

# Identification of genes preferentially methylated in hepatitis C virus-related hepatocellular carcinoma

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Chronic infections by hepatitis B virus (HBV) and hepatitis C virus (HCV) appear to be the most significant causes of hepatocellular carcinoma (HCC). Aberrant promoter methylation is known to be deeply involved in cancer, including in HCC. In this study, we analyzed aberrant promoter methylation by methylated DNA immunoprecipitation-on-chip analysis on a genome-wide scale in six HCCs including three HBV-related and three HCV-related HCCs, six matched noncancerous liver tissues, and three normal liver tissues. Candidate genes with promoter methylation were detected more frequently in HCV-related HCC. Candidate genes methylated preferentially to HBV-related or HCV-related HCCs were detected and selected, and methylation levels of the selected genes were validated by quantitative methylation analysis using MALDI-TOF mass spectrometry using 125 liver tissue samples, including 61 HCCs (28 HBV-related HCCs and 33 HCV-related HCCs) and 59 matched noncancerous livers, and five normal livers. Among analyzed genes, preferential methylation in HBV-related HCC was validated in one gene only. However, 15 genes were found to be methylated preferentially in HCV-related HCC, which was independent from age. Hierarchical clustering of HCC using these genes stratified HCV-related HCC as a cluster of frequently methylated samples. The 15 genes included genes inhibitory to cancer-related signaling such as RAS/RAF/ERK and Wnt/ $\beta$ -catenin pathways. Methylation of dual specificity phosphatase 4 (*DUSP4*), cytochrome P450, family 24, subfamily A, polypeptide 1 (*CYP24A1*), and natriuretic peptide receptor A (*NPR1*) significantly correlated with recurrence-free survival. It was indicated that genes methylated preferentially in HCV-related HCC exist, and that DNA methylation might play an important role in HCV-related HCC by silencing cancer-related pathway inhibitors, and might perhaps be useful as a prognostic marker. (*Cancer Sci* 2010; 101: 1501–1510)

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer mortality in the world, accounting for 598 000 deaths in 2002.<sup>(1)</sup> Among numerous causative factors suggested, chronic infection caused by hepatitis B virus (HBV) and/or hepatitis C virus (HCV) has been defined as the main risk factor for HCC development. Chronic HBV infection is the predominant risk factor for HCC in South-East Asia and Africa, whereas chronic HCV infection is predominant in Western countries and Japan.<sup>(2)</sup> HBV, DNA-containing virus, and HCV, RNA-containing virus, belong to two different viral families, Hepadnaviridae and Flaviviridae, respectively.<sup>(3)</sup> They show different characteristics in carcinogenesis and clinical presentation of HCC. It is reported that about 10% of HBV-carriers grow into chronic hepatitis patients, 20% of which transform into liver cirrhosis (LC) and HCC. Hepatitis B virus can also cause HCC in the absence of LC through the pathway of integration into the human genome.<sup>(4,5)</sup> On the other hand, more than 60% of HCV-carriers

grow into LC and mostly develop to HCC over several decades. The onset of HCV-related HCC is later than that of HBV-related HCC.<sup>(4,5)</sup>

Hepatocellular carcinoma is caused by genetic or epigenetic alterations.<sup>(6–8)</sup> The genetic alterations include chromosomal instability with point mutations and deletions. Chromosomal instability of *CDKN2A* and *TP53* loci was observed in HCC, and mutations of *AXIN1* and *PIK3CA* were more frequently seen in HBV-related HCC than in HCV-related HCC.<sup>(9)</sup>

Aberrant DNA methylation of promoter CpG islands has been described as one of the major epigenetic alterations in human cancers,<sup>(10,11)</sup> including HCC.<sup>(12,13)</sup> It has been reported that methylation of promoter CpG islands of the *p16* tumor suppressor gene occur in 73% of HCC tissues,<sup>(14)</sup> and in 56% of HBV-related HCC and 84% of HCV-related HCC.<sup>(15)</sup> Yang *et al.* reported that methylation of suppressor of cytokine signaling 1 (*SOC1*), adenomatous polyposis coli (*APC*), and *p15* was more frequently observed in HCV-related HCC than in HBV-related HCC.<sup>(16)</sup> This may be a consequence of the involvement of different genetic/epigenetic pathways in the process of carcinogenesis in HBV- and HCV-related HCC, and the occurrence of HBV-related HCC mainly through chromosomal instability, whereas HCV-related HCC may be more involved with aberrant DNA methylation,<sup>(17,18)</sup> and there may be genes methylated preferentially to HCV-related HCC.

To enable high-resolution mapping of DNA methylation, we reported methylated DNA immunoprecipitation (MeDIP)-chip analysis on short oligonucleotide array using unbiased amplification of MeDIP products by *in vitro* transcription,<sup>(19)</sup> and applied the method to genome-wide DNA methylation analysis of colorectal cancer.<sup>(20)</sup> In the present study, we analyzed aberrant promoter methylation in six HCC clinical samples (including three HBV-related HCCs and three HCV-related HCCs) and noncancerous tissues on a genome-wide scale by the method. Candidate regions of promoter methylation preferentially to HBV-related HCC and HCV-related HCC were selected, and the methylation levels of these genes were measured quantitatively using MALDI-TOF mass spectrometry (MassARRAY).

## Materials and Methods

**Clinical samples and cell lines.** Twenty-eight HBV-related HCC and 26 matched noncancerous liver tissues, and 33 HCV-related HCC and 33 matched noncancerous liver tissues, were obtained from HCC patients, and five normal liver samples were obtained from colorectal cancer patients with liver metastasis, who underwent surgical liver resection at Tokyo University

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**Table 1. Clinicopathological features in HBV- and HCV-related HCC patients**

Features	HBV+ (n = 28)	HCV+ (n = 33)	Normal liver (n = 5)	P-value
Gender				
Male/female	23/5	20/13	4/1	0.093
Age (years)				
Mean ± SE	58.1 ± 1.9	67.1 ± 1.3	59.8 ± 2.2	<0.0001*
Background				
Normal	1	0		0.44
Hepatitis	13	13		
Liver cirrhosis	14	20		
Tumor size (cm)				
<3.0	7	15		0.18
≥3.0	20	18		
NA	1			
Differentiation				
Well	8	15		0.29
Moderate	16	16		
Poor	4	2		
AFP (ng/mL)				
>60	10	14		0.61
<60	18	19		
PIVKAll (mAU/mL)				
>40	15	14		0.45
<40	13	19		

\*P < 0.05. Background, surrounding liver tissue around hepatocellular carcinoma (HCC). AFP, alpha fetoprotein; HBV+, serum hepatitis B virus surface antigen (+); HCV+, serum anti-hepatitis C virus antibody (+); NA, information not available; PIVKAll, protein induced by vitamin K absence or antagonist II.

