

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

So Hyun Shin¹, Baek-hui Kim²,
Ja-June Jang³, Kyung Suk Suh⁴,
and Gyeong Hoon Kang^{1,3}

Laboratory of Epigenetics¹, Cancer Research Institute and Brain Korea 2nd Stage, Seoul National University, Seoul; Department of Pathology², Korea University Medical School, Seoul; Departments of Pathology³, and Surgery⁴, Seoul National University College of Medicine, Seoul, Korea

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Address for Correspondence:
Gyeong Hoon Kang, M.D.

Department of Pathology, Seoul National University College of Medicine, 101 Daehang-no, Jongno-gu, Seoul 110-744, Korea
Tel: +82.2-2072-3312, Fax: +82.2-743-5530
E-mail: ghkang@snu.ac.kr

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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

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 inactivation of tumor suppressor genes or tumor-related genes in human cancers, and occurs in virtually all types of human cancers (6). In addition to gene inactivation, promoter CpG island hypermethylation has received attention for its potential utility as a biomarker for tumor detection or prediction of prognosis or response of tumor cells to chemotherapeutic agents (7). Hepatocellular carcinoma (HCC) is one of the human cancers in which researchers are actively studying epigenetic changes. More than 113 genes have been found to be hypermethylated in HCC in a cancer-specific manner and some of them have been

demonstrated to have a close association between hypermethylation and poor prognosis for HCC patients, supporting the potential usefulness of DNA methylation markers as prognostic markers (8-10).

However, the number of genes that have been identified to date as harboring hypermethylation in their promoter CpG island loci is far fewer for HCC than for colon cancer or gastric cancer, which can be attributed to fewer attempts to perform genome-wide methylation analysis compared with colon cancer or gastric cancer. Considering that about 400 genes may be inactivated through promoter CpG island hypermethylation in colorectal cancers (11), there remain many genes that may be hypermethylated in HCC.

Among the various technologies available for large-scale analysis of DNA methylation, the GoldenGate methylation solution is a powerful and accurate screening tool for analyzing methylation status in 807 genes (12). With the advent of this technique, we were able to analyze the methylation status of 687 promoter CpG island loci in liver cancer. The aim of the present study was to identify additional genes that are hypermethylated in HCC in a cancer-specific manner that have not yet been reported as such in the literature thus far.

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 3×10⁵/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).

Tissue samples

Fresh-frozen samples of HCC and paired non-neoplastic liver tissues were obtained from patients (n=5) who underwent curative resection for HCC at the Seoul National University Hospital in 2008. In addition, after microscopic examination of HCC samples from patients who underwent curative resection at the Seoul National University Hospital from 2001 to 2002, we selected 50 cases of HCC that had sufficient amounts of archival neoplastic and non-neoplastic tissues for the DNA methylation study. For the bead array study requiring fresh tissue samples, we obtained informed consent from the patients. The Institutional Review Board approved this study.

Methylation microarray

DNA methylation analysis of individual genes was performed using GoldenGate Methylation Solution utilizing the current Cancer Panel I platform (Illumina, San Diego, CA, USA), which probes 1,505 CpG loci selected from 807 genes. Using this method, oligonucleotide primers query putative methylation sites in

