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Identification of Epigenetically Inactivated Genes in Human Hepatocellular Carcinoma by Integrative Analyses of Methylation Profiling and Pharmacological Unmasking

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Key Words
DNA methylation · Epigenetic alterations · Gene hypermethylation · Methylation profiling · Tumor suppressor genes

Abstract
Objectives: DNA methylation-dependent transcriptional inactivation of tumor suppressor genes (TSGs) is critical for the pathogenesis of hepatocellular carcinoma (HCC). This study identifies potential TSGs in HCCs using methylation profiling and pharmacological unmasking of methylated TSGs. ***Methods:*** Methylation profiling was performed on 22 pairs of HCCs and their corresponding noncancerous liver tissues using the Infinium HumanMethylation27 BeadChip. We also determined the gene reexpression after treatment with 5-aza-2′-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) in 5 HCC cell lines. ***Results:*** We selected CpGs that exhibited a significant increase in methylation in HCC tissues compared with that of the noncancerous control group. Two hundred and thirteen CpGs on different gene promoters with a mean difference in the β value ≥0.15 and a value of $p < 0.05$ were selected. Of the 213 genes, 45 genes were upregulated in 3 or more HCC cell lines with multiplier value of differences ≥2.0 after 5-Aza-dC and TSA treatment. ***Conclusions:*** We identified several potential TSGs that participate in transcription inactivation through epigenetic interactions in HCC. The results of this study are important for the understanding of functionally important epigenetic alterations in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer [1]. Several risk factors contribute to the development of HCC, including chronic infection due to hepatitis virus, alcohol intake, nonalcoholic fatty liver disease, hemochromatosis, α_1-antitrypsin deficiency, Wilson's disease and aflatoxin exposure. Regardless of the etiology, chronic liver damage causes genetic and epigenetic alterations, which play an important role in hepatocarcinogenesis [2].

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Recent technical advancement in genomic sequencing and comprehensive methylation analyses give us a profound insight into the molecular events that drive carcinogenesis, including HCC [3–8]. Several studies have noted abnormal DNA methylation in HCC; DNA methylation of specific sequences on the promoters of tumor suppressor genes (TSGs) is a key epigenetic alteration that marks HCC pathogenesis [2, 9]. Therefore, it is essential to determine unique methylation events that are linked to transcriptional inactivation and HCC pathology. Currently, the correlation between individual DNA methylation and transcription of its corresponding genes has not been fully clarified in HCC. Understanding this phenomenon will provide important insights into HCC pathology [10] and may provide a venue for novel therapeutics [11].

In this study, we have addressed this issue by performing a comprehensive analysis of hypermethylated genes within HCC promoters. To achieve this, we used the Infinium HumanMethylation27 BeadChip (Illumina, San Diego, Calif., USA) to target 27,578 CpGs within the promoters of 14,474 genes in patients with HCC, and compared the results with those of their corresponding noncancerous control group. Using pharmacological agents, we also determined the genes that were upregulated due to DNA demethylation and histone acetylation in HCC cell lines. Here, we report a systematic and integrative analysis of epigenetically inactivated genes in human HCC.

Materials and Methods

Patients

In this study, 22 pairs of HCCs and their corresponding noncancerous liver tissues were taken from 19 men and 3 women ranging in age from 53 to 79 years (median, 71 years). The patient cohort had different etiological profiles. Of the 22 study patients, 1 patient was positive for hepatitis B virus surface antigen, 9 were positive for hepatitis C virus antibody, and 12 patients were negative for both. Among these 12 patients, 4 patients consumed alcohol (>20 g/day). One, 5, 7 and 9 patients had stage F1, F2, F3 and F4 liver fibrosis, respectively. The median tumor size was 3.0 cm (25th–75th percentiles: 2.6–8.0 cm). The tumors were at different stages of differentiation. Of the 22 tumor samples, 9 tumors were well differentiated, 12 were moderately differentiated, and 1 was poorly differentiated. Written informed consent was obtained from all patients, and necessary approvals were obtained from the institutional review boards of the institution involved.