Hospital from August 1998 to June 2006, with written informed consent. Clinicopathological information is shown in Table 1. Tissues were kept frozen at  $-80^{\circ}\text{C}$  until use. Normal liver tissue was extracted with enough margins from cancer and it was confirmed microscopically that no cancer cells were included. Five liver cancer cell lines, Huh7, Huh6, HepG2, Alex, and Hep3B, were obtained from the American Tissue Culture Collection (Manassas, VA, USA). Human peripheral lymphocyte DNA was obtained from Coriell Cell Repositories (Camden, NJ, USA). DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), and RNA using TRIzol (Invitrogen, Carlsbad, CA, USA). This study was certified by Ethics Committee of the University of Tokyo.

**5-Aza-2'-deoxycytidine and Trichostatin A treatment.** Liver cancer cell lines were seeded at a density of  $3 \times 10^5$  cells/10-cm dish on Day 0, and exposed to 1, 3, or 10  $\mu\text{M}$  5-aza-2'-deoxycytidine on Days 1, 2, and 3. Trichostatin A treatment was performed on Day 3 at a dose of 300 nM. Medium was changed every 24 h, and cells were harvested on Day 4.

**Methylated DNA immunoprecipitation-chip analysis.** Methylated DNA immunoprecipitation-chip using Human Promoter 1.0R tiling array (Affymetrix, Santa Clara, CA, USA), which covered 199 543 165 bp and 4 071 296 CpG sites around over 25 500 promoter regions, was performed as we previously reported.<sup>(19,20)</sup> Briefly, 6  $\mu\text{g}$  each of genomic DNA of six clinical HCC samples, six matched noncancerous liver tissues, and three normal livers were fragmented by sonication, and immunoprecipitated by anti 5-methylcytosine monoclonal antibody (kindly supplied by Dr K. Watanabe, Toray Research Center, Tokyo, Japan). MeDIP and input samples underwent unbiased amplification by *in vitro* transcription. Amplified cRNA was converted into cDNA. cDNA was hybridized by promoter tiling array twice for both MeDIP and input samples (i.e. 60 arrays in

total for the 15 samples). Within a window of 550 bp, the duplicated data from MeDIP DNA was compared with duplicated data from input DNA using the Wilcoxon rank sum test to calculate P-values to detect candidate methylation sites. MeDIP-chip data is available at GEO datasets (Nagae *et al.*, #GSE19665).

**Bisulfite treatment.** Bisulfite conversion of DNA was performed as previously described.<sup>(21)</sup> After ultrasonic fragmentation of DNA in 30 s, 1  $\mu\text{g}$  of DNA was denatured in 0.3 N NaOH and then subjected to 15 cycles of 30 s at  $95^{\circ}\text{C}$  and 15-min incubation in 3.6 M sodium bisulfite and 0.6 mM hydroquinone at  $50^{\circ}\text{C}$ . The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI, USA), desulfonated in 0.3 N NaOH at room temperature for 5 min, and then purified by ethanol precipitation. Finally, bisulfite-treated DNA was dissolved in 80  $\mu\text{L}$  of distilled water.

**Quantitative methylation analysis.** Methylation levels were measured using MassARRAY (Sequenom, San Diego, CA, USA).<sup>(20,22)</sup> Bisulfite-treated DNA was amplified by PCR, PCR product was transcribed by *in vitro* transcription (IVT) and the RNA was cleaved by RNaseA. Unmethylated cytosine was converted to uracil by bisulfite treatment, that is thymine in PCR product and finally adenine (A) in IVT product. Methylated cytosine was not converted, that is cytosine in PCR product, and finally guanine (G) in IVT product. Methylation status was determined by the mass difference between A and G in cleaved RNA product. Quantitative methylation scores were obtained at each analytic unit of a cleaved product, referred to as "CpG unit", which could comprise one to several CpG sites.

Primers were designed to include no CpG site or only one CpG site in the 5' region of primers; these are shown in Supporting information, Table S1. Human peripheral lymphocyte DNA was used as diploid human DNA, and amplified with the GenomiPhi v2 DNA amplification kit (GE Healthcare Lifescience, Piscataway, NJ, USA). The amplified DNA was not methylated at all in any CpG sites, and was used as unmethylated (0%) control. The amplified DNA was also methylated by SssI methylase (New England Biolabs, Beverly, MA, USA) and used as a fully methylated (100%) control. By mixing the 0% and 100% control samples, partially methylated control samples (25%, 50%, and 75%) were generated.<sup>(20)</sup>

**Expression microarray analysis.** mRNA expression in 10 HCCs and the 10 matched noncancerous liver samples was analyzed by GeneChip Human Genome U133 plus 2.0 oligonucleotide arrays (Affymetrix). For global normalization, the average signal in an array was made equal to 100. Expression array data for the 20 samples is available at GEO datasets (#GSE19665).

**Quantitative PCR analysis.** Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously reported.<sup>(23)</sup> cDNA was synthesized from 1  $\mu\text{g}$  of total RNA treated with DNase I (Ambion, Austin, TX, USA) with a Superscript III kit (Invitrogen). Real-time RT-PCR was done with SYBR Green PCR Core Reagents (PE Biosystems, Foster City, CA, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The quantity of mRNA of each gene in a sample was measured by comparing with standard samples that contained  $10^1$  to  $10^6$  copies of the genes, and was normalized to that of actin, beta (*ACTB*). The PCR primers and conditions are shown in Supporting information, Table S2.

**Statistical analysis.** Correlation between HBV- or HCV-related HCC and clinicopathological features was analyzed by Fisher's exact test for all except age, for which the *t*-test was used. Methylation levels of HBV- and HCV-related HCC were compared by *t*-test. The frequency of methylation was compared with clinicopathological features by Fisher's exact test. Fisher's exact test and *t*-test were performed with JMP 8 software (<http://www.jmp.com>). Hierarchical clustering was analyzed based on uncentered correlation and average linkage clustering algorithm in sample direction using Cluster 3.0

software based on DNA methylation level. The dendrogram and heat map were constructed using TreeView software (<http://rana.lbl.gov/EisenSoftware.htm>). Disease-free survival analysis by Kaplan–Meier method was performed with JMP 8 software and *P*-values were calculated by log-rank test. The end of the follow-up period was 60 months from the primary surgery and the mean follow-up time of the cases was 51 months (*n* = 58). HCC recurrence was the primary end point and deaths by other causes were censored.