Table 1. Primers for methylation-specific polymerase chain reaction

Gene	Forward primer	Reverse primer	Tm (°C)	Product (bp)
ADAMTS12	TTTATTTATATTCGTCGAAAGCG	ACGACTACAAAACCTACCCGCG	61	135
ADCYAP1	GGTTTGGTTAGTTATTGGGCGTC	CCCTAAATTAACAACACTTAACGACAACCG	59	118
CTSL	TTTGGGATAGTTAGTAATAAGTTACGAATCGC	AACTCTACTTCTAAACCTCGAATCTAAATTG	57	150
DST	TTTTTTAGTAGGGGGTTGCGTATAGC	CCGAATCCCCAAAAACGACG	59	103
F2R	TTTTTATATTTTAGGAGGGTCGAGAC	TTCCTCTAACACCGTTAATTCG	60	124
FGF3	GGAGTTTTTTGTCGTCGTTTTTCGC	CGCCGCCGATAACTAATATCCG	59	116
FLT3	GGAGTTTCGGGGTCGTC	CCCCAAAAACAAAACCGAAAACG	59	134
FLT4	CGTATTGTTCCGGGTGATCGGAC	AAACGAAAACCGAAAACGAAACCG	59	105
FZD9	CGGGCGGATATTATTAGGTTCCGGTTTTATTC	CCCCCGAAACGAAACTCCG	60	123
GP1BB	TAGCGGGTGTTCGAGTGTTTC	CTAACCAAAAATAACCAAAAACCGCG	59	138
HIC2	GTTTGGGTTTTCGGTTTTTTTCGC	CGAAACCTACGAAAACGAAACCG	59	115
HOXA9	CGGGCGTTTTTCGTTTTAGGC	AAATCCGTCCCAACGAAACCG	60	122
HS3ST2	GGGAGCGTTCGAGTCGTTTC	CACAATACCAAAAATCCCGAAAACAACG	59	117
ICA1	GTCGTTGCGTTGCGTTGC	ACGTCAACGTCAACCGAAAACG	59	130
IGF2AS	CGGTTGCGTCGGGTC	TAAACGCACCCCGATAAACTCG	59	131
IHH	TTATTCGGACGTTTGTAGTTTTCGTAGC	GAACGAAAACATAACCGAAAACCG	59	111
IRAK3	CGTGTTTTTAGGGTTTTGTGTCGTC	CGAACCCCGAAAACGAAACG	59	108
IRF5	AATTGAGTATTGTAGCGGGAGGTAC	CTCCAAAAAATACCAACGACGACG	61	116
MCM2	TTTGAGTTGTGATTCGTTTATTC	CCGACGACACTTACTAATACGTA	58	139
MLF1	TTGTTAGGGTAGCGGCGTATTGTTTTTC	AAACGATACCCCGGAAATAAAAAATACG	60	113
NGFR	TAGTTAGAGCGAGTCGAGTCGC	CTCGACTTCCAACCTCGATCCG	60	109
NOTCH3	GGTAGGGATTCGGGAGGTC	GCCAACTTCGCGGAAATAAAACG	59	120
NPY	AAAGAAGGAAAGTAGGGATCGGGTATTGTTTC	CTAAAACCGCGTAAACGAAACAACG	59	120
PDGFRB	TTATATTTTGAAGCAACGGGGC	AAACAAAAAATAAACCGGTACGT	62	139
PGF	CGGGTTGATCGGGCGTTTC	GCGCCTAAATTTCAAAAACCTAAACCGAAAACG	61	103
PLAGL1	GCGGCGATGATAAGAGTTTTCGC	GACCCCAACCGACCCG	61	107
PTCH2	GTAGTTGTTATTCGAGTTTTGTTGTCGTTTC	CGCTCCGTAACCGTAAACCG	59	134
RBP1	GATTTTTTCGTAGGTTTTGTGCG	ACCGATACTACGCGAATAATAAACG	59	114
SH3BP2	TTTCGGGATTCGGGTCGC	CCCCAAAAACGCGTAAACG	60	108
TAL1	ATTTGGTTGAGGAGGTAATCGC	CCGACAAACTATCTAAACATTTTCG	62	108
TESK2	GTAGGCGTTTTAGTTTTTCGCGTTTC	TACCATAACCCACAATCGAAACCG	62	134
TIAM1	TTTTTACGGGTTTTCGCGTTTC	GACGCTACTCTACCGACCG	59	110
TNFRSF10C	TAAGGGGTGAAGGAGCGTTTTTTATC	ACGCCGTCTAAATCTAAATCG	59	104
WNT2	TTAGGCGTAGTATTTTTTTTCGTTGCGTTTC	CCAATTCGCCAAACGCAACG	59	115
ZP3	GGGTTGTCGTTTTTCGATGTGAC	TCAACTAACTACGAAACGACCATAACCG	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 μ g 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-

