ORIGINAL ARTICLE
Oncology & Hematology

JKMS

http://dx.doi.org/10.3346/jkms.2012.27.6.594 • J Korean Med Sci 2012; 27: 594-604

Pharmacological Unmasking Microarray Approach-Based Discovery of Novel DNA Methylation Markers for Hepatocellular Carcinoma

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Received: 21 November 2011
Accepted: 23 February 2012

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This study was supported by a Mid-career Researcher Program through NRF grant funded by the Ministry of Education, Science and Technology (MEST) (2011-0015646) and a Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by MEST (2009-0093820).

DNA methylation is one of the main epigenetic mechanisms and hypermethylation of CpG islands at tumor suppressor genes switches off these genes. To find novel DNA methylation markers in hepatocellular carcinoma (HCC), we performed pharmacological unmasking (treatment with 5-aza-2´-deoxycytidine or trichostatin A) followed by microarray analysis in HCC cell lines. Of the 239 promoter CpG island loci hypermethylated in HCC cell lines (as revealed by methylation-specific PCR), 221 loci were found to be hypermethylated in HCC or nonneoplastic liver tissues. Thirty-three loci showed a 20% higher methylation frequency in tumors than in adjacent nonneoplastic tissues. Correlation of individual cancer-related methylation markers with clinicopathological features of HCC patients (n = 95) revealed that the number of hypermethylated genes in HCC tumors was higher in older than in younger patients. Univariate and multivariate survival analysis revealed that the *HIST1H2AE* methylation status is closely correlated with the patient's overall survival ($P = 0.022$ and $P = 0.010$, respectively). In conclusion, we identified 221 novel DNA methylation markers for HCC. One promising prognostic marker, *HIST1H2AE*, should be further validated in the prognostication of HCC patients.

Key Words: CpG Islands; DNA Methylation; Carcinoma, Hepatocellular; Microarray; Prognosis

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).

pISSN 1011-8934
eISSN 1598-6357

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 3×10^5 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 non-neoplastic 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-glutamyltranspeptidase (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 RNeasy 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 hypermethylation-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

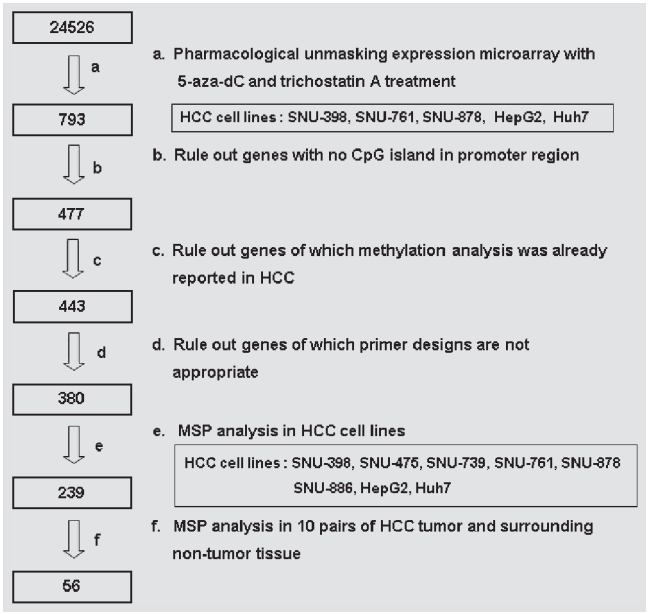

Fig. 1. Flow chart for selection of candidate genes. Screening of candidate tumor suppressor genes (TSGs) was performed in 5 hepatocellular carcinoma (HCC) cell lines treated with 5 μM 5-aza-2′-deoxycytidine (AZA) or 300 nM trichostatin A (TSA) by using a 24,526-oligonucleotide mRNA microarray. We obtained 793 candidates whose gene expression did not increase with TSA treatment (< 1.4-fold) but increased more than 2-fold after AZA treatment. We excluded genes that do not harbor CpG islands in their promoters or whose methylation status in HCC tumors had already been reported in the literature. We further excluded genes for which adequate oligonucleotide primers could not be designed by using the MSPprimer or MethPrimer software programs. As a result, we selected 380 genes to be examined for their methylation status in HCC cell lines by using methylation-specific PCR (MSP).