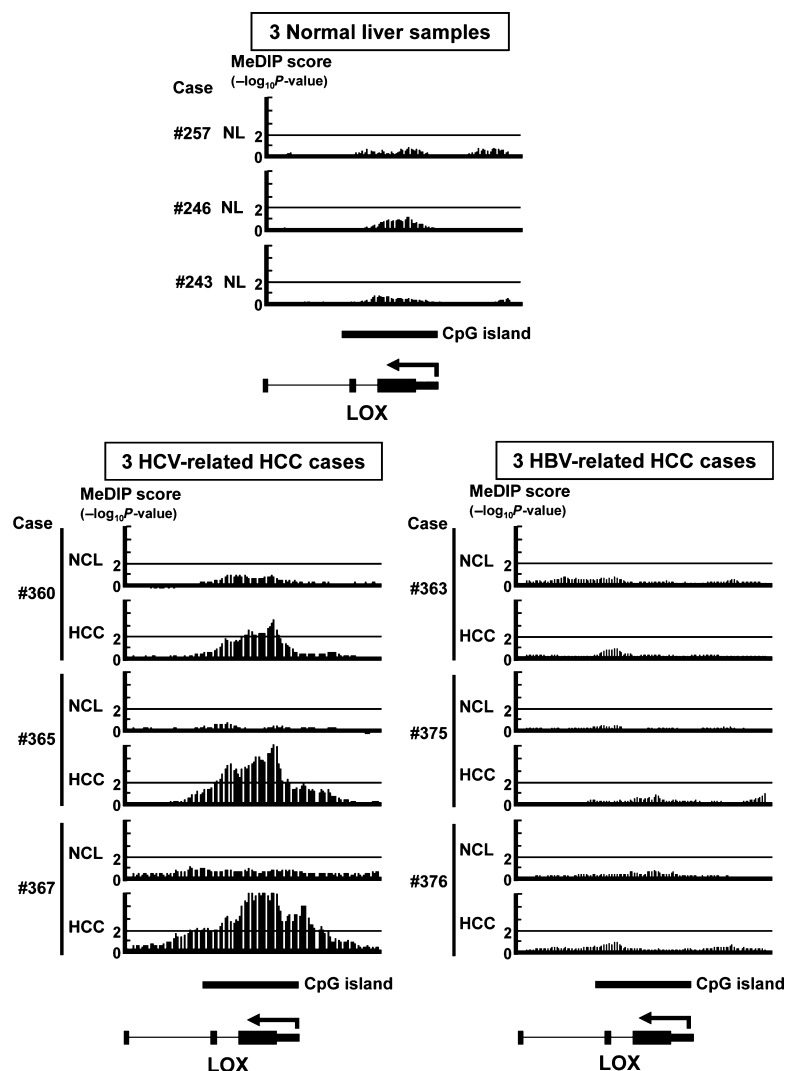
## Results

**Methylated DNA immunoprecipitation-chip analysis.** Six clinical HCC samples and their matched noncancerous liver tissues and three normal livers were analyzed by MeDIP-chip. Genes that possessed regions with  $P < 10^{-2}$  within 1 kb  $\pm$  transcription start site were regarded as candidate genes with promoter methylation (Fig. 1).

Among candidate genes with promoter methylation, 1421 genes were methylated in at least two of six HCCs but not in any matched noncancerous liver tissues nor in any normal liver

tissues (Fig. 2a). Among these 1421 genes, 666 genes showed methylation only in HCV-related HCC, 696 genes showed methylation in both HBV- and HCV-related HCC, and 59 genes showed methylation only in HBV-related HCC. Candidate methylation was detected more frequently in HCV-related HCC than in HBV-related HCC.

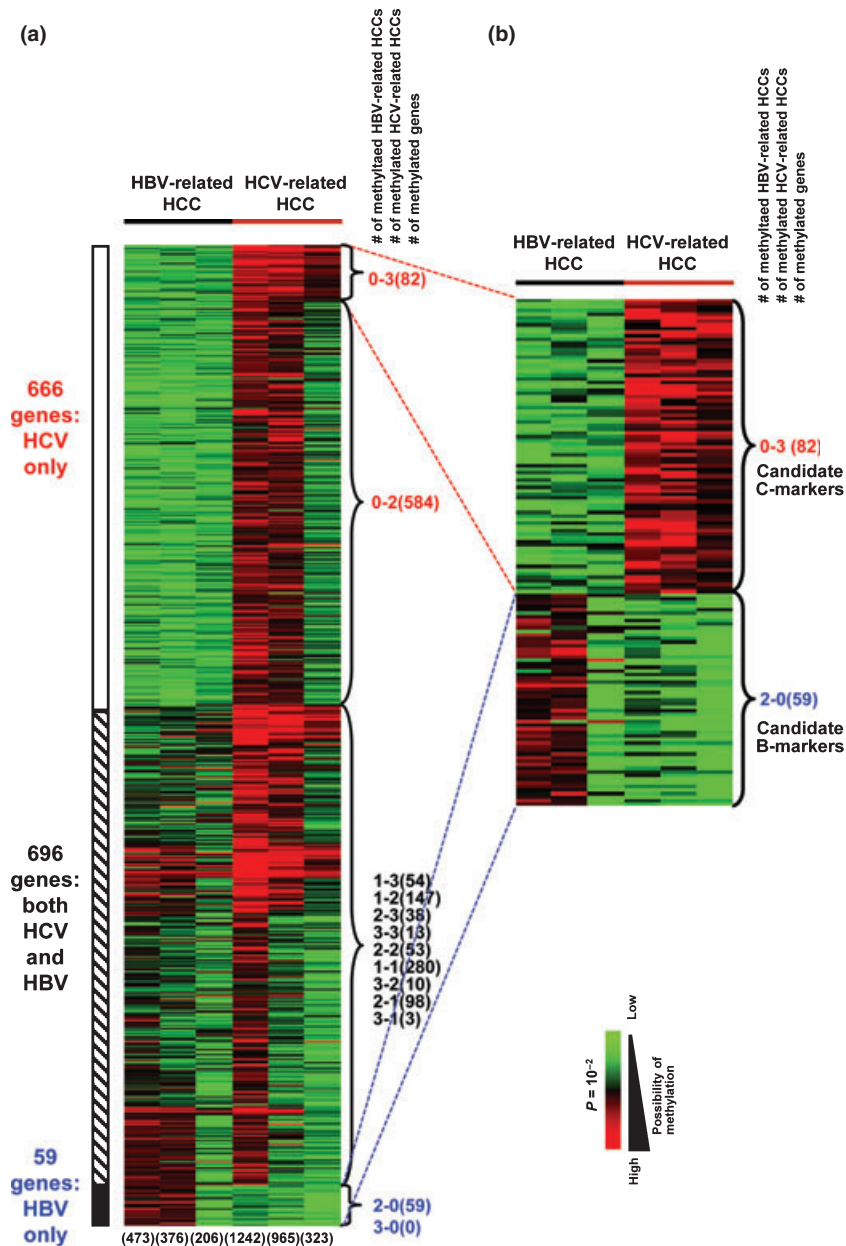
Among 666 candidate genes with methylation only in HCV-related HCC, 82 genes were methylated in three HCV-related HCCs but no HBV-related HCC, and were regarded as candidate genes methylated preferentially in HCV-related HCC (C-markers, hereafter) (Fig. 2b). Among 59 candidate genes with methylation only in HBV-related HCC, however, we did not detect any genes methylated in three HBV-related HCCs but no HCV-related HCC. Instead, the 59 genes methylated in two of three HBV-related HCCs but no HCV-related HCC were regarded as candidate genes methylated preferentially in HBV-related HCC (B-markers, hereafter) (Fig. 2b). Among these 82 and 59 genes, 26 and 14 candidate C- and B-markers, respectively, were selected for further analysis (Supporting information Fig. S1 and Supporting information Table S3).



