermethylation. Second, the expression of these genes might be regulated by the methylation status of CpG island shores rather than promoter CpG islands (28). Lastly, AZA might induce degradation of retinoblastoma protein (pRb) through the MDM2-dependent proteasome pathway. Because loss of pRb protein induces a significant decrease in recruitment of G9a and SUV39H1 to histones around the promoter of target genes and thus reduces H3K9 di- and tri-methylation, pRb degradation might result in re-expression of several silenced genes (Dr. Wei-Guo Zhu, personal communication).

In the present study, of the 380 genes containing CpG island loci in their promoter sequences and for which MSP oligonucleotide primers could be designed, 239 genes were found to be methylated in at least 1 HCC cell line. The remaining genes were found to be unmethylated in all HCC cell lines. The causes of selective predisposition to methylation are at present unclear. However, genome architecture has been recently proposed to strongly correlate with predisposition to DNA methylation. Specifically, genes with low frequency of retrotransposons (ALU and LINE-1) near the transcription start site or regulated by Polycomb-group proteins were significantly correlated with methylation status, whereas LINE-1 was not.

We analyzed 95 HCC samples for their methylation status in 33 CpG island loci in their promoter sequences and found that HCC tumors from older patients harbored more methylated genes than HCC tumors from younger patients. In addition, we found that 2 DNA methylation markers (HIST1H2AE and EFEMP2) are closely associated with poor prognosis of HCC patients. HIST1H2AE is a member of the histone H2A family. Histones are responsible for nucleosome structure and their appropriate balance is required for the correct assembly of chromatin as well as for proper cell division and growth. H2A, one of the core histones, has nonallelic variants that are related by simple amino acid substitution (33). It is not clear whether these variants have functional signif-

### Table 3. Univariable analysis of clinicopathological characteristics with regard to survival of hepatocellular carcinoma (HCC) patients. The P value was determined by the Kaplan-Meier method

| Characteristics                  | No. of patients | 3-yr survival rate (%) | 5-yr survival rate (%) | P value |
|----------------------------------|-----------------|------------------------|------------------------|---------|
| Sex                              |                 |                        |                        |         |
| M                                | 71              | 56                     | 43                     | 0.782   |
| F                                | 19              | 45                     | 45                     |         |
| Age (yr)                         |                 |                        |                        |         |
| < 55                             | 44              | 41                     | 33                     | 0.014   |
| ≥ 55                             | 46              | 66                     | 53                     |         |
| Tumor size (cm)                  |                 |                        |                        | < 0.001 |
| < 6                              | 41              | 72                     | 61                     |         |
| ≥ 6                              | 47              | 38                     | 28                     |         |
| Background liver                 |                 |                        |                        |         |
| Cirrhosis                        | 60              | 46                     | 35                     | 0.037   |
| Chronic hepatitis                | 30              | 69                     | 60                     |         |
| Child-Pugh score                 |                 |                        |                        | 0.136   |
| A                                | 81              | 57                     | 47                     |         |
| B                                | 9               | 27                     | 14                     |         |
| C                                | 0               | -                      | -                      |         |
| GGT (IU/L)                       |                 |                        |                        |         |
| < 56                             | 44              | 65                     | 59                     | 0.012   |
| > 56                             | 46              | 44                     | 30                     |         |
| Microscopic invasion             |                 |                        |                        | 0.014   |
| Absent                           | 37              | 60                     | 60                     |         |
| Present                          | 51              | 46                     | 33                     |         |
| HIST1H2AE                        |                 |                        |                        | 0.022   |
| Unmethylated                     | 76              | 57                     | 47                     |         |
| Methylated                       | 14              | 35                     | 26                     |         |
| EFEMP2                           |                 |                        |                        | 0.080   |
| Unmethylated                     | 77              | 58                     | 45                     |         |
| Methylated                       | 13              | 31                     | 31                     |         |
| Histologic grade (Edmondson-Steiner) |         |                        |                        | 0.408   |
| 1, 2                             | 52              | 60                     | 45                     |         |
| 3, 4                             | 23              | 44                     | 44                     |         |
| AFP (ng/mL)                      |                 |                        |                        | 0.091   |
| < 100                            | 47              | 59                     | 50                     |         |
| > 100                            | 39              | 46                     | 32                     |         |

### Table 4. Multivariate analysis of factors associated with poor survival in HCC patients

|                      | β   | SE  | Hazard ratio (95% confidence interval) | P value |
|----------------------|-----|-----|--------------------------------------|---------|
| Tumor size           | 0.913 | 0.359 | 2.491 (1.231-5.037)                 | 0.011   |
| GGT                  | 0.746 | 0.328 | 2.109 (1.109-4.012)                 | 0.023   |
| Microscopic vascular invasion | 0.668 | 0.359 | 1.951 (0.966-3.941)                 | 0.062   |
| EFEMP2 methylation   | 0.962 | 0.413 | 2.618 (1.166-5.878)                 | 0.020   |
| HIST1H2AE methylation | 0.982 | 0.380 | 2.670 (1.268-5.624)                 | 0.010   |
icance and how the expression of individual histone genes is controlled. Among the 16 replication-dependent histone H2a genes, *HIST1H2AE* is included in the largest histone gene cluster (HIST1, including 55 histone genes) located on human chromosome 6 (34, 35). Previous studies have found that the expression of histone H2a genes is related to histone gene clusters with different promoters and different transcription factor binding sites in mouse hepatoma cell lines. However, the structure in the promoters and transcription binding sites responsible for such differential expression has not been determined yet. On the other hand, it has been reported that the H2a genes are regulated by histone H3K9 acetylation levels in the promoter regions (36). Similarly, we suggest that HIST1H2AE transcription might be influenced by histone acetylation based on the experimental results obtained by RT-PCR, using AZA-, TSA- or combined AZA/TSA-treated cell lines. The combinatory treatment showed to act synergistically in the reexpression of HIST1H2AE.

EFEMP2, also known as fibulin-4, is a novel extracellular matrix protein that belongs to the fibulin protein family (fibulin1-7). EFEMP2 is involved in the stabilization and organization of the extracellular matrix. Moreover, missense mutations of EFEMP2 (G169A) cause human genetic disorders (37-39). However, the role of EFEMP2 in driving tumorigenesis is still unclear and requires further study. A recent study has reported limited EFEMP2 methylation in melanoma cell lines (40). In contrast, our data have demonstrated a correlation of its methylation status with poor survival of HCC patients.

In conclusion, we have identified 221 novel DNA methylation markers for HCC by using a pharmacological unmasking microarray approach along with MSP. Of these, 55 DNA methylation markers are more frequently methylated in HCC than in adjacent nonneoplastic liver tissues. The biological functions of these genes are associated with neuroptide signaling pathways and positive regulation of amine transport. HIST1H2AE methylation is strongly associated with poor prognosis of HCC patients. A future independent study is required to validate HIST1H2AE methylation as a prognostic marker for HCC.

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