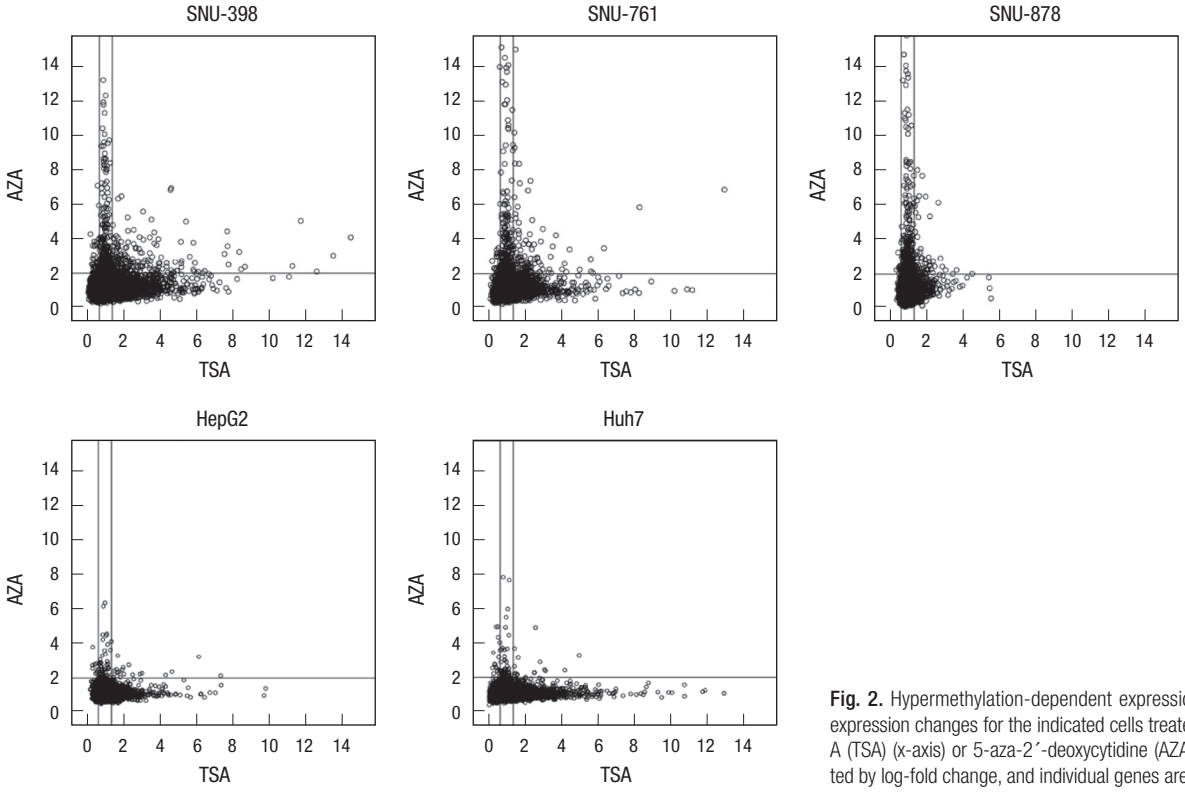

Fig. 2. Hypermethylation-dependent expression changes. Gene expression changes for the indicated cells treated with trichostatin A (TSA) (x-axis) or 5-aza-2′-deoxycytidine (AZA) (y-axis) are plotted by log-fold change, and individual genes are shown in circles.

http://dx.doi.org/10.3346/jkms.2012.27.6.594

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 MSPprimer 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).

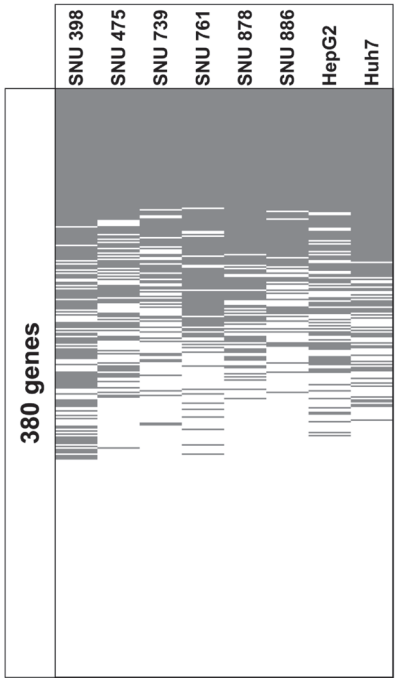

Fig. 3. Methylation-specific PCR (MSP) analysis of 380 selected genes in 8 hepatocellular carcinoma (HCC) cell lines. The methylated and unmethylated status is indicated by a gray and a white box, respectively.