Table 2. Primers for quantitative RT-PCR

Gene	Forward primer	Reverse primer	Tm (°C)	Product (bp)
ADAMTS12	TGCAATTCACATTGTTGTGG	GTGAGGCTGACACATTCCTG	59	253
ADCYAP1	GATCTTCACGGACAGCTA AG	GTTTGGATAGAACACACGAGC	60	226
DST	AGCAAAGGACGCATACTGAC	ATTTGGCTCTACTCCTGAAC	56	270
FGF3	TTGGAGATAACGGCAGTGGGA	CTCCAGGTTATCCGGGCTCT	56	448
FLT3	GAGGACTTGAATGTGCTTACA	TCCACAGTAATATCCATATGA	55	308
FZD9	TGCCCTCTCTGGCTACCTG	GGGCACCGTGTAGAGGATGG	62	164
GAPDH	CAATGACCCCTTCATTGACC	TGGAAGATGGTGATGGGATT	55	135
GP1BB	CCTGCAAACCTCGACAGGAC	CAGAGTTTGGAAAGGGAGACG	59	231
HIC2	TGCGAGAAGACCTACAAGGA	AGCTGGCACTCGTAAGGTTT	59	251
HOXA9	GCGCCTTCTCTGAAAACAAT	CCAGATCTTGACCTGCCTCT	60	242
HS3ST2	GGATCCCTTGCTTGAAAAA	TGCAACTCAAGTTGGGAAAG	59	302
IRAK3	TTTGAATGCAGCCAGTCTGA	GCATTGCTTATGGAGCCAAT	59	365
IRF5	CAGGGAGCTTCTCTCTGAGG	AAGAGTTCCACCTGCTCCTG	68	237
MLF1	GGTTTTTCAGGCCTCAACTC	AACTCACTTTGCCACTCC	64	245
NOTCH3	TGTGGACGAGTGTCTATCG	AATGTCCACCTCGCAATAGG	60	238
NPY	AACCTCATCACCAGGCAGAG	CTGCATGCATTGGTAGGATG	60	220
PDGFRB	GGGCTAGACACGGGAGAATA	GATCATAGGGGACAGGCAGT	59	250
PTCH2	GTGGCAAAGTGCTCTTTCTG	TCCAGGACTTCCCATAGAG	55	301
RBP1	GTTGGGAAGGAGTTTGAGGA	GCTCACACATCCTGCTGATT	59	258
SH3BP2	ATCCACATCAGCAAGAAGCA	GAGTCATCCTCATCGTCGTG	68	251
TAL1	AAGAGGAGACCTTCCCCCTA	CCTCCTCCTCTGGTCATT	66	247
TIAM1	AAGACGTACTCAGGCCATGTCC	GACCCAAATGTCCAGTCAG	61	252
TNFRSF10C	GATTACACCAACGCTTCCAA	TTGGCACCAAAATCTTCAAC	59	248
WNT2	CAGACGCAAGGGGTTAATA	CACATCTGGATGTCGGTGAC	61	202
ZP3	CAGAATGCCTCCCTTATCA	ATCTGGGCTCTGCTCAGTA	60	210

ered to be potential novel methylation markers for HCC. This gene filtering approach is depicted in Fig. 1.

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 Meth-Primer (<http://www.urogene.org/methprimer>). However, for one gene (*HOXB2*), 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*, *FGF3*, *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*, *PGE*, *NGFR*, *FLT4*, and *ICAI*) 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).

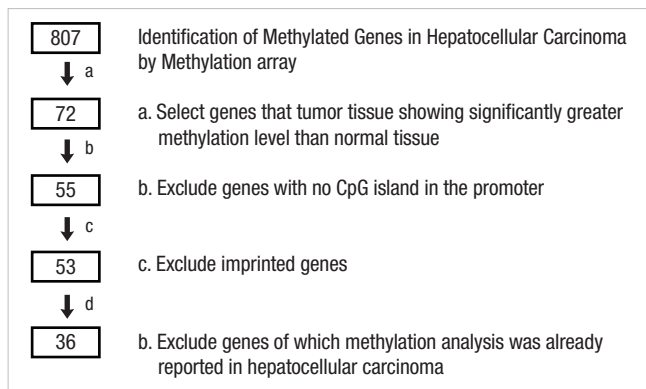


Fig. 1. Flow chart for selection of candidate methylation markers. We used 5 paired hepatocellular carcinoma/normal tissues to screen for candidate methylation markers using a methylation array. We obtained 72 candidates that showed significant hypermethylation in hepatocellular carcinoma tissues. We 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.