**Fig. 1.** Representative data from methylated DNA immunoprecipitation (MeDIP)-chip analysis. NCL, noncancerous liver tissue; NL, normal liver. MeDIP-chip score (*y*-axis) was shown by  $-\log_{10}P$ -value, and regions with score  $>2$  (i.e.  $P < 10^{-2}$ ) were regarded to be candidate methylation regions. *LOX* possessed regions with  $P < 10^{-2}$  within 1 kb  $\pm$  transcription start site, and was regarded as a candidate gene with promoter methylation in three hepatitis C virus (HCV)-related hepatocellular carcinomas (HCCs), but not in the remaining samples. Clinical information for six HCC cases used for MeDIP-chip analysis are shown at the bottom.

Case #	Sex	Age	Poor/moderate/well	Tumor size (cm)
#360	Male	66	Moderate	2.7
#365	Female	71	Moderate	6.5
#367	Male	72	Moderate	8.0

Case #	Sex	Age	Poor/moderate/well	Tumor size (cm)
#363	Male	64	Poor	10
#375	Male	68	Moderate	5.5
#376	Male	57	Moderate	12



**Fig. 2.** Heat map of genes methylated in methylated DNA immunoprecipitation (MeDIP)-chip. (a) Heat map of 1421 genes that were methylated in at least two of six hepatocellular carcinomas (HCCs) but unmethylated in matched noncancerous liver tissues and normal liver tissues. The six numbers at the bottom show the number of genes methylated in each case. The three numbers at the right side of heat map show the numbers of hepatitis B virus (HBV)- and hepatitis C virus (HCV)-related HCC cases with methylation, and the number of genes included (in parentheses). (b) Heat map of 82 candidate C-marker genes and 59 candidate B-marker genes.

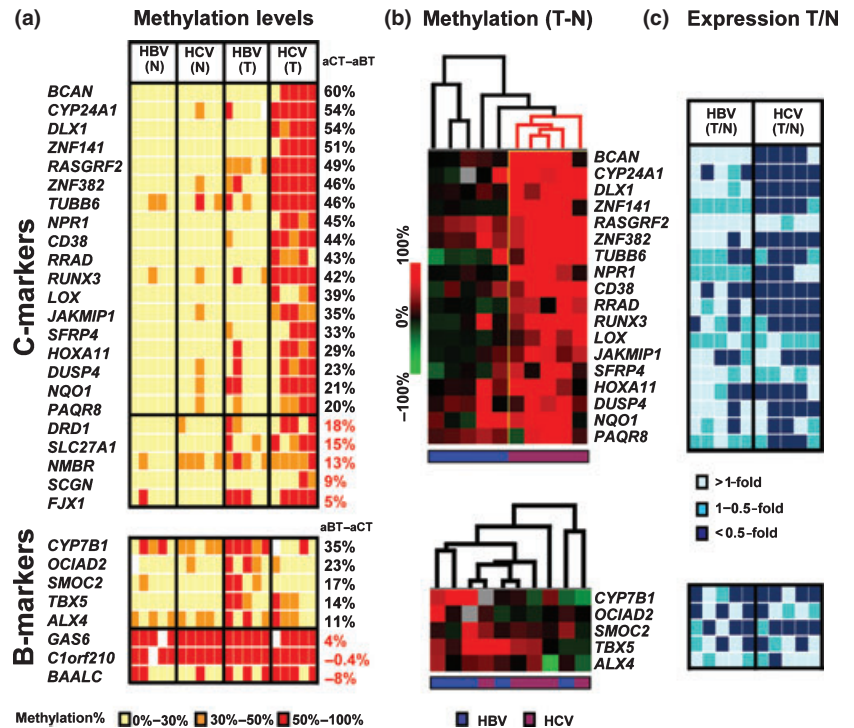
**Quantitative methylation analysis in clinical samples.** We first analyzed methylation levels of control samples (0%, 25%, 50%, 75%, 100% methylation) by MassARRAY and validated the quantitativity of each primer set (Supporting information Fig. S2). Among 40 genes, primer sets for 31 genes including 23 candidate C-markers and eight candidate B-markers were validated in regard to quantitativity and used for further analysis (Supporting information Fig. S1). The amplicons of 31 analyzed regions included 6902 bp and 334 CpG units derived from 548 CpG sites. Methylation scores were measurable at 200 CpG units derived from 310 CpG sites by MassARRAY (Supporting information Table S1).