Methylation Analysis Using HumanMethylation27 BeadChip

Genomic DNA was extracted from frozen tissues as described previously [12]. After confirming the quality and concentration of DNA, 1 μg of genomic DNA was treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, Calif., USA). Whole genome amplification of DNA, enzymatic fragmentation and isopropanol precipitation were performed according to the manufacturer's instructions (Infinium Methylation Assay, Manual Protocol, Rev.A). The DNA fragment was applied onto the HumanMethylation27 BeadChip array and hybridized overnight. Then, the array was scanned with the Illumina iScan SQ scanner (iScan Control software v.3.3.28) and the intensities of the images were captured using GenomeStudio (v.2011.1) and Methylation Module (v.1.9.0) software. The β value representing the methylation levels was calculated as the ratio of the signal intensity of the methylated allele divided by the sum of the signal intensity of the unmethylated and methylated allele + 100. Each β value was accompanied by a detection p value, which indicated statistical significance against the background. Only β values with a detection value of $p < 0.05$ were included in the data analysis. All samples showed a CpG coverage of >95% in this analysis.

Cell Culture and 5-Aza-2-Deoxycytidine and Trichostatin A Treatment

HCC, HLE, HLF, HepG2, Huh7 and PLC/PRF/5 cell lines were purchased from the Japanese Collection of Research Bioresources Bank at the National Institute of Biomedical Innovation (Osaka, Japan) and American Type Culture Collection (Manassas, Va., USA). To perform the pharmacological unmasking procedure, $1–5 \times 10^5$ cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, Mo., USA) with either 5 or 10% fetal bovine serum (Gibco, Life Sciences Technologies, St. Clara, Calif., USA) in 10-cm culture dishes for 24 h. The cell lines were then treated with 1 μM 5-aza-2′-deoxycytidine (5-Aza-dC; Sigma) for 72 h, followed by an additional 24-hour treatment with 100 nM trichostatin A (TSA; Wako, Osaka, Japan). Finally, the cells were harvested and studied for DNA methylation and RNA expression.

DNA and RNA Extraction, Combined Bisulfite Restriction Analysis and Array-Based Analysis of Reactivated Genes

DNA and RNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, Calif., USA) and RNeasy Mini Kit (Qiagen), respectively. To confirm DNA demethylation in response to 5-Aza-dC and TSA treatment, we performed COBRA (combined bisulfite restriction analysis) to determine the methylation status of the promoters on the *CDKN2A, GSTP1, HIC1, RIZ1, SOCS1* and *RASSF1A* genes [12, 13].

To elucidate the effects of DNA demethylation on transcription further, before and after 5-Aza-dC and TSA treatment, RNA samples were subjected to expression microarray analysis using Agilent SurePrint G3 human GE 8x60K v2 (Agilent Technology, St. Clara, Calif., USA). This system targets about 50,599 transcripts including 11,912 large intergenic noncoding RNAs and transcripts with uncertain coding potential. Data were analyzed with Agilent Feature Extraction software and the multiples of differentially expressed genes before and after treatment were calculated. Multiplier values of differences ≥2.0 were scored as upregulated genes, while those of differences ≤0.5 represented downregulated genes. The value between 0.5 and 2.0 was considered insignificant or unaltered gene expression.

Selection of Differentially Methylated and Reactivated Genes after 5-Aza-dC and TSA Treatment

Initially, we selected CpGs within promoters that showed increased methylation in HCC tissues compared with those in the

noncancerous livers, with the mean difference in a β value ≥0.15. The final data were analyzed with the Mann-Whitney U test with the false discovery rate controlled with the Benjamini-Hochberg procedure. The extracted CpGs in the hypermethylated promoters (corrected $p < 0.05$) were considered differentially methylated (hypermethylated DM-CpGs).

