Identification of Novel Methylation Markers in Hepatocellular Carcinoma using a Methylation Array

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INTRODUCTION

A CpG island is an approximately 1-kb DNA sequence with a high density of CpG dinucleotides. About 70% of human genes are known to harbor CpG islands in their promoter sequences (1, 2). In normal cells, promoter CpG islands are usually protected from aberrant hypermethylation, except for those on imprinting genes or genes of inactivated X chromosomes (3, 4). However, in association with carcinogenesis, hundreds of promoter CpG islands undergo aberrant hypermethylation, which represses gene transcription of active genes or enforces suppression of already inactive genes (5). Promoter CpG island hypermethylation has become recognized as an important mechanism for inactivating tumor suppressor genes or tumor-related genes in human cancers of various tissues. Gene inactivation in association with promoter CpG island hypermethylation has been reported to be four times more frequent than genetic changes in human colorectal cancers. Hepatocellular carcinoma is also one of the human cancer types in which aberrant promoter CpG island hypermethylation is frequently found. However, the number of genes identified to date as hypermethylated for hepatocellular carcinoma (HCC) is fewer than that for colorectal cancer or gastric cancer, which can be attributed to fewer attempts to perform genome-wide methylation profiling for HCC. In the present study, we used bead-array technology and coupled methylation-specific PCR to identify new genes showing cancer-specific methylation in HCC. Twenty-four new genes have been identified as hypermethylated at their promoter CpG island loci in a cancer-specific manner. Of these, TNFRSF10C, HOXA9, NPY, and IRF5 were frequently hypermethylated in hepatocellular carcinoma tissue samples and their methylation was found to be closely associated with inactivation of gene expression. Further study will be required to elucidate the clinicopathological implications of these newly found DNA methylation markers in hepatocellular carcinoma.

Key Words: Bead Array; CpG Islands; DNA Methylation; Carcinoma, Hepatocellular
MATERIALS AND METHODS

Cell lines and 5-aza-dC treatment
We used eight different human HCC cell lines (SNU398, SNU475, SNU739, SNU761, SNU878, SNU886, HepG2, and Huh7) obtained from the Korean Cell Line Bank (Seoul, Korea). All of the cell lines except Huh7 were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Huh7 was grown in DMEM medium supplemented with 10% fetal bovine serum. All cell lines were cultured in a humidified 37°C, 5% CO2 incubator. The cell lines were seeded at $3 \times 10^5$/mL in their respective culture media and treated with 1 µM and 5 µM 5-aza-dC (Sigma Chemical Co., St. Louis, MO, USA) for 96 hr; media and drugs were replaced every 24 hr. As a control, cell lines were mock-treated in parallel with the addition of an equal volume of PBS without the drug. We prepared total RNA using the RNeasy Mini kit (Qiagen, Valencia, CA, USA).