**Comparison of promoter methylation with mRNA expression of the candidate genes.** For methylation validation, promoter methylation levels of the 31 genes were measured by MassARRAY for the six pairs of HCC and matched noncancerous livers used in MeDIP, as well as four additional pairs. The average methylation rates in HBV- and HCV-related HCC were compared. Eighteen of the 23 candidate C-markers showed a methylation

difference >20% (Fig. 3a). Using the methylation increase value of HCC compared to the matched noncancerous livers, hierarchical clustering analysis stratified a group of five HCV-related HCCs as samples with higher methylation (Fig. 3b). Expression of these genes was analyzed in 10 pairs of HCC by expression microarrays, and we observed a decrease of expression to <0.5-fold mainly in HCV-related HCC samples, suggesting gene silencing by promoter methylation (Fig. 3c). To confirm gene silencing by promoter methylation, methylation levels in five liver tumor cell lines were analyzed by MassARRAY (Fig. 4a). If the methylation level of a gene was >75% in a cell line, the expression level of the gene in the methylated cell line and its re-expression after 5-aza-2'-deoxycytidine/Trichostatin A treatment were analyzed by real-time RT-PCR. All analyzed genes showed very low expression level in methylated cell lines, and showed up-regulation after 5-aza-2'-deoxycytidine treatment alone or with Trichostatin A treatment, suggesting that the genes were silenced by promoter methylation in HCC (Fig. 4b).



**Fig. 3.** Relationship between promoter methylation and mRNA expression of the candidate genes. (a) Methylation levels measured by MassARRAY in 10 pairs of hepatocellular carcinoma (HCC) (T) and matched noncancerous liver samples (N) including the six pairs analyzed by methylated DNA immunoprecipitation (MeDIP). Yellow, orange, red, white boxes: methylation rate 0–30%, 30–50%, 50–100%, no data, respectively. aCT, aBT: average methylation level of hepatitis C virus (HCV)-related HCC, and that of hepatitis B virus (HBV)-related HCC, respectively. (b) Heat map of the 18 genes with aCT–aBT values  $\geq 20\%$  (upper) and six genes with aBT–aCT values  $\geq 10\%$  (lower). Methylation data was shown by methylation increase in the HCC sample compared to matched noncancerous liver. Blue, HBV-related HCC. Violet, HCV-related HCC. (c) Levels of mRNA expression detected by GeneChip in 10 pairs of HCC and matched noncancerous liver. Light-blue, blue, dark-blue: >1-fold, 0.5–1-fold, <0.5-fold expression (calculated using GeneChip scores) in HCC compared to matched noncancerous liver, respectively.



On the other hand, only two of eight candidate B-markers showed >20% as a difference of methylation rate (Fig. 3a). Though five of the eight genes showing a difference >10% were used as tentative B-markers for clustering and expression analyzes (Fig. 3b,c), we did not observe a cluster of methylated HBV-related HCC nor decreased expression preferentially to HBV-related methylation.

**DNA methylation status in 125 clinical samples.** Methylation levels were measured by MassARRAY in an expanded number of samples, that is 33 pairs of HCV-related HCC and matched noncancerous liver tissues, 28 HBV-related HCCs and 26 matched noncancerous liver samples, and five normal liver samples (Fig. 5a). All the 18 validated C-markers were significantly more methylated in HCV-related HCC compared with HBV-related HCC ( $P < 0.05$ ,  $t$ -test). As for the five tentative B-markers, however, only two genes, cytochrome P450, family 7, subfamily B, polypeptide 1 (*CYP7B1*) and T-box 5 (*TBX5*), showed significantly more frequent hypermethylation in HBV-related HCC ( $P < 0.05$ ,  $t$ -test).

Since HCV-related HCC patients were significantly older (Table 1), methylation levels were compared between age-matched (51–69 years) HBV- and HCV-related HCC samples. Among 18 C-markers, 15 markers still showed significant difference of methylation levels (Supporting information Table S4), and were therefore considered to be preferentially methylated in HCV-related HCCs independent from age. Among the two B-markers, *CYP7B1* and *TBX5*, only *CYP7B1* showed significant difference of methylation in comparison to age-matched HCCs.

Hierarchical clustering of 59 HCC samples was performed using the methylation difference values in the 59 HCC samples compared to the matched noncancerous liver samples (Fig. 5b). When the 15 age-independent C-markers were used, a cluster of frequently methylated HCC was detected, which was significantly correlated to HCV-related HCC ( $P < 10^{-5}$ ,  $\chi^2$ -test). The cluster and its significant correlation to HCV-related HCC indicated that genes methylated preferentially in HCV-related HCC exist, including the 15 identified C-markers at least.

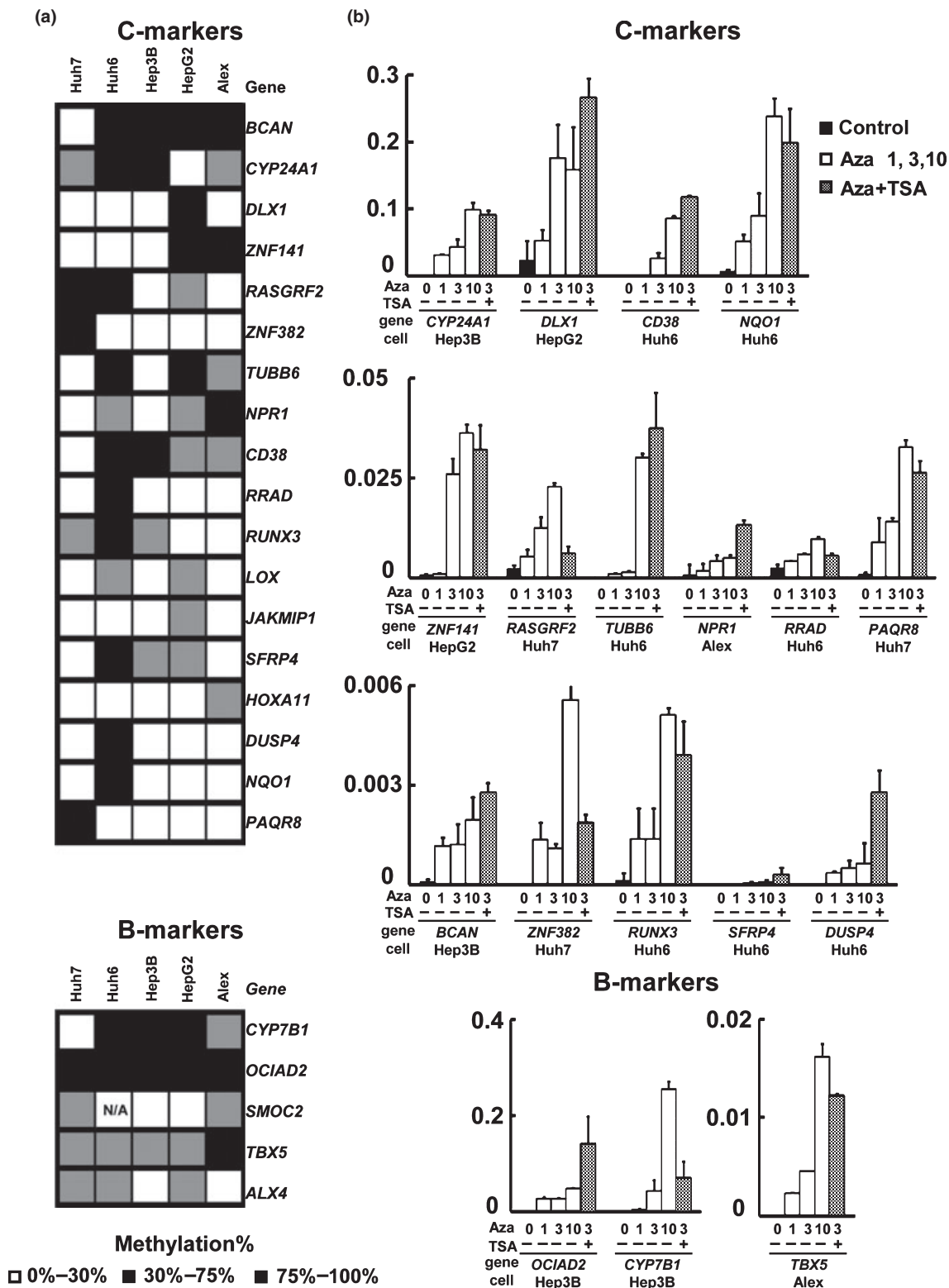
**Correlation between DNA methylation status and clinicopathological features.** DNA methylation status of the 15