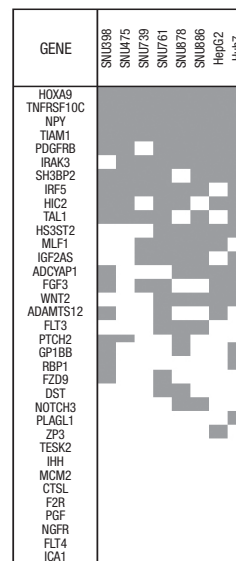


Fig. 3. Summary of methylation-specific PCR for hepatocellular carcinoma (HCC) cell lines. We examined eight HCC cell lines for methylation status of the 35 genes and found that 26 of the genes (*HOXA9*, *TNFRSF10C*, *NPY*, *TIAM1*, *PDGFRB*, *IRAK3*, *SH3BP2*, *IRF5*, *HIC2*, *TAL1*, *HS3ST2*, *MLF1*, *IGF2AS*, *ADCYAP1*, *FGF3*, *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.

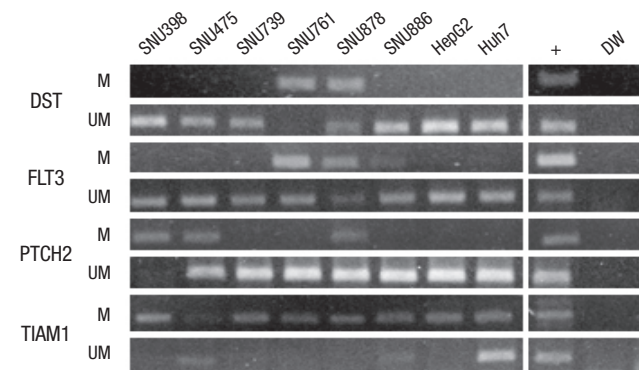


Fig. 2. Representative examples of MSP analysis of *DST*, *FLT3*, *PTCH2* and *TIAM1* in 8 HCC cell lines. DNA extracted from 8 HCC cell lines were amplified with primers specific to the methylated (M) or unmethylated (UM) CpG islands of each gene after modification with sodium bisulfite. +, positive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

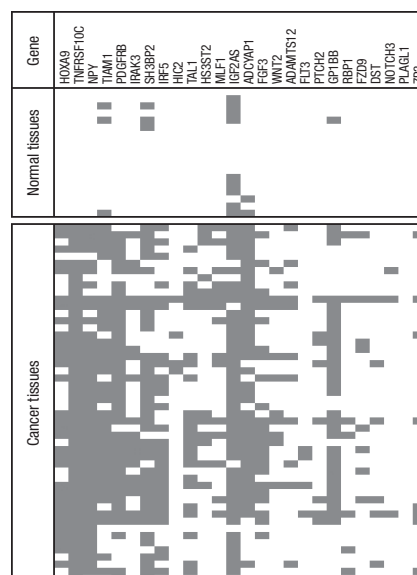
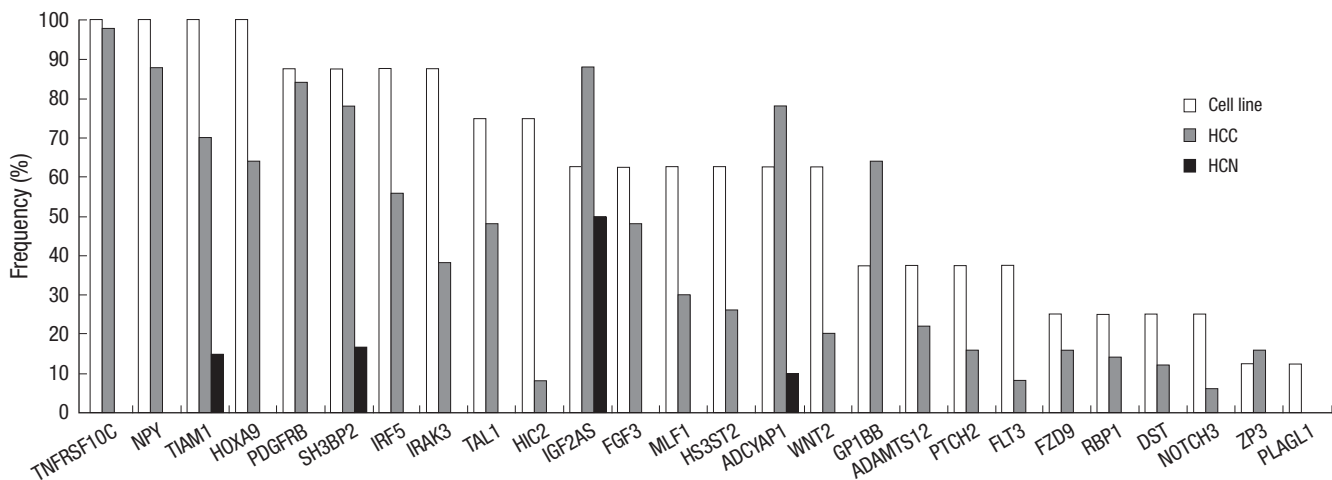