Table 1. Primers for methylation-specific polymerase chain reaction

| Gene   | Forward primer | Reverse primer | Tm (°C) | Product (bp) |
|--------|----------------|----------------|---------|--------------|
| ADAMTS12 | TTTATTTTATATTTCGTCGAAAGCG | ACGACTCAAAAACTACCGCG | 61      | 135          |
| ADCAP1 | GGGTTGTTATTGATTTGCGTCG | CCCCCAATAACACTACCGAACAACG | 59      | 118          |
| CTSL   | TTTGCGATATGAATATATCTGCACTGC | AACCTTACCTTAAACCTGCGTTTACGC | 57      | 115          |
| DST    | TTTTTATGATGATGGTTTTTGCATGAC | CCAGAATCCCAAAAAAGGACG | 59      | 103          |
| F2R    | TTTTTATTTTAGAGGGGTTTTCGAGAC | TTCTCTCATAACCGTTACTTCG | 60      | 124          |
| FG3    | GGGATGTTCGGTCTGGTTTTCG | CCGCCGCGTAAACTTTACCG | 59      | 116          |
| FLT3   | GGAGTTGCGGGTGTCGC | CCCCAAAACAAAAAGGACGACCGAAACG | 59      | 134          |
| FLT4   | CGATTGTGGCCGGTATGCAGGC | AAACCGAACCGGAAAAAGGACG | 59      | 105          |
| FZD9   | CGGCGGGATTATTTTATTTCGAGTGTCG | CCCCCGAACGGAAACTCTCG | 60      | 123          |
| GP1BB  | TAGCGGCGTTGCGAGTGTC | CTTCAACAAAAATACCGGAAAAACG | 59      | 138          |
| HIC2   | GGGTTGTGTTGGCTGGTTTTTTCG | CGGAGCTGAAAGGAGGACG | 59      | 115          |
| HOXA9  | GGGCGGTTTGGCGTTTTATTCG | AAACCTCGCGAAAAAGGACG | 60      | 122          |
| HS3ST2 | GGAGGGTTCGATGATTTTCGC | CATCACTACAAAAATACCGGAAAAACG | 59      | 117          |
| ID1A   | GCTGATGCGCTGGTCGCG | ACSTCGAATGAGCAACCGAAG | 59      | 130          |
| IG2AS  | CGGGGTTGCTGGCTGGTCG | TAAACGGCGCGCAAACGACG | 59      | 131          |
| IHH    | TTATGATGCATGTTGATGTC | GAACGGAAAAAATACCGGAAAAACG | 59      | 111          |
| IRAK3  | CGGTTTTTGGCTTTGTTTGTGC | CGGAGCAGCTCTAAGCTTACAAG | 60      | 116          |
| IRF5   | AATTGAGTATTGAGCGGGCAGATC | CTCAAAAAAAATACGCAAAAGAAG | 59      | 108          |
| MCM2   | TTTGAGTTGTGCTTTTGTTCG | CGGAGCAGCCTCTAAGCTTACAAG | 58      | 139          |
| MLF1   | TTATGATGCATGTTGATGTC | GAACGGAAAAAATACCGGAAAAACG | 60      | 113          |
| NF1    | TTAGGATACCGCGAGGTCG | CTGATTGCTCAACCTGACGATGC | 60      | 109          |
| NOTCH3 | GTGAGGGTTGGGGGAGGTCG | GCAAACTCTGGCAGAAAGGACG | 59      | 120          |
| NPY    | AAAAGAAGAGAGAGAGAGAGGACG | CTAACGCTGAAAGGAGGACG | 59      | 120          |
| PDGFBR | TTAATGATGCAGGCTGGGCCG | AAACGAAAAAATACCGGTTAC | 62      | 139          |
| PFG    | CGGGCGGTTGCTGGCTGGTCG | CGGAGCAGCCTCTAAGCTTACAAG | 61      | 103          |
| PLASL1 | GCCGGCGTTGATAGAGAGTTCGCG | GACCGCGAATGCGCGCG | 61      | 107          |
| PTCH2  | GTATTTGCTGATATACTGGGCG | GCCATGCAAACGCGGAA | 59      | 134          |
| RBB1   | GATTATTTGCTGATATTTTGTCG | AGGATATAGCTGGAAATACGAAAGAAG | 59      | 114          |
| SH3BP2 | TTTAGGTTGTTTCGCGGC | CCCGCGGAAATGCGGAA | 59      | 108          |
| TAL1   | ATTTGAGGAGGGGATACG | GCAGAAAAATACCTAAAGGCTT | 62      | 108          |
| TESK2  | TAGGGCATTGTTTTCGCGGTCG | TACCATAGCCTAAGCACG | 62      | 134          |
| TIAM1  | TTTTTAGGTTGTTTCGCGGC | GACGCGTATTGCTAAGC | 59      | 110          |
| TNFRSF10C | TAAAGGGTGAAGAGGCGGTTTATTCG | ACGCGCGTCCTAAAGTATG | 59      | 104          |
| WNT2   | TTGAGGTTGATTTTTTTCGCGGTCG | CCAATTCCAAAACGCAGGAAAG | 59      | 115          |
| ZP3    | GGGTTGCTGCTGGTCTGTTAC | TCAACTGAACTGAAAGCCACATC | 59      | 106          |
bisulfite-converted genomic DNA. Bisulfite modification of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Similar to methylation-specific PCR, two primer sets are designed for each CpG target; one primer set corresponds to the unmethylated, bisulfite-converted sequence (uracil) while the other corresponds to the unconverted sequence (5-methyl cytosine). The methylation level (beta value) is determined by the ratio of the fluorescent signals from methylated and unmethylated alleles. The beta value identifies the level of DNA methylation at a CpG site, ranging from 0 in the case of almost all unmethylated sites to 1 for completely methylated sites (12). Of 1,505 CpG sites (selected from 807 genes) included in GoldenGate Methylation Cancer Panel I, 1,044 of the CpG sites are located within CpG islands, and 461 are located outside of CpG islands. Comparing the average beta values for the 1,044 CpG sites between HCC and non-neoplastic liver tissues, cancer-specific hypermethylation was determined to be present when the average beta value for a CpG site was significantly greater ($P<0.05$) for the HCC samples (n=5) than for the non-neoplastic liver tissues (n=5).