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-

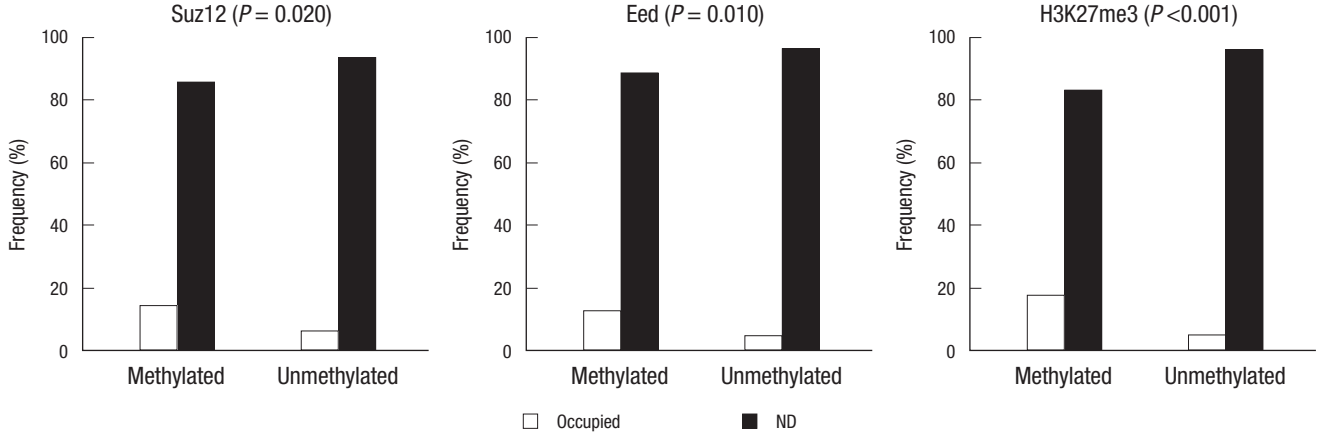

Fig. 4. Frequency of H3K27me3 modification and occupancy rate of SUZ12 and EED in methylated (n = 239) and unmethylated genes (n = 141) in human embryonic stem cells. The chi-squared test was conducted to analyze the significance of the association.

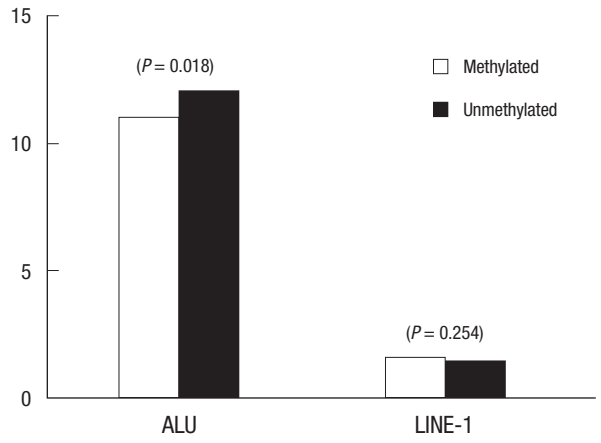

Fig. 5. Comparison of *ALU* and *LINE-1* repeats between methylated and unmethylated genes. For *ALU* counting, the promoter sequence of a specific gene was divided into 20 bins of 1-kb sequence each (10 bins upstream and 10 bins downstream of each gene transcription start site), and the presence of *ALU* was annotated for each bin. We counted bins containing *ALU* within a 1-kb sequence. For *LINE-1* counting, the promoter sequence of a specific gene was divided into 7 bins of 1-kb sequence each (2 bins upstream and 5 bins downstream of each gene transcription site), and the presence of *LINE-1* was annotated for each bin. Bins containing *LINE-1* within a 1-kb sequence were counted. Student's t-test was performed to determine the statistical significance of the difference of means between 2 groups.