To determine the genes inactivated through DNA methylation, we performed pharmacological unmasking in HCC cell lines. Gene expression levels that increased over 2-fold in response to 5-Aza-dC and TSA treatment (multiplier value of differences ≥2.0) in at least 3 cell lines were considered as reactivated genes. Thus, genes with hypermethylated DM-CpGs that were transcriptionally reactivated through 5-Aza-dC and TSA treatment are candidates for genes that are inactivated through epigenetic mechanisms in human HCC.

All statistical analyses were conducted using the JMP version 9.0 software (SAS Institute Inc., Cary, N.C., USA). After calculating two-sided p values, $p < 0.05$ was considered statistically significant.

Results

Identification of Hypermethylated Genes That Were Reactivated after 5-Aza-dC and TSA Treatment in HCC

We selected hypermethylated CpGs based on the two criteria. First, CpGs must show increased methylation compared with noncancerous livers, with a difference in the β value ≥0.15. Second, the increase must be statistically significant (corrected $p < 0.05$) after the Mann-Whitney U test with the false discovery rate control. Overall, 213 CpGs in 213 different gene promoters met the above criteria. The mean distance of the location of the selected CpGs from the transcription start site was 269.5 bp (95% confidence interval 232.2–306.8 bp). We also performed an epigenetic unmasking procedure with 5-Aza-dC and TSA treatment, which induced DNA demethylation and histone acetylation in the 5 liver cancer cell lines. The optimal treatment conditions were determined by analyzing the demethylation status of 6 TSG promoters, *CDKN2A, GSTP1, HIC1, RIZ1, SOCS1* and *RASSF1A*, which were known to carry abnormal methylation in human HCC, after treatment (fig. 1) [12]. Then, we selected the transcripts that were upregulated (multiplier value of differences ≥2.0) after 5-Aza-dC and TSA treatment. In total, 2,412 transcripts were upregulated in at least 3 HCC cell lines. Of these, 317 transcripts were upregulated in all 5 cell lines, 711 transcripts were upregulated in 4 cell lines, and 1,384 transcripts were upregulated in 3 cell lines. Furthermore, among the 2,412 upregulated transcripts, 45 transcripts were derived from the genes carrying hypermethylated CpGs within the promoters in human HCC tissues. Detailed information regarding these 45 genes is listed in table 1.

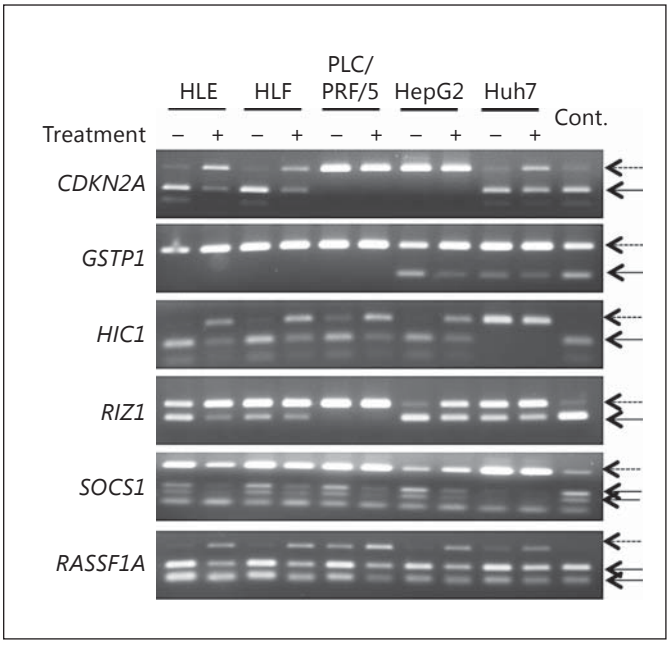

Fig. 1. DNA methylation status of 5 HCC cell lines before and after treatment with 5-Aza-dC and TSA by COBRA. – = Before treatment with 5-Aza-dC and TSA; + = after treatment with 5-Aza-dC and TSA; Cont. = a positive control of methylated DNA sample that was treated with CpG methylase (CpGenome™ universal methylated DNA; Chemicon International, Inc., Temecula, Calif., USA). The solid and dashed arrows indicate the methylated and unmethylated allele, respectively.