**Methylation-specific polymerase (MSP) chain reaction**

For MSP analysis, DNA was extracted following a standard phenol-chloroform extraction method. Bisulfite modification of genomic DNA was carried out using the EZ DNA methylation Kit (Zymo Research). Primers were designed using two web sites, MSPPrimer (http://www.mspprimer.org) and MethPrimer (http://www.urogene.org/methprimer). Primer sequences and PCR conditions are shown in Table 1. MSP was performed as previously described (13).

**Quantitative RT-PCR**

Total RNA was prepared using the RNeasy (Qiagen) kit according to the manufacturer’s protocols. A total of 5 μg of RNA was reverse transcribed using Oligo dT and Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR amplification reactions were performed using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with a 7300 Real-Time PCR System (Applied Biosystems). The expression levels of the genes were normalized to the expression of GAPDH. Primer sequences and PCR conditions are shown in Table 2.

**RESULTS**

**Microarray analysis of methylated genes**

The methylation profiles of five pairs of HCC and non-neoplastic liver tissues were analyzed for 807 genes using the Illumina GoldenGate Methylation Solution. In total, 72 annotated genes (81 CpG sites) were found to be hypermethylated in tumor tissues. After excluding genes without a CpG island in the promoter (18 genes), imprinted genes (2 genes), and genes for which methylation of their promoter CpG island loci has already been reported for HCC (17 genes), we explored DNA hypermethylation for the remaining 36 genes using MSP. These 36 genes were consid-

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**Table 2. Primers for quantitative RT-PCR**