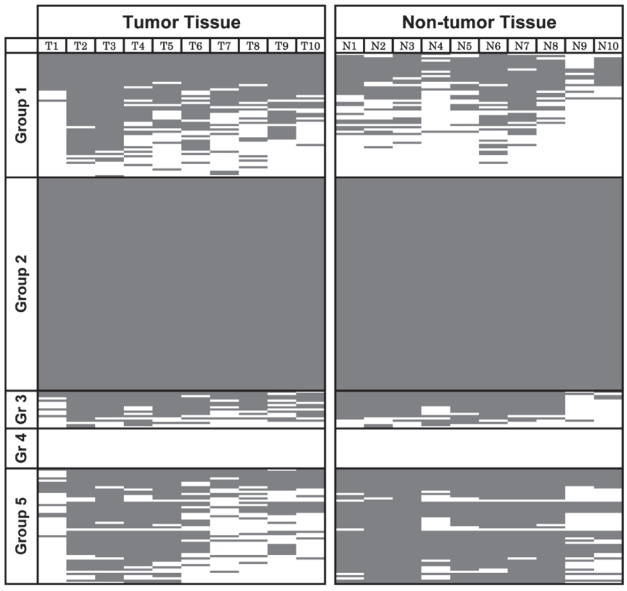

Fig. 6. Methylation-specific PCR (MSP) analysis of 239 genes in 10 pairs of tumor and surrounding nontumor tissue. The methylated and unmethylated status is indicated by a gray and a white box, respectively. Genes were divided into subgroups based on the methylation pattern in hepatocellular carcinoma (HCC) tumors and surrounding nonneoplastic liver tissues (see main text).

Table 1. Functional annotation analysis of classified genes. Gene ontology of hepatocellular carcinoma (HCC) tumors, surrounding nontumor tissues, and cell lines based on their DNA methylation patterns

Category	Term	*P* value
Group 0 (MF of genes: not methylated in cell lines)	Gene expression	1.E-02
	Antiapoptosis	2.E-02
	Nucleosome assembly	2.E-02
	Cellular macromolecule metabolic process	3.E-02
	Positive regulation of transmission of nerve impulse	3.E-02
Group 1 (MF of genes: HCC > NL)	Neuropeptide signaling pathway	3.E-02
	Positive regulation of amine transport	4.E-02
	Acid secretion	5.E-02
Group 2 (MF of genes: 100% in both HCC and NL)	DNA methylation during gametogenesis	3.E-04
	Reproductive process in a multicellular organism	8.E-04
	Developmental process	2.E-03
	Positive regulation of translation	4.E-03
	DNA methylation	4.E-03
Group 3 (MF of genes: HCC = NL)	Regulation of steroid metabolic process	3.E-02
Group 5 (MF of genes: HCC < NL)	Nucleosome assembly	2.E-03
	Chromatin assembly	2.E-03
	Protein-DNA complex assembly	2.E-03
	Chromatin assembly or disassembly	6.E-03
	Tissue development	1.E-02

Group 4: genes did not show any significant association with specific biologic functions. MF, methylation frequency; HCC, hepatocellular carcinoma; NL, normal liver.

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-

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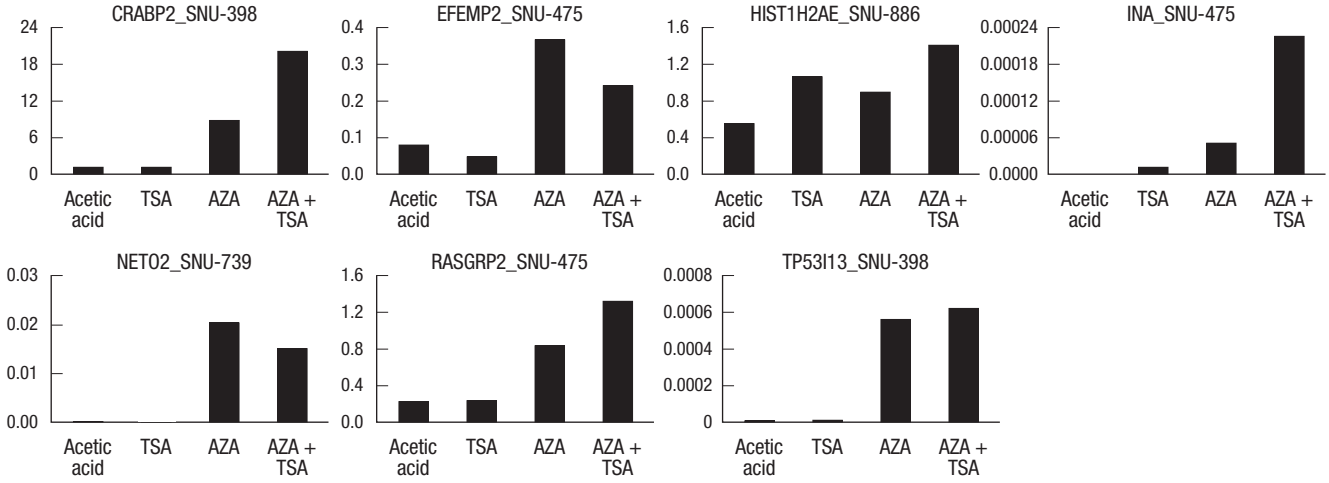