Discussion

TSG inactivation through DNA methylation is one of the most important mechanisms that drive human hepatocarcinogenesis [14]. In this study, we comprehensively analyzed alterations in promoter DNA methylation of human HCCs and integrated these results with those obtained from the pharmacological unmasking of 5 different HCC cell lines. We have successfully identified a set of genes that are strong candidates for epigenetically inactivated TSGs in HCCs.

At first, we obtained the methylation profile of HCCs and their corresponding noncancerous liver tissues with the Infinium HumanMethylation27 BeadChip array. Through this analysis, we selected the hypermethylated

Table 1. Candidate genes with transcriptional inactivation through DNA methylation in HCC

Ch.	Gene symbol	Annotation	Gene product	UCSC RefGene	β value difference
19	ZNF154	zinc ion binding; transcription factor	zinc finger protein 154	ZNF154	0.2910
19	TNFSF9	tumor necrosis factor receptor binding	tumor necrosis factor superfamily; member 9	TNFSF9	0.2239
13	EFNB2	ephrin receptor binding; cell differentiation; cell-cell signaling; nervous system development	ephrin B2	EFNB2	0.2146
11	FLJ33790		hypothetical protein LOC283212	KLHL35	0.2097
2	LOXL3	copper ion binding; oxidoreductase activity; scavenger receptor activity	lysyl oxidase-like 3 precursor	LOXL3	0.2053
11	OVOL1	putative transcription factor: zinc ion binding	OVO-like 1 binding protein	OVOL1	0.2031
10	NKX6-2	transcription factor activity	NK6 transcription factor related; locus 2	NKX6-2	0.2009
11	LRFN4	leucine-rich repeat and fibronectin type-III domain-containing 4 variant	leucine-rich repeat and fibronectin type-III domain-containing 4	LRFN4	0.2007
3	RBP1	retinol binding; vitamin A metabolism	retinol binding protein 1; cellular	RBP1	0.1994
X	FLJ14503	catalytic activity	hypothetical protein LOC256714	MAP7D2	0.1930
1	TRIM58	synonyms: BIA2; DKFZp434C091	tripartite motif-containing 58	TRIM58	0.1924
11	MAPK8IP1	mitogen-activated protein kinase scaffold activity; protein kinase inhibitor activity; regulation of the JNK cascade	mitogen-activated protein kinase 8-interacting protein 1	MAPK8IP1	0.1897
4	AFAP	actin filament-associated protein; 110 kDa	actin filament-associated protein	AFAP1	0.1866
5	APC	β-catenin binding; Wnt receptor signaling pathway	adenomatosis polyposis coli	APC	0.1842
2	FRZB	cell differentiation; negative regulation of Wnt receptor signaling pathway	frizzled-related protein	FRZB	0.1838
17	RND2	small GTPase-mediated signal transduction	Rho family GTPase 2	RND2	0.1807
2	TACSTD1	human epithelial glycoprotein-2; surface marker	tumor-associated calcium signal transducer 1 precursor	EPCAM	0.1789
6	BMP6	transforming growth factor-β; growth factor activity; cell differentiation	bone morphogenetic protein 6 precursor	BMP6	0.1784
14	SLC22A17	potent brain-type organic ion transporter	solute carrier family 22 (organic cation transporter); member 17 isoform a	SLC22A17	0.1748
11	UCP2	mitochondrial transport	uncoupling protein 2	UCP2	0.1726
X	SYN1	actin binding; transporter activity; go_process: neurotransmitter secretion	synapsin I isoform Ib	SYN1	0.1725
2	BOLL	RNA binding; spermatogenesis; cell differentiation; regulation of translation	boule isoform 2	BOLL	0.1710
19	ZNF177	negative regulation of transcription from RNA polymerase II promoter	zinc finger protein 177	ZNF177	0.1679
10	ALOX5	oxidoreductase activity; arachidonate 5-lipoxygenase activity; inflammatory response	arachidonate 5-lipoxygenase	ALOX5	0.1663
3	CLDN11	tight junction; calcium-independent cell-cell adhesion	claudin 11	CLDN11	0.1657
3	MCF2L2	synonyms: FLJ42509; KIAA0861	Rho family guanine-nucleotide exchange factor	MCF2L2	0.1640
7	AEBP1	transcription factor activity; cell adhesion; muscle development	adipocyte enhancer binding protein 1 precursor	AEBP1	0.1636