| Gene    | Forward primer | Reverse primer | Tm (°C) | Product (bp) |
|---------|----------------|----------------|---------|--------------|
| ADAMTS12| TGCAATCCACATTGTGTGG | GTGAGGCTGACACATTCTG | 59      | 253          |
| ADCAIP1 | GATCCCTACAGGAACTCAAG | GTTTGAGATBAAACACAGGAC | 60      | 226          |
| D2T     | AGCAAGAGACGCATCTGAC | AATTGGCTACTCTCGGAC | 56      | 270          |
| F9G3    | TGTGGATAGCTGCTCCAGTA | CCAGATCTGCGGAGCTTCC | 56      | 448          |
| FLT3    | AGACTGTAGTTCCTCAAGC | TCCCAAGTAATTCCTCATG | 55      | 308          |
| F2D9    | TGGCCCTCTTCGCTGATCT | GGGCCAGCTGCTAGAGAGT | 62      | 164          |
| GAPDH   | CAATGGCCCTCATTGACCC | TGGAAGATGGTGATGGGATT | 55      | 135          |
| H2F     | TGGCGAAGAGACCTCAAGGA | AGGTGGACCTCTGAAATTT | 59      | 251          |
| HDX9    | GGCGCCTCTCTGAAACAT | CCAAGATCTGCTGCTTCT | 60      | 242          |
| HSSST2  | GGATCCCTCCTGGTGAAAAA | TGGCAACTCAAGGTGGAAA | 59      | 302          |
| IRF5    | TTTGAAATGCAAGGAGTCGTA | GCAATGGCTTATGGAAGCAA | 59      | 365          |
| MLF1    | CAGGGAGCTTCCTCCTGAGG | AAGATCTCCACTCCTGCTT | 68      | 237          |
| NOTCH3  | GSGCTTCAAGGCTCCTATCG | AATGTCACCTGGGAAATG | 64      | 245          |
| NPY     | AACCTCTATTACAGGAGAGA | CTGAATGGCTGATGAGATG | 60      | 220          |
| PDGFRB  | GGGCTAGACGGGAGGATA | GATCATAGGCGAGAGAGA | 59      | 250          |
| PTOH2   | GTGGAAAGTGGCCTCTTCGG | TCCCAAGACTCTCTATGAG | 55      | 301          |
| RBP1    | GTGGGAAGAGGGTTGGGAGA | GCTCAGACACTGGTCGATT | 59      | 258          |
| SH3BP2  | ATCCACATCAGGAAAGGCAA | GAATGCTCATCCCTGCTT | 68      | 251          |
| T1L     | AAGGAGAGACCTCCCTCCA | CCTCCCTCCCTGCTGCTT | 66      | 247          |
| T1AM1   | AAGGAGCTCTACGGCCATGCC | GACCCAAATGTCGCACTG | 61      | 252          |
| TNRRI10C| GTATCACCAACCGCTTCAA | TGGCAGAAATCTCCTTAC | 59      | 248          |
| WNT2    | CAGAGGCAAGGAGGTTTATA | CAAACATGCTGCTGCTG | 61      | 202          |
| ZP3     | CAGAATGGCCCTCCCATGCA | ATCGGGCTCTGGTCAAGCT | 61      | 210          |
Validation of the methylation status of candidate genes in the HCC cell lines using MSP
For MSP analysis, we attempted to design primers for the 36 genes using both MSPPrimer (http://www.mspprimer.org) and MethPrimer (http://www.urogene.org/methprimer). However, for one gene (HOXA9), neither tool was able to design primers, because it has a short CpG island in the promoter. Therefore, we examined the methylation status of the remaining 35 genes in the 8 HCC cell lines. It was found that 26 of the genes (HOXA9, TNFRSF10C, NPY, TIAM1, PDGFRB, IRAK3, SH3BP2, IRF5, HIC2, TAL1, HS3ST2, MLF1, IGF2AS, ADCYAP1, FGFR3, WNT2, ADAMTS12, FLT3, PTCH2, GP1BB, RBP1, FZD9, DST, NOTCH3, PLAGL1, and ZP3) were methylated in at least one HCC cell line (26 of 35; 74.3%). The remaining 9 genes (TESK2, IHH, MCM2, CTSL, F2R, PGF, NGFR, FLT4, and ICA1) were not found to be methylated in the HCC cell lines using the MSP assay (Figs. 2, 3).

Confirmation of hypermethylation of newly developed candidate genes in liver tissues
To determine whether the genes that were hypermethylated in the HCC cell lines were hypermethylated in a cancer-specific manner, we analyzed the methylation status of the 26 genes in 18 normal liver samples and 50 primary HCC samples using methylation-specific PCR. Of the candidate 26 genes that showed methylation in the cell lines, promoter methylation was detected in 25 of the genes in primary tumor tissues. In contrast, only five genes (5 of 26; 19.2%) were methylated in normal tissues. Moreover, all but one of these genes (IGF2AS) were methylated at low frequencies in the non-neoplastic liver tissues (Fig. 4).

Fig. 1. Flow chart for selection of candidate methylation markers. We used 5 paired hepatocellular carcinoma/normal tissue samples to screen for candidate methylation markers using a methylation array. We obtained 72 candidates that showed significant hypermethylation in hepatocellular carcinoma tissues. We removed genes with no CpG island loci in their promoters, imprinted genes, and genes for which methylation status was already known for hepatocellular carcinoma. Thus, we selected 36 genes to further examine for methylation analysis using methylation-specific PCR.

Fig. 2. Representative examples of MSP analysis of DST, FLT3, PTCH2 and TIAM1 in 8 HCC cell lines. DNA extracted from 8 HCC cell lines were amplified with primers specific to the methylated (M) or unmethylated (UM) CpG islands of each gene after modification with sodium bisulfite. +, positive control; DW, distilled water. Positive controls for methylated MSP and unmethylated DNA are M.SssI-treated placental DNA and whole-genome amplified DNA, respectively.