Fig. 7. Effect of 5-aza-2′-deoxycytidine (AZA) and trichostatin A (TSA) on gene expression. RNA was isolated from hepatocellular carcinoma (HCC) cell lines left untreated or treated with AZA, TSA, or a combination of AZA and TSA. mRNA was reverse-transcribed, and gene expression was quantitated by real-time PCR and normalized to *GAPDH* expression.

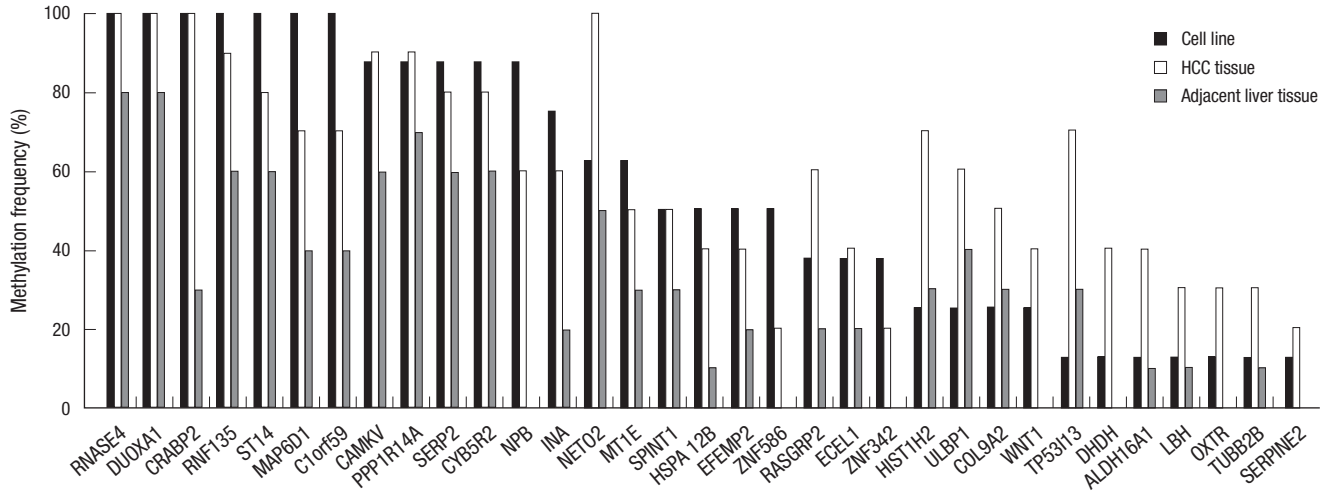

Fig. 8. Methylation frequencies of 33 DNA methylation markers in HCC cell lines (n = 8) and tissue samples (10 paired HCC and non-neoplastic liver tissue samples). Methylation frequencies of these DNA methylation markers in HCC tissue samples were higher than those of non-neoplastic liver tissue samples: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