C-markers was compared with clinicopathological features including gender, age, background liver tissue, tumor size, tumor differentiation, serum alpha fetoprotein, and serum protein induced by vitamin K absence or antagonist II (PIVKAI) (Supporting information Table S5). Methylation of cytochrome P450, family 24, subfamily A, polypeptide 1 (*CYP24A1*), distal-less homeobox 1 (*DLX1*), natriuretic peptide receptor A (*NPR1*), secreted frizzled-related protein 4 (*SFRP4*), dual specificity phosphatase (*DUSP4*), and progesterin and adipoQ receptor family member VIII (*PAQR8*) significantly correlated with older age (>60 years). As for gender, female correlated with *SFRP4* methylation ( $P = 0.03$ , Fisher's exact test). The other features did not correlate with the methylation of any C-markers.

Regarding prognosis, correlation between disease-free survival and methylation statuses of the 15 C-markers was analyzed by the Kaplan–Meier method. When whole HCC cases were analyzed, HCCs with *DUSP4* methylation and *CYP24A1* methylation showed significant correlation with survival without recurrence ( $P = 0.038$  and  $0.013$ , respectively, log-rank test) (Fig. 6a), and methylation of three other genes, zinc finger protein 141 (*ZNF141*), Ras-related associated with diabetes (*RRAD*), and *SFRP4*, showed tendency toward better prognosis ( $P = 0.084$ ,  $0.052$ , and  $0.088$ , respectively) (Supporting information Table S6). Gene methylation did not correlate with poorer prognosis. When correlation was analyzed among HCC-related HCCs only, methylation of *DUSP4* and *NPR1* significantly correlated with better prognosis ( $P = 0.042$  and  $0.007$ , respectively) (Fig. 6b). *CYP24A1* methylation tended to correlate with better prognosis among HCV-related HCCs ( $P = 0.050$ ) (Supporting information Table S6). There was no significant difference between prognoses of HBV-related and HCV-related HCCs (Supporting information Fig. S3).

## Discussion

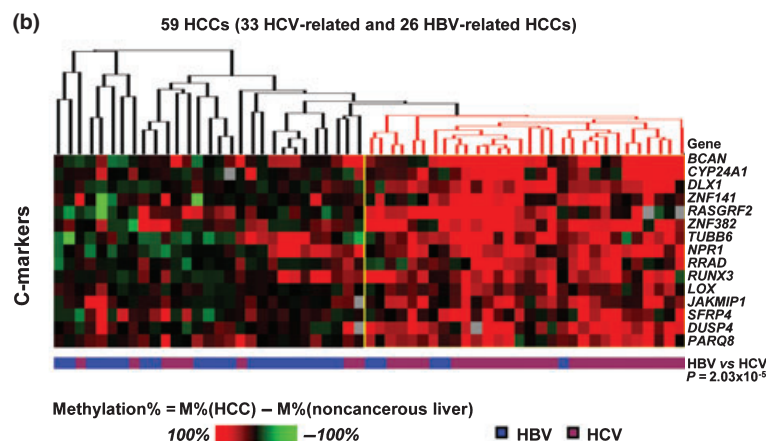
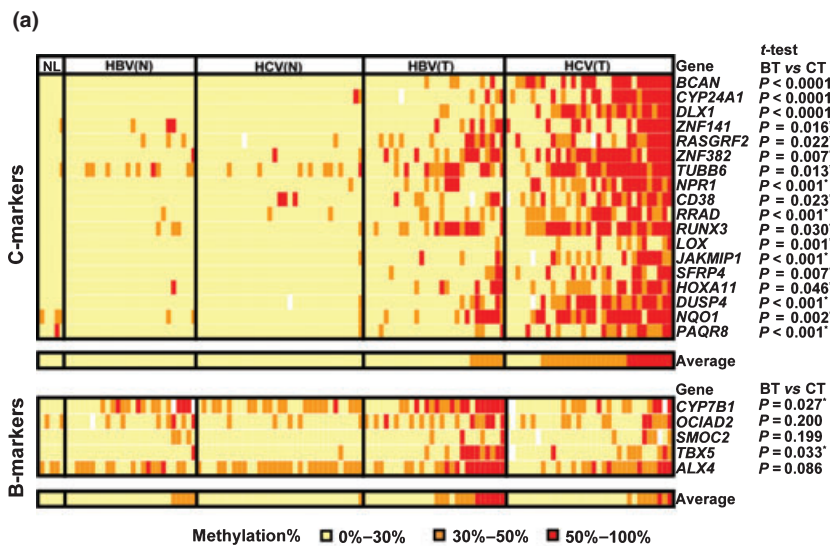
Our results demonstrated that HCV-related HCC and HBV-related HCC could be classified into different groups according to DNA methylation information. It was indicated that C-markers exist, at least 15 genes, and that DNA methylation might be