Fig. 3. Summary of methylation-specific PCR for hepatocellular carcinoma (HCC) cell lines. We examined eight HCC cell lines for methylation status of the 35 genes and found that 26 of the genes (HOXA9, TNFRSF10C, NPY, TIAM1, PDGFRB, IRAK3, SH3BP2, IRF5, HIC2, TAL1, HS3ST2, MLF1, IGF2AS, ADCYAP1, FGFR3, WNT2, ADAMTS12, FLT3, PTCH2, GP1BB, RBP1, FZD9, DST, NOTCH3, PLAGL1, and ZP3) were methylated in at least one HCC cell line. Data are color-coded as follows: gray fill indicates the presence of methylation, whereas white fill indicates the absence of methylation.

Fig. 4. Summary of methylation-specific PCR results in hepatocellular carcinoma (n=50) and non-neoplastic liver tissue samples (n=18). Out of 26 genes showing methylation in HCC cell lines, promoter methylation was detected in 25 genes in primary tumor tissues. Data are color-coded as follows: gray fill indicates the presence of methylation, while white fill indicates absence of methylation.
Thus, the vast majority of the genes were found to show cancer-specific methylation at frequencies of 6%–98% (Fig. 5). Thus, 24 new cancer-specific CpG island loci were identified through our approach (Table 3).

**Gene expression and induction after 5-aza-dC treatment in HCC cell lines**

For hypermethylated and transcriptionally silenced genes, the DNA demethylating agent 5-aza-dC is known to induce gene re-expression (14, 15). To identify whether promoter CpG island hypermethylation was closely associated with gene expression in the 24 newly identified cancer-specific methylated genes, we treated the 8 HCC cell lines with 5-aza-dC (1 and 5 µM for 96 hr) and performed quantitative RT-PCR of the mock-treated and 5-aza-dC treated cell lines.

**Table 3. New cancer-specific methylated genes**

| Genes          | Gene name                      | Loci                  | Function                                                                 |
|----------------|--------------------------------|-----------------------|--------------------------------------------------------------------------|
| ADAMTS12       | ADAM metallopeptidase with thrombospondin type 1 motif, 12 | 5q35                  | Metal ion binding, metalloendopeptidase activity, zinc ion binding       |
| ADCYAP1        | Adenylate cyclase activating polypeptide 1 | 18p11                | Hormone activity, neuropeptide hormone activity                           |
| DST            | Dystonin isoform 1B            | 6p12−p11              | Actin binding, calcium ion binding, integrin binding, protein binding     |
| FGFR3          | Fibroblast growth factor 3     | 11q13                 | Growth factor activity, protein binding                                  |
| FLT3           | Fms-related tyrosine kinase 3  | 13q12                 | ATP binding, nucleotide binding, receptor activity                       |
| FZD9           | Frizzled 9                     | 7q11.23               | G-protein coupled receptor activity, Wnt receptor activity               |
| GP1BB          | Glycoprotein Ib (platelet), beta polypeptide | 22q11.21             | Protein binding, transmembrane receptor activity                        |
| HIC2           | Hypermethylated in cancer 2    | 22q11.21              | DNA binding, metal ion binding                                          |
| HDX9           | Homeobox protein A9 isomor b   | 7p15–p14              | Protein binding, sequence-specific DNA binding, transcription factor activity |
| HS3ST2         | Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 2 | 16p12                | Sulfotransferase activity, transferase activity                        |
| IRAK3          | Interleukin-1 receptor-associated kinase 3 | 12q14.3              | ATP binding, identical protein binding, magnesium ion binding           |
| IRF5           | Interferon regulatory factor 5 isomor b | 7q32                 | RNA polymerase III transcription factor activity                        |
| MLF1           | Myeloid leukemia factor 1      | 3q25.1                | Protein binding, protein domain specific binding                        |
| NOTCH3         | Notch homolog 3                | 19p13.2–p13.1         | Calcium ion binding, receptor activity                                  |
| PDGFRB         | Platelet-derived growth factor receptor beta | 5q31−q32             | ATP binding, nucleotide binding, platelet activating factor receptor activity; protein binding; |
| PTCH2          | Patched 2                      | 1p33–p34              | Hedgehog receptor activity                                               |
| RBP1           | Retinol binding protein 1      | 3q23                  | Lipid binding, retinal binding, retinol binding                         |
| SH3BP2         | SH3-domain binding protein 2   | 4p16.3                | SH3/SH2 adaptor activity                                                |
| TAL1           | T-cell acute lymphocytic leukemia 1 | 1p32                 | DNA binding, transcription regulator activity                            |
| TIAM1          | T-cell lymphoma invasion and metastasis 1 | 21q22.11            | Rho guanyl-nucleotide exchange factor activity                          |
| TNFRSF10C      | Tumor necrosis factor receptor superfamily, member 10c | 8p22–p21            | GPI anchor binding, transmembrane receptor activity                     |
| WNT2           | Wingless-type MMTV integration site family member 2 | 7q31                 | Extracellular matrix structural constituent, signal transducer activity |
| ZP3            | Similar to Zona pellucida sperm-binding protein 3 | 7q11.23          | Acrosin binding, receptor activity                                      |