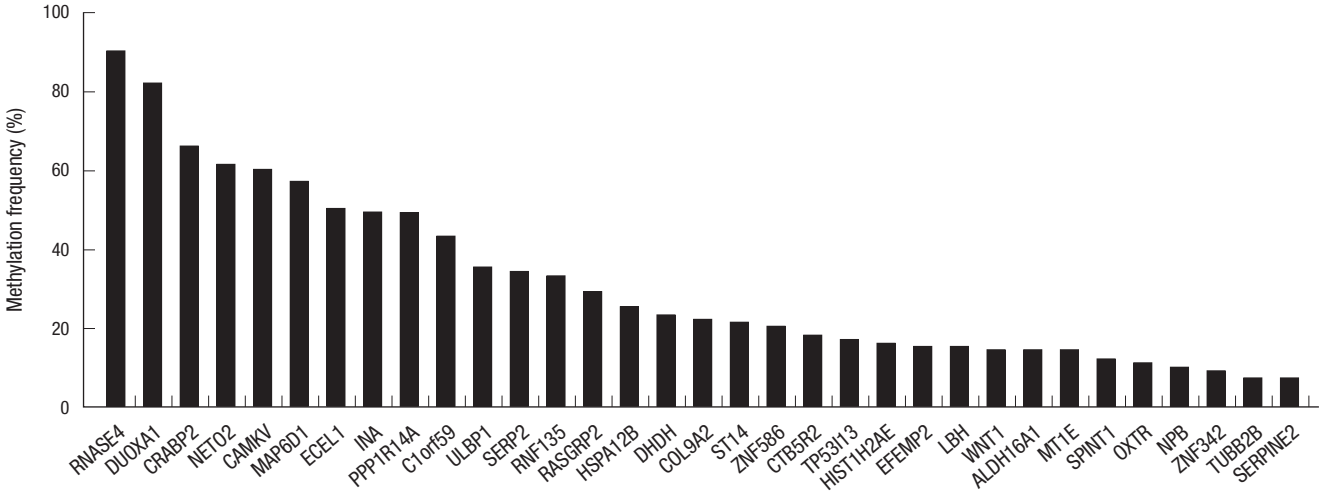

Fig. 9. Methylation frequencies of 33 DNA methylation markers in hepatocellular carcinoma (HCC) tissue samples (n = 95). DNA methylation markers were distributed along the x-axis according to the decreasing order of methylation frequency.