Fig. 4. Summary of methylation-specific PCR results in hepatocellular carcinoma (n=50) and non-neoplastic liver tissue samples (n=18). Out of 26 genes showing methylation in HCC cell lines, promoter methylation was detected in 25 genes in primary tumor tissues. Data are color-coded as follows: gray fill indicates the presence of methylation, while white fill indicates absence of methylation.

Table 3. New cancer-specific methylated genes

Genes	Gene name	Loci	Function
ADAMTS12	ADAM metalloproteinase 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 1eB	6p12–p11	Actin binding, calcium ion binding, integrin binding, protein binding
FGF3	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
HOXA9	Homeobox protein A9 isoform 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 isoform 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
NPY	Neuropeptide Y	7p15.1	Calcium channel regulator activity, neuropeptide hormone 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).

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 μ M for 96 hr) and performed quantitative RT-PCR of the mock-treated and 5-aza-dC treated cell lines. *TNFRSF10C*, *NPY*, *TIAM1*, and *HOXA9* were methylated in all cell lines and showed up-regulation of mRNA expression in all cell lines with 5-aza-dC, although *TIAM1* was not re-expressed in two cell lines, SNU-878 and Huh7. Most of the methylated genes exhibited re-expression after 5-aza-dC treatment; in contrast, SNU-886 in *PDGFRB*; SNU-878 in *IRF5*; SNU-398, HepG2, and Huh7 in *HIC2*; HepG2 and Huh7 in *HS3ST2*; SNU-878 in *ADCYAP1*; and SNU-886 in *NOTCH3* were