Fig. 5. Comparison of hypermethylation frequencies for 26 genes. Hepatocellular carcinoma (HCC) cell lines (white column), HCC tissue samples (gray column), and non-neoplastic liver tissue samples (HCN, black column).
down-regulated by 5-aza-dC. In addition, HepG2 and SNU-761 in WNT2, HepG2 in ADAMTS12, and SNU-878 in FLT3 were not re-expressed (Fig. 6). In such cases, histone modification may be related to transcription suppression (16, 17).

**DISCUSSION**

Although many studies have reported aberrant hypermethylation of genes in HCC, for example, identifying E-cadherin, RASSF1A, GSTP, SOCS1, SFRP1, and PTEN as tumor suppressor gene silenced by hypermethylation, most of these studies were limited to analysis of a single or a few specific genes (8-10). Therefore, the true extent of promoter CpG island hypermethylation in HCC remains largely unknown. With advancements in microarray technology, the number of genes found to be hypermethylated in HCC in a cancer-specific manner is expected to increase exponentially, leading to a better understanding of epigenetic modulation of tumor-related genes in hepatocarcinogenesis. The GoldenGate bead-array technology incorporates a strong analytical methylation platform and provides reliable and highly reproducible data (12). Here, we present identification of new genes demonstrating cancer-specific methylation in HCC through the use of bead-array technology. The fidelity of the bead-array results was confirmed using methylation-specific PCR for genes selected according to our criteria (Fig. 1). As a result of the present approach, 24 genes were newly identified as cancer-specific methylation loci for HCC.

Table 3 summarizes the functions of the genes that were newly identified to be hypermethylated in a cancer-specific manner in HCC. TNFRSF10C, HOXA9, NPY, and IRF5 were frequently methylated in our panel of cell lines and HCC tissues and their methylation was correlated with low expression levels or silencing.
ing. These genes were re-expressed in the majority of the cell lines after 5-aza-dC treatment. Therefore, these genes are considered to be repressed by DNA hypermethylation in their promoter CpG islands. Although it is unclear whether TNFRSF10C plays a pro-apoptotic role or an anti-apoptotic role in tumors, hypermethylation of TNFRSF10C has been reported in various human cancers, including breast, lung, prostate, and neuroblastomas (18). HOXA9 encodes a DNA-binding transcription factor that may regulate gene expression, morphogenesis, and differentiation. Expression of HOXA9 proteins is spatially and temporally regulated during embryonic development (19). HOXA9 promoter CpG island methylation has been reported in ovary and lung cancer (20, 21). NPY is involved in cell motion and cell proliferation as well as neuropeptide hormone activity (22, 23).

In prostate cancer, low NPY expression is closely associated with aggressive clinical behavior (24). In another recent study, NPY was shown to be frequently methylated in neuroblastomas (25). IRF5, a member of the interferon regulatory factor family, is known to be related to innate immune system activity and is also a critical regulator of DNA damage-induced apoptosis (26). Because IRF5 is critical for induction of apoptosis, IRF5 deficiency predisposes cells to tumorigenic transformation (27). However, methylation of IRF5 in cancer has rarely been reported.