**Fig. 4.** Re-expression analysis by treatment with 5-aza-2'-deoxycytidine/Trichostatin A. (a) Methylation levels in five liver tumor cell lines. White, 0–30%; gray, 30–75%; black, 75–100%. (b) Gene expression levels analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Gene re-expression was analyzed in cell lines with gene methylation level >75% if any, and normalized to that of *ACTB*. All the analyzed genes showed very low expression level in methylated cell lines, and showed up-regulation after 5-aza-2'-deoxycytidine treatment or with additional Trichostatin A treatment. Aza 1, 3, 10 refers to treatment by 1, 3, 10  $\mu$ M of 5-aza-2'-deoxycytidine. TSA+, 300 nM of Trichostatin A.

an important cause of hepatic carcinogenesis resulting from HCV infection. HCV-related HCC patients were significantly older than patients with HBV-related HCC or metastatic liver

tumor (Table 1), so age-dependent methylation may have to be considered (Supporting information Table S5).<sup>(24)</sup> But 15 among 18 C-marker genes were methylated in age-matched



**Fig. 5.** Analysis of DNA promoter methylation in an expanded number of hepatocellular carcinoma (HCC) cases. (a) Methylation analysis of 125 liver samples including 33 pairs of hepatitis C virus (HCV)-related HCC and matched noncancerous livers, 28 hepatitis B virus (HBV)-related HCCs and 26 HBV-related matched noncancerous livers, and five normal liver samples. BT, HBV-related HCC; CT, HCV-related HCC; NL, normal liver. Average methylation rate of all markers in each sample is shown at the bottom. Significant difference of methylation level between HBV- and HCV-related HCC was analyzed by *t*-test (right). (b) Heat map of methylation increase in 15 age-independent C-markers in 59 HCCs compared to matched noncancerous liver tissues. Red dendrogram, a cluster of HCC with frequent methylation.

HCV-related HCC significantly more than in HBV-related HCC (Supporting information Table S3). As for HBV, only one gene, *CYP7B1*, was found to be methylated in age-matched HBV-related HCC significantly more than in HCV-related HCC, and involvement of promoter methylation was suggested to be smaller in this study. But detection of candidate methylation genes was started using three HBV-related and three HCV-related HCCs, and further study is necessary to clarify the involvement and importance of DNA methylation in HBV-related HCC, including the absence/existence of B-markers.

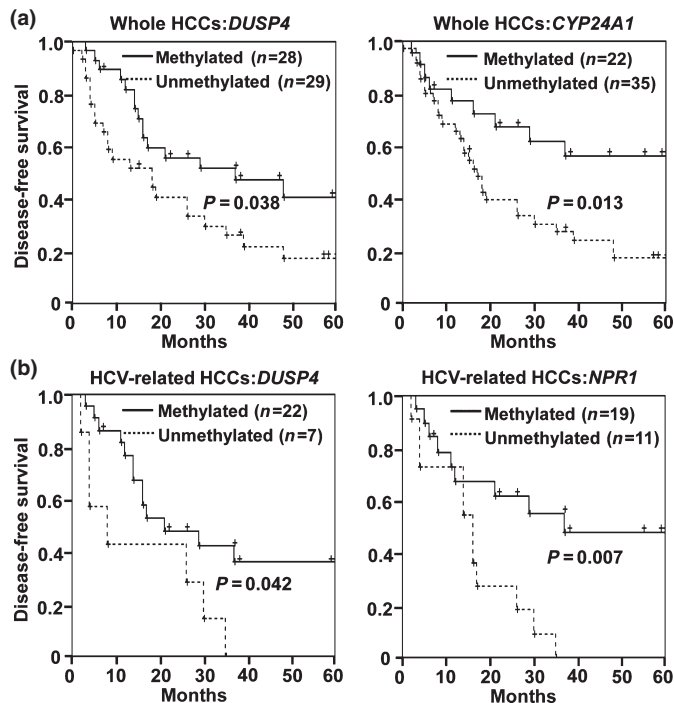
The 15 genes identified as age-independent C-markers included brevican (*BCAN*), *CYP24A1*, *DLX1*, *ZNF141*, Ras protein-specific guanine nucleotide-releasing factor 2 (*RASGRF2*), *ZNF382*, tubulin, beta 6 (*TUBB6*), *NPR1*, *RRAD*, *RUNX3*, lysyl oxidase (*LOX*), janus kinase and microtubule interacting protein 1 (*JAKMIP1*), *SFRP4*, *DUSP4*, and *PAQR8*. Genes inhibitory to Ras/Raf/ERK signaling (*NPR1*, *DUSP4*, *LOX*, and *RRAD*) and inhibitory to Wnt/ $\beta$ -catenin signaling (*SFRP4* and *RUNX3*) were included, suggesting that while methylation of some C-markers may be passenger methylation, methylation of a part of C-markers might contribute to genesis of HCC by disrupting cancer-related pathways through the silencing of a variety of genes.

The Ras/Raf/ERK signaling cascade is frequently deregulated in tumorigenic diseases, promoting cell proliferation and transformation.<sup>(25)</sup> While oncogenic mutation of Ras or Raf was reported to be rare in HCC,<sup>(26)</sup> MAPK/ERK activity level was increased in 58–75% of HCC.<sup>(27,28)</sup> It was also reported that Ras

and its downstream effector were activated in all HCC, and that at least one of the genes involved in the inhibition of the Ras pathway was affected by methylation in all HCC.<sup>(29)</sup> Correlation of increase of Ras/Raf/ERK activity to HCV-related HCC, however, has not been reported. When HCV core gene was co-transfected, *HRAS*-transfected rat embryonic fibroblasts showed more accelerated growth compared with c-myc-transfected rat embryonic fibroblasts, suggesting a possible mechanism of HCV core protein cooperating with Ras in tumorigenesis.<sup>(30)</sup> *NPR1* has an inhibitory role against the Ca<sup>2+</sup>/CaM/PKC/MAPK/ERK pathway through inhibiting its upstream Ca<sup>2+</sup>/Calmodulin.<sup>(31)</sup> *DUSP4* inactivates the Ras pathway by dephosphorylation of ERK and increasing protein kinase C (PKC).<sup>(32)</sup> *LOX* inhibits the transforming activity of Ras in NIH3T3 fibroblasts by inhibiting activities of Akt, Erk, and nuclear factor- $\kappa$ B.<sup>(33,34)</sup> *RRAD* has been reported to be a mediator that inhibits cardiac hypertrophy through down-regulation of CaMKII as calcium-calmodulin-dependent kinase in Ras the pathway.<sup>(35)</sup> Methylation of these Ras inhibitors, involved specifically in HCV-related HCC, might play a role in Ras signaling activation and the carcinogenesis of HCC with HCV infection.