Table 2. Correlation of promoter hypermethylated genes with clinical parameters

Parameter	RNASE4	DUOXA1	CRABP2	NET02	CAMKV	MAP6D1	ECEL1	INA	PPP1R14A	C1orf59	ULBP1	SERP2	RNF135	RASGRP2	HSPA12B	DHDH	COL9A2	ST14	ZNF586	CYB5R2	TP53I13	HIST1H2AE	EFEMP2	LBH	ALDH16A1	MT1E	WNT1	SPINT1	OXTR	NPB	ZNF342	SERPINE2	TUBB2B
Age (yr)																																	
< 55 (n = 47)	43	40	31	23	28	21	22	25	23	14	12	14	13	16	12	4	7	9	9	4	9	6	6	5	7	9	4	2	3	6	1	1	3
≥ 55 (n = 48)	47	42	35	38	32	35	28	24	26	29	23	20	20	13	13	19	15	12	11	14	8	10	9	10	7	5	10	10	8	4	8	6	4
P value	0.20	0.73	0.46	0.00	0.47	0.01	0.26	0.76	0.61	0.00	0.02	0.23	0.15	0.46	0.86	0.00	0.06	0.49	0.65	0.01	0.75	0.29	0.42	0.17	0.97	0.23	0.09	0.02	0.12	0.52	0.03	0.11	0.72
Gender																																	
M (n = 75)	71	64	52	50	49	45	37	39	36	33	25	25	26	21	15	19	19	17	15	15	11	11	9	10	9	11	12	9	9	7	8	6	5
F (n = 20)	19	18	14	11	11	11	13	10	13	10	10	9	7	8	10	4	3	4	5	3	6	5	6	5	5	3	2	3	2	3	1	1	2
P value	1.0	0.73	0.95	0.33	0.39	0.69	0.21	0.87	0.18	0.63	0.17	0.33	0.98	0.30	0.01	0.77	0.39	1.0	0.76	0.76	0.18	0.32	0.08	0.30	0.16	1.0	0.73	0.71	1.0	0.43	0.68	1.0	0.64
Tumor size																																	
< 6 cm (n = 45)	45	37	32	30	28	25	24	25	25	22	18	20	16	15	9	13	10	9	12	10	9	8	7	7	8	8	8	6	5	4	5	5	4
≥ 6 cm (n = 48)	43	43	32	29	31	29	25	23	22	20	15	13	15	13	14	8	10	12	6	8	6	7	7	7	6	6	6	6	5	6	4	1	3
P value	0.06	0.31	0.64	0.53	0.81	0.63	0.90	0.46	0.35	0.48	0.38	0.08	0.66	0.51	0.31	0.16	0.87	0.56	0.08	0.50	0.33	0.68	0.90	0.90	0.48	0.48	0.48	0.90	1.0	0.74	0.73	0.10	0.71
AFP																																	
< 100 (n = 48)	45	43	34	35	32	34	22	23	22	22	17	18	18	7	8	17	12	10	9	10	6	6	8	8	8	7	10	10	5	4	7	6	4
≥ 100 (n = 43)	41	36	28	22	24	21	25	23	25	18	15	15	12	20	15	5	9	10	10	7	11	9	7	6	6	7	3	2	6	6	2	1	2
P value	1.0	0.41	0.56	0.03	0.29	0.03	0.24	0.60	0.24	0.70	0.96	0.80	0.33	0.00	0.05	0.01	0.65	0.78	0.60	0.58	0.11	0.28	0.96	0.72	0.72	0.82	0.06	0.02	0.61	0.51	0.11	0.07	0.48
GGT																																	
< 56 (n = 48)	46	43	35	34	31	28	32	30	30	25	21	20	19	17	15	14	12	13	13	9	11	9	9	9	8	9	9	6	7	5	6	6	4
≥ 56 (n = 47)	44	39	31	27	29	28	18	19	19	18	14	14	14	12	10	9	10	8	7	9	6	7	6	6	6	5	5	6	4	5	3	1	3
P value	0.68	0.35	0.46	0.17	0.77	0.90	0.01	0.03	0.03	0.18	0.16	0.23	0.32	0.30	0.27	0.25	0.67	0.24	0.15	0.96	0.20	0.62	0.42	0.42	0.59	0.26	0.26	0.97	0.36	1.0	0.49	0.11	1.0
Child-Pugh classification																																	
A (n = 86)	81	77	61	58	56	51	46	46	46	40	32	32	31	27	22	21	19	21	19	15	16	14	14	13	12	14	12	10	10	9	7	6	6
B (n = 9)	9	5	5	3	4	5	4	3	3	3	3	2	2	2	3	2	3	0	1	3	1	2	1	2	2	0	2	2	1	1	2	1	1
P value	1.0	0.02	0.45	0.07	0.28	1.0	0.73	0.31	0.31	0.51	1.0	0.48	0.49	0.72	0.69	1.0	0.43	0.20	0.68	0.36	1.0	0.64	1.0	0.63	0.62	0.35	0.62	0.32	1.0	1.0	0.20	0.51	0.51
Background liver																																	
Cirrhotic (n = 62)	60	52	39	40	36	36	34	35	34	28	22	21	20	18	19	14	10	14	11	9	14	12	12	11	6	10	9	8	8	7	5	4	5
Noncirrhotic (n = 33)	30	30	27	21	24	20	16	14	15	15	13	13	13	11	6	9	12	7	9	9	3	4	3	4	8	4	5	4	3	3	4	3	2
P value	0.34	0.53	0.06	0.93	0.16	0.81	0.55	0.19	0.38	0.98	0.71	0.59	0.49	0.66	0.19	0.61	0.03	0.88	0.28	0.13	0.10	0.37	0.19	0.47	0.07	0.76	1.0	1.0	0.74	1.0	0.71	0.69	1.0
Histologic grade																																	
1, 2 (n = 52)	49	44	35	35	36	32	29	26	26	23	21	22	18	13	14	13	14	10	9	10	7	8	9	9	8	7	7	6	5	6	4	2	4
3, 4 (n = 23)	25	21	13	10	12	12	9	9	9	6	6	6	5	9	3	2	2	6	8	3	5	2	2	1	2	3	1	2	2	3	0	1	0
P value	0.55	1.0	0.19	0.02	0.07	0.26	0.10	0.25	0.25	0.09	0.16	0.12	0.19	0.32	0.14	0.12	0.06	0.63	0.15	0.53	0.51	0.48	0.49	0.15	0.48	1.0	0.26	1.0	1.0	1.0	0.30	1.0	0.30
Microscopic vascular invasion	37	32	29	29	27	23	25	22	23	24	18	17	14	14	13	13	9	6	6	12	6	6	7	7	9	7	6	6	8	1	4	5	4
Absent (n = 38)	51	49	36	31	33	31	25	27	26	19	17	17	19	15	12	10	13	14	12	6	11	10	8	8	5	7	8	6	3	8	5	2	3
Present (n = 55)	0.65	0.54	0.26	0.05	0.27	0.69	0.05	0.40	0.21	0.01	0.11	0.17	0.82	0.33	0.19	0.08	1.0	0.26	0.47	0.01	0.61	0.76	0.62	0.62	0.05	0.45	0.87	0.54	0.05	0.08	0.82	0.12	0.36
P value																																	
Frequency (%)	95	86	69	64	63	59	53	52	52	45	37	36	35	31	26	24	23	22	21	19	18	17	16	16	15	15	15	13	12	11	9	7	7