Recently, the role of chromatin structure and epigenetic alterations in controlling gene transcriptional activity during embryonic stem (ES) cell self-renewal and differentiation has been intensively investigated (28, 29). Recent studies have attempted to identify the relationships between DNA methylation, histone modifications, and promoter occupancy of pluripotent regulators such as Pcg in regulation gene expression in ES cells (30). Gene promoter chromatin patterns, including Pcg-mediated repressive histone modifications, may render certain genes vulnerable to DNA hypermethylation. These changes may enhance the likelihood of tumor initiation and progression from cell transformation (30). To identify whether polycomb repressive complex 2 (PRC2) occupancy information obtained from embryonic stem cells may predict the vulnerability of individual CpG island loci to hypermethylation, we compared the average methylation level (beta value) of each CpG island locus between normal tissues and tumor tissues and correlated it with PRC2 occupancy. In PRC2-positive CpG islands, the methylation level of the tumor group was higher than the methylation level of the normal group. In addition, the differences between the two groups were larger when the CpG island loci were occupied by two or more components of the PRC2 complex. However, there were no differences among PRC2-negative CpG islands (Supplementary Table 1). Thus, PRC2 may indicate that these genes are preferred substrates for targeted methylation.

In addition to hypermethylation of promoter CpG island loci, some hypomethylated CpG sites were also identified through comparison of methylation levels (bead array-determined beta values) of CpG sites in HCC. CpG sites showing hypomethylation in a cancer-related manner appear to be different from those that were hypermethylated with respect to their relationships with CpG island loci. In the present study, we found that 69 genes (83 CpG sites) were significantly hypomethylated in tumor tissues. Of these 83 CpG sites, only 23 sites (27.7%) were located in promoter CpG island loci, in contrast with the 63 of 81 (77.8%) hypermethylated CpG sites that were located in CpG island loci (Supplementary Table 2). The identified hypomethylated genes tend to be involved in biological processes of immunity, neuronal activity, lipid metabolism, and transport, whereas the hypermethylated genes tend to be involved in nucleic acid metabolism, cell proliferation, differentiation, and the cell cycle. For example, IL16, involved in chemokine-mediated immunity, was significantly hypomethylated in HCC compared to non-neoplastic liver tissues (P<0.003). GABRA5, with neurotransmitter receptor activity, was hypomethylated (P<0.001). APOC1, which processes lipid metabolism and transport, was hypomethylated (P<0.001). In addition, certain oncogenes, including HGF, BLK, and PGR, were hypomethylated in HCC. Interestingly, hypomethylation of histone deacetylase 1 (HDAC1), which plays a key role in the regulation of eukaryotic gene repression, was prominent and had the largest significant difference in methylation levels between HCC and non-neoplastic liver tissues (P<0.001).

Supplementary Table 2. The summarization of the number of CpG sites undergoing hypermethylation or hypomethylation in association with cancerization according to their genomic location. CpG sites located in CpG island loci tend to undergo hypermethylation in association with cancerization whereas CpG sites located in non-CpG island tend to become hypomethylated

|                | Hypermethylation | Hypomethylation |
|----------------|------------------|-----------------|
| Promoter       |                  |                 |
| CpG island     | 39               | 19              |
| Non-CpG island | 15               | 38              |
| First exon     |                  |                 |
| CpG island     | 17               | 4               |
| Non-CpG island | 3                | 17              |
| Second or distal exon or intron |     |                 |
| CpG island     | 7                | 0               |
| Non-CpG island | 0                | 5               |
| Total CpG sites | 81 (72 genes)   | 83 (69 genes)  |
In summary, we have identified 24 novel cancer-specific methylation markers for HCC using array-based methylation profiling and coupled MSP. Of these, TNRFSF10C, HOXA9, NPY, and IRF5 were frequently hypermethylated in HCC tissues and their promoter hypermethylation was correlated with inactivation of gene expression in cell lines. The clinicopathologic implications of these newly identified DNA methylation markers will need to be further investigated in large-scale studies of HCC samples.

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