Table 1 (continued)

Ch.	Gene symbol	Annotation	Gene product	UCSC RefGene	β value difference
19	PBX4	embryonic development; regulation of transcription	pre-B cell leukemia transcription factor 4	PBX4	0.1631
16	SMPD3	sphingomyelin phosphodiesterase activity; cell cycle	sphingomyelin phosphodiesterase 3; neutral membrane	SMPD3	0.1624
11	THY1	T cell receptor signaling pathway; negative regulation of apoptosis, cell migration, protein kinase activity, T cell receptor signaling pathway	Thy-1 cell surface antigen	THY1	0.1621
7	FSCN1	cell proliferation; actin cytoskeleton organization	fascin 1	FSCN1	0.1617
8	RBM35A	nucleic acid binding	hypothetical protein LOC54845 isoform 1	ESRP1	0.1614
10	ADAM8	metalloendopeptidase activity	ADAM metallopeptidase domain 8 precursor	ADAM8	0.1613
2	NRP2	vascular endothelial growth factor receptor activity; cell adhesion; cell differentiation; nervous system development	neuropilin 2 isoform 2 precursor	NRP2	0.1611
12	WIF1	Wnt receptor signaling pathway	Wnt inhibitory factor-1 precursor	WIF1	0.1608
16	SOCS1	negative regulation of JAK-STAT cascade	suppressor of cytokine signaling 1	SOCS1	0.1589
14	CHGA	calcium ion binding; blood pressure regulation	chromogranin A precursor	CHGA	0.1579
1	KIF17	microtubule motor activity; microtubule-based movement	kinesin family member 17	KIF17	0.1575
11	CDKN1C	p57KIP2; Beckwith-Wiedemann syndrome; cyclin-dependent protein kinase inhibitor activity	cyclin-dependent kinase inhibitor 1C	CDKN1C	0.1574
20	C20orf100	regulation of transcription	chromosome 20 open reading frame 100	TOX2	0.1571
20	SALL4	sal *(Drosophila)*-like 4; zinc ion binding; go_function: metal ion binding; regulation of transcription	sal-like 4	SALL4	0.1568
20	GDAP1L1	synonyms: dJ881L22.1; DKFZp761K228; dJ995J12.1.1	ganglioside-induced differentiation-associated protein 1-like 1	GDAP1L1	0.1549
2	TFCP2L1	transcription factor activity; regulation of transcription from RNA polymerase II promoter	LBP-9	TFCP2L1	0.1533
9	CDKN2A	cyclin-dependent kinase 4 inhibitor p16-INK4; cyclin-dependent kinase inhibitor 2A; p14ARF	cyclin-dependent kinase inhibitor 2A isoform 3	CDKN2A	0.1513
X	TMSL8	thymosin β; cytoskeleton; actin binding	thymosin-like 8	TMSB15A	0.1504

The 45 genes that showed upregulation in 3 or more cell lines and hypermethylation on the promoters in human HCCs were listed. The β value difference denotes the difference in the mean β value between HCCs and noncancerous livers. Ch. = Chromosome; Annotation = the gene annotation provided by the National Center for Biotechnology Information (NCBI); Product = the name of the gene product by the NCBI; UCSC RefGene = the gene names by the University of California, Santa Cruz (UCSC) Genome Bioinformatics.

genes with a mean β value ≥1.5 in HCCs compared to that of noncancerous liver tissues. To obtain differentially methylated genes, the β values between the two groups were assessed with the Mann-Whitney U test with false discovery rate controlled using the Benjamini-Hochberg procedure. To confirm the reactivation of hypermethylated genes after pharmacological unmasking, we performed a comprehensive analysis of gene reactivation after 5-Aza-dC and TSA treatment in human HCC. In conclusion, the 45 hypermethylated genes that turned on transcription in 3 or more HCC cell lines were considered as key candidates for epigenetically inactivated genes during hepatocarcinogenesis.