AFP, alpha-fetoprotein; GGT, serum γ-glutamyltransferase.

http://dx.doi.org/10.3346/jkms.2012.27.6.594

pression, we selected 7 genes (*CRABP2*, *EFEMP2*, *HIST1H2AE*, *INA*, *NETO2*, *RASGRP2*, and *TP53I13*) 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 nonneoplastic 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 *DUOXA1* (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-Meir 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

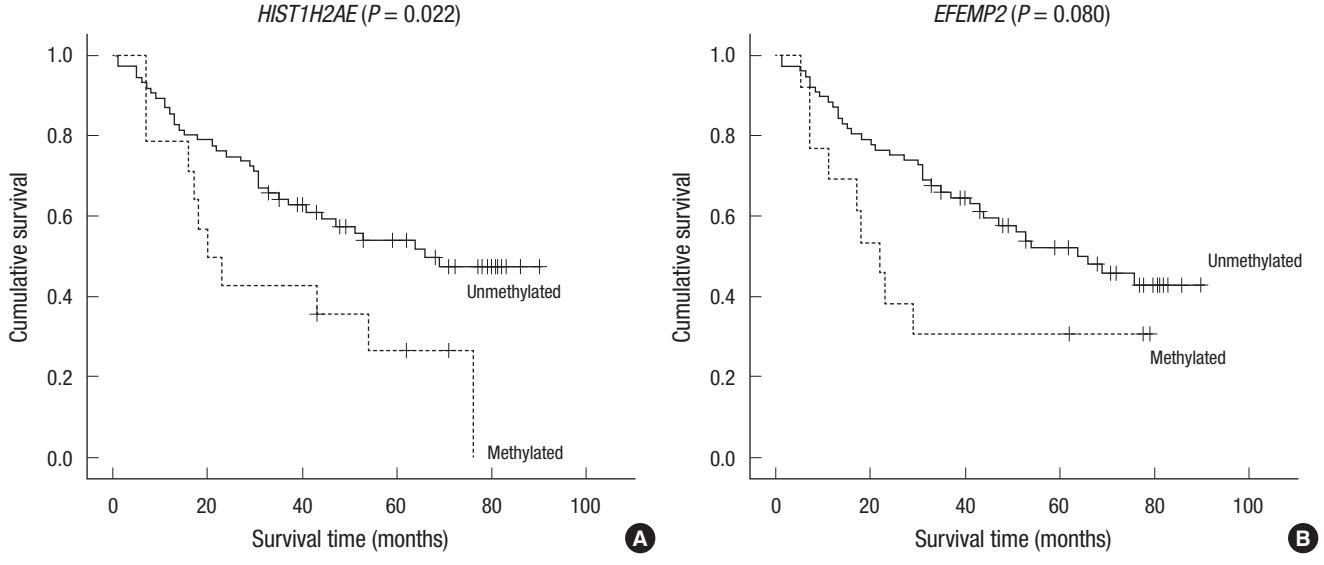

Fig. 10. Kaplan-Meier survival curves of 90 hepatocellular carcinoma (HCC) patients. Correlation of (**A**) *HIST1H2AE*, and (**B**) *EFEMP2* methylation status with overall survival.

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				0.782
M	71	56	43	
F	19	45	45	
Age (yr)				0.014
< 55	44	41	33	
≥ 55	46	66	53	
Tumor size (cm)				< 0.001
< 6	41	72	61	
≥ 6	47	38	28	
Background liver				0.037
Cirrhosis	60	46	35	
Chronic hepatitis	30	69	60	
Child-Pugh score				0.136
A	81	57	47	
B	9	27	14	
C	0	-	-	
GGT (IU/L)				0.012
< 56	44	65	59	
> 56	46	44	30	
Microscopic invasion				0.014
Absent	37	68	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	

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 mechanisms by which 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 reduced H3K9 di- and tri-methylation, pRb degradation might result in re-expression of several silenced genes (Dr. Wei-Guo Zhu, personal communication).

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

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 protein binding are more prone to DNA methylation (29, 30). When we matched these data with ours, we also could observe that *ALU* and Polycomb-group proteins were significantly correlated with methylated status, whereas *LINE-1* was not.

We analyzed 95 HCC samples for their methylation status in 33 CpG island loci 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 (31, 32). 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-

http://dx.doi.org/10.3346/jkms.2012.27.6.594

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 neuropeptide 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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