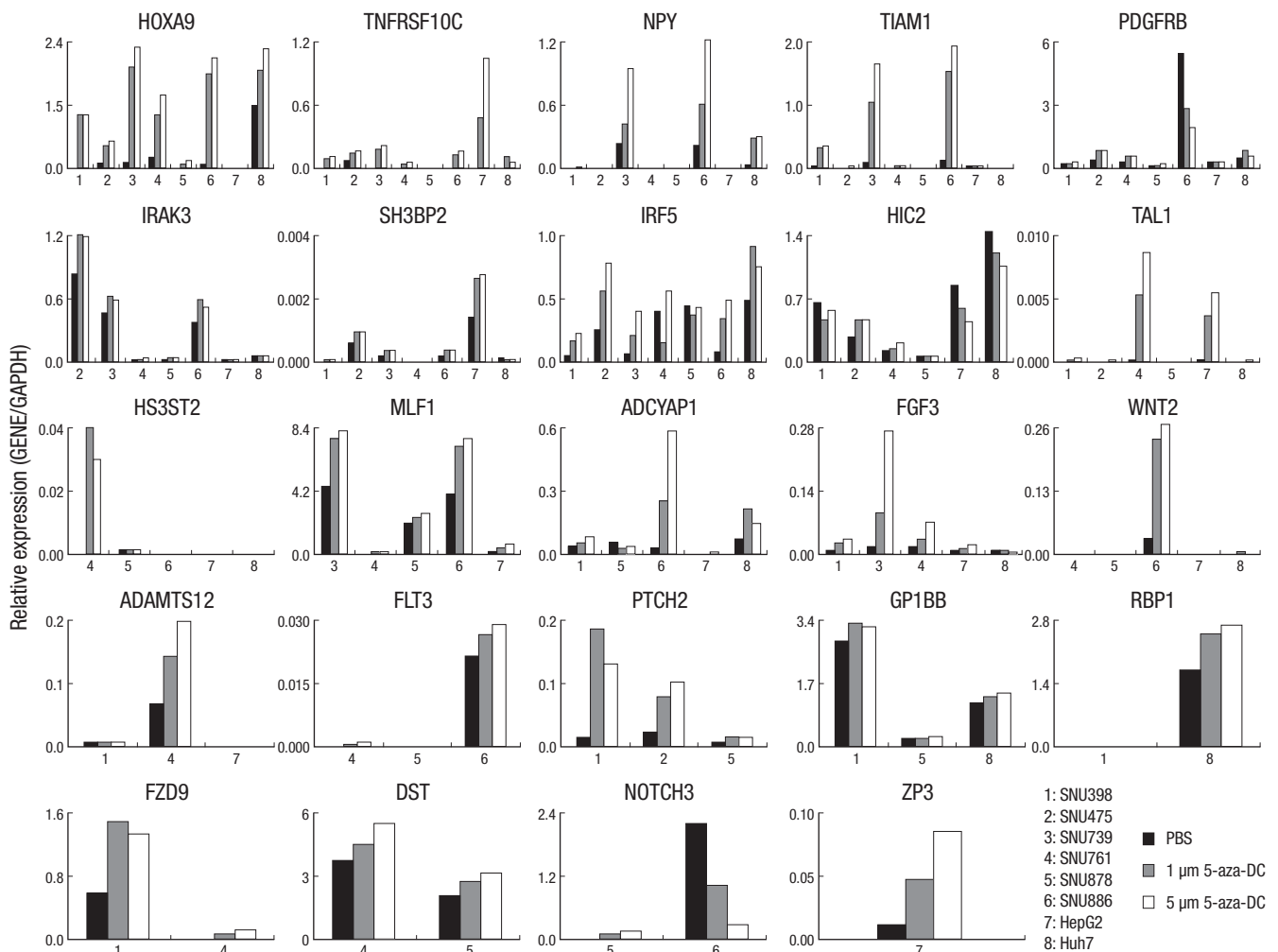


Fig. 6. Analysis of mRNA expression of candidate genes in hepatocellular carcinoma cell lines by quantitative RT-PCR. Cells were either mock-treated (PBS) or treated with 5-aza-dC (1AZA [1 μ M] or 5AZA [5 μ M] for 96 hr) as indicated. The expression patterns and re-expression patterns for each gene after 5-aza-dC treatment are shown for the cell lines that were methylated. Numbers along the horizontal axis indicate the cell lines. Gene expression levels were determined by quantitative RT-PCR and normalized to GAPDH levels.

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 ex-

ponentially, 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 silenc-

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 tis-

sues 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

Supplementary Table 1. The average methylation level (beta value) of each CpG island locus between non-neoplastic tissues and HCC and correlated it with polycomb repressive complex 2 (PRC2) occupancy. The value of PRC2 occupancy represents the number of occupied components of PRC2 in the gene promoter

	The number of genes	Beta value (mean)	
		Non-neoplastic liver	HCC
PRC2 occupancy=0	407	0.326	0.348
PRC2 occupancy \geq 1	132	0.244	0.315
PRC2 occupancy=1	41	0.245	0.277
PRC2 occupancy=2	34	0.231	0.319
PRC2 occupancy=3	57	0.253	0.339

	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, *TNFRSF10C*, *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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