The aberrant Wnt/ $\beta$ -catenin signaling and increased nuclear expression of  $\beta$ -catenin have been detected in cancer,<sup>(36)</sup> including HCC.<sup>(37)</sup> Though a correlation between the Wnt/ $\beta$ -catenin pathway and HCV infection is still unknown, it was reported that HCV core protein could induce Huh7 liver cancer cell line proliferation by transcriptional up-regulation of Wnt-1.<sup>(38)</sup> *SFRP4* is a secreted protein competing with the frizzled receptor





**Fig. 6.** Disease-free survival analysis by Kaplan–Meier method. Methylated, methylation level >30%. Unmethylated, methylation level ≤30%. (a) Analysis among whole hepatocellular carcinoma (HCC) cases. Methylation of *DUSP4* and *CYP24A1* significantly correlated with survival without recurrence. (b) Analysis among hepatitis C virus (HCV)-related HCCs. Methylation of *DUSP4* and *NPR1* significantly correlated with survival without recurrence.

for Wnt ligands and antagonizes the Wnt signaling pathway.<sup>(39)</sup> Wnt signaling up-regulated by APC inactivation or  $\beta$ -catenin activation was reported to be attenuated by *RUNX3* through its interaction with the  $\beta$ -catenin/TCF4 complex.<sup>(40)</sup> Methylation of *SFRP4* and *RUNX3* may also contribute to the genesis of HCV-related HCC, involving Wnt signal activation.

C-markers also included the detoxification enzyme genes. *CYP24A1* was included in 15 age-independent C-markers; NAD(P)H dehydrogenase, quinone 1 (*NQO1*) was included in 18 C-markers and its hypermethylation was reported in HCC.<sup>(41)</sup> These two genes belong to the phase I/II xenobiotic-metabolizing enzyme family and these enzymes play an important role in protecting cells from cytotoxic and carcinogenic agents.<sup>(42,43)</sup> Disruption of the detoxification enzymes might cause excessive reactive oxygen species and result in the initiation of HCC.<sup>(41)</sup>

The reason why genes, at least C-markers, were preferentially methylated in HCV-related HCC is still unknown. HCV core protein was reported to down-regulate expression of E-cadherin correlated with CpG island methylation of E-cadherin promoter through activation of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3B (DNMT3B) in the HCV core protein-expressing HepG2 cell line.<sup>(44)</sup> Frequent methylation may occur through activation of DNMT by HCV core protein, or it may be explained by the longer infection

period. HCV patients develop HCC with chronic infection for several decades. HBV can cause HCC at earlier onsets, in the absence of LC through integration into the human genome.<sup>(4,5,45)</sup> Actually HCV-related HCC patients were significantly older than HBV-related HCC patients (Table 1), so methylation of C-markers was confirmed in age-matched patients (Supporting information Table S4). But a longer period from infection could give more chances to methylation alteration and still should be considered.

Methylation of three C-markers, *DUSP4*, *NPR1*, and *CYP24A1*, correlated with recurrence-free survival (Fig. 6). Two possibilities may be suggested. First, this may be because C-markers in this study were extracted as genes methylated in tumors only but not in background liver tissues. HCC recurrence could be caused either by intrahepatic metastasis or multicentric occurrence.<sup>(46)</sup> Specific gene expression patterns in cancerous tissues of HCC could predict early recurrence,<sup>(47)</sup> whereas expression in the background tissues has also been studied based on the idea of field cancerization and multicentric carcinogenesis.<sup>(48,49)</sup> Genes methylated in tumor only are considered to contribute to the genesis of the resected tumor and its intrahepatic metastasis, but not to the genesis of the multicentric recurrent tumor. The correlation with better prognosis might suggest the importance of multicentric recurrence, and methylation alteration accumulated in the background liver as well as tumor might be a determinant of early multicentric recurrence. Second, accumulation of DNA methylation may contribute to carcinogenesis but cause HCC with better prognosis, while HCCs due to other mechanisms, for example chromosomal instability, may perhaps cause cancer with poorer prognosis; colorectal cancer with highly frequent methylation correlated with microsatellite instability and therefore with better prognosis.<sup>(20,50)</sup> Additional studies are necessary to investigate these possibilities, and to clarify the prognostic significance of C-marker methylation itself using a larger cohort of patients.

In summary, genome-wide MeDIP-chip analysis of clinical samples and quantitative DNA methylation analysis using MassARRAY were performed, and it was found that at least genes that methylate preferentially in HCV-related HCC exist. Promoter methylation might play an important role in HCV-related HCC, possibly by inactivating inhibitors for cancer-related pathway, and might perhaps be useful as a prognostic marker.

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## Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Primer sequences for methylation analysis.

**Table S2.** Reverse transcription-polymerase chain reaction (RT-PCR) primers.

**Table S3.** Candidate C- and B-markers and their MeDIP scores.

**Table S4.** Methylation levels in age-matched HBV- and HCV-related HCCs.

**Table S5.** Association between gene methylation and clinicopathological features.

**Table S6.** Association between disease-free survival and methylation.

**Fig. S1.** The numbers of marker genes detected or selected in each experimental step.

**Fig. S2.** (a) Demonstration of raw methylation data of HCT116 and control samples analyzed by MassARRAY. CpG sites represented by circles in dotted line were not analyzed because the mass of cleaved RNA product was out of the measurable range. (b) Validation of the quantitative analysis and revision of the methylation score. Raw data acquired by MassARRAY was revised as follows: (i) by duplicative analyzes of control samples (0%, 25%, 50%, 75%, 100%): correlation coefficient ( $R^2$ ) and standard curve were calculated at each CpG unit. CpG units with  $R^2 \leq 0.9$  were excluded for further analysis. Primer pairs whose amplicon contained less than three CpG units with  $R^2 > 0.9$  were also excluded. (ii) Raw methylation scores at each CpG unit were revised by standard curve. (iii) Methylation rate (%) of the genes/loci was decided by calculating weighted average of revised methylation score of CpG units, considering the number of CpG sites in each CpG unit.

**Fig. S3.** Disease-free survival analysis by Kaplan–Meier method to compare hepatitis B virus (HBV)- and hepatitis C virus (HCV)-related HCCs. There was no significant difference in recurrence.

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