Previously, we have reported that a set of 8 TSGs showed abnormal methylation in early HCC [15]. The number of these methylated TSGs in the hepatitis tissue correlated with the time to HCC occurrence in chronic hepatitis C patients. Among the 8 TSGs, the *APC*, *CDKN2A* and SOCS1 genes were also identified as transcriptionally inactivated TSGs by DNA methylation through the presented selection. In addition, a recent report identified *sphingomyelin phosphodiesterase 3* (*SMPD3*) as a TSG in HCC using HumanMethylation27 BeadChip and array-based reexpression profiling [16]. Another gene called the *Wnt inhibitory factor-1 precursor (WIF1)* also reportedly undergoes epigenetic silencing in HCC [17]. Notably, both of these genes were selected as epigenetically inactivated in HCC through our analysis. Together, these studies validate the robustness our data and methodology used to obtain the present data.

Interestingly, several potential TSGs, which were identified as epigenetically inactivated genes in this study, have not been reported in human HCCs but in other cancers. For example, the *frizzled-related protein (FRZB)* gene that regulates Wnt signaling shows hypermethylation in bladder cancer [18]. More importantly, our analysis identified *FRZB* as a potential TSG in HCC as indicated by its inactivation through DNA methylation. Another gene, *claudin 11 (CLDN11)* – an epigenetic biomarker of melanoma [19, 20] – was found having a hypermethylated promoter in our study. We have also identified the *cyclin-dependent kinase inhibitor 1C* (*CDKN1C)* as an epigenetically inactivated gene in HCCs. The loss of the *CDKN1C* gene product, p57(KIP2), correlates with a poor prognosis in HCC patients [21]. Therefore, it is possible that p57(KIP2) downregulation in HCCs may be due to an epigenetic interaction.

Our analyses have also revealed that increased methylation occurs in certain genes coding stem cell markers such as the *Sal-like protein 4 (SALL4)* and the *tumor-associated calcium signal transducer 1 (TACSTD1/EPCAM)* in a subset of HCCs. Studies have shown that increased expression of hepatic progenitor markers such as SALL4 and TACSTD1 are associated with a poor prognosis for cancer, including HCC [22, 23]. Until now, the biological implication of the hypermethylation of these progenitor cell markers in HCC was controversial owing to the reported lack of SALL4 expression in noncancerous liver [24]. However, we observed an increase in both SALL4 and TACSTD1 expression levels after the pharmacological unmasking procedure in HCC cell lines. Hypermethylated SALL4 reactivates in response to 5-Aza-dC treatment in the acute promyelocytic leukemia cell line and is responsible for aggressive tumors [25]. These studies indicate that further analyses of the epigenetic regulation of SALL4 and TACSTD1 in HCC should be required for the development of epigenetic-based therapies for HCC.

In this study, we identified several potential TSGs that are inactivated by epigenetic interactions in HCC. Since we analyzed a limited number of cell lines for pharmacological unmasking, many other epigenetically inactivated TSGs in HCC still need to be discovered [26, 27]. For example, the *GSTP1* and *RUNX3* genes, which have abnormal methylation in HCCs, were not listed because only 1–2 cell lines out of the 5 HCC cell lines analyzed showed abnormal methylation of these genes [15]. Nonetheless, the data presented here are important to understand what kinds of TSGs are inactivated through epigenetic mechanisms during HCC onset and progression.

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