Identification of novel aberrant methylation of *BASP1* and *SRD5A2* for early diagnosis of hepatocellular carcinoma by genome-wide search

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Abstract. A genome-wide study using expression profiles of 12,600 genes was conducted to identify methylated genes that could be used for early diagnosis of hepatocellular carcinoma (HCC). Of the 12,600 genes examined, we identified 23 genes with significantly lower expression levels in HCC tissues than in non-HCC liver tissues by our statistical and CpG mapping tests. Of these 23 genes, methylation analysis by direct sequencing with bisulfite treatment determined 4 genes that were aberrantly methylated in 20 HCC samples of TNM stages I and II. Further methylation analysis of the 4 genes by quantitative sequencing with 20 HCCs and the corresponding non-tumor liver tissues from an independent cohort of HCC patients revealed that 2 genes, *BASP1* and *SRD5A2*, were aberrantly methylated in only HCC tissues, though not in any corresponding non-tumor liver tissues. Notably, in the cohort we found that *BASP1* or *SRD5A2* were aberrantly methylated when a cut-off value of 30% in the methylation rate was used, in all cases of 11 HCCs of TNM stages I and II, of 10 well-differentiated HCCs and of 4 small HCCs <2 cm in maximum diameter, but in none of the 20 corresponding non-HCC livers. Methylation-specific PCR for *BASP1* and *SRD5A2* reproduced the same results observed by direct sequencing. These results indicate that *BASP1* and *SRD5A2* might serve as useful biomarkers for early diagnosis of HCC.

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Introduction

Hepatocellular carcinoma (HCC), which mainly develops from chronic liver diseases (chronic hepatitis and liver cirrhosis) with persistent infection of the hepatitis C virus (HCV) and hepatitis B virus (HBV), represents a major international health problem due to an increasing incidence in many countries (1,2). HCC is also one of the most common fatal cancers worldwide due to the fact that most cases are first diagnosed at an advanced stage since there is no reliable diagnostic test available for detecting HCC among high-risk individuals with chronic hepatitis and liver cirrhosis (1,2). Therefore, a simple and sensitive system for early diagnosis of HCC is urgently needed to improve the outcome of patients with HCC.

Previously, several investigators have proposed that epigenetic inactivation of gene expression linked to aberrant methylation on CpG islands is a fundamental contributor to carcinogenesis and cancer progression (3-5). Previous clinical studies of the association between abnormal methylation on CpG islands and carcinogenesis have been conducted in various cancers including HCCs (6-11), supporting the concept that detection of methylation on particular genes in cancer cells may be useful for cancer diagnosis. However, it remains unclear if hypermethylation detection of those genes can be applied to routine clinical use for diagnosis of cancer because previous studies have been limited to looking at only tumor suppressor genes or genes associated with cell proliferation and death (6-11), which should be commonly related to carcinogenesis in various organs and tissues. This genome-wide study was conducted to identify methylated genes suitable for use in early diagnosis of HCC in HCV-infected patients and to establish an easy-to-use methylation-specific PCR (MSP) system to measure the methylation status of such genes.

Materials and methods

Patients and gene expression database. For identification of novel HCC-specific methylation genes, we examined a 12,600-gene expression database (12) using the Affymetrix Human Genome U95A Gene Chip® (Affymetrix, Inc., Santa Clara, CA), with surgical specimens of 76 tumor and 16 non-tumor liver tissues in 76 patients who underwent surgical treatment for HCC at Yamaguchi University Hospital between May 1997 and August 2000. To examine the promoter methylation status of candidate genes in HCC samples that were selected *in silico*, we used genomic DNA from liver tissue samples of 40 HCC patients independent of the above 76 HCC patients who were subjected to DNA microarray analysis. Subsequently, the 40 HCC patients were divided into 2 subgroups; first round methylation profiling group and second round methylation profiling group (Table I). The first group consisted of 20 tumor samples from 20 patients with HCC of TNM stages I and II. The second group was composed of 20 tumor samples and the corresponding non-tumor liver samples (histologically determined as 12 liver cirrhosis, 7 chronic hepatitis and 1 normal liver) from 20 HCC patients. All patients underwent surgical treatment for HCC at Yamaguchi University Hospital between April 2001 and May 2003. The clinicopathological characteristics of these patients based on the International Union against Cancer TNM classification (13) are summarized in Table I. Written informed consent was obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi University School of Medicine. Histopathological diagnosis of HCC was made after surgery for each case.

Filtering of HCC-specific methylation candidate genes using the gene expression database. To filter HCC-specific methylation genes out of the ~12,600 in the gene expression database (12), we first selected genes whose signal strength (expression level) was specifically and significantly reduced to half or less in HCC tissues against non-tumor liver tissues by comparing gene expression levels between 76 HCC and 16 non-tumor liver tissues ($p<0.05$). The CpG islands on these genes should be most predominantly methylated in HCC. Then, among the down-regulated genes in HCC tissues, we selected genes carrying CpG islands in the regions, including promoter and exon 1 (defined as 1,500 bp upstream to 2,200 bp downstream from exon 1 start site, which also included up to 4,000 bp downstream for *CYP2E1* which has no CpG island in the promoter) by CpG mapping analysis using the CpGPlot/CpGReport/Isochore, a public tool at The European Bioinformatics Institute (EBI) in the European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/emboss/cpgplot/). The parameters were set as Obs/Exp (the minimum average observed to expected ratio of C plus G to CpG) = 0.6, MinPC (the minimum average percentage of G plus C) = 50, and Length (the minimum length that a CpG island has to be) = 100 bp.

Analysis of DNA methylation. For the candidate genes selected by filtering *in silico*, 1 μg of genomic DNA was extracted from tumor tissues of 20 patients with HCC of TNM stages I and II (first round methylation profiling group), as summarized in Table I and 3 commercially available normal liver tissues derived from Asian individuals used as a reference (Male 1/Female 2, Age: 62.7±6.1; Tissue Transformation Technologies, Inc., Edison, NJ). These extracted DNAs were treated with bisulfite (BIS) for 5 h at 50˚C to convert unmethylated cytosine to uracil, as described previously (14-16). BIS-treated DNA was quantified as described previously (16) and then was prepared with 75 μl of 10 mM Tris-HCl-0.1 mM EDTA solution. After BIS processing, the region including CpG islands on the candidate genes was amplified by PCR and the methylation status was examined by direct-sequencing using dideoxy chain termination method, using BIS-treated DNA for a template. During this first round methylation profiling, genes unmethylated or methylated poorly at CpG positions were eliminated from the gene candidate group. Furthermore, of the genes hypermethylated at CpG positions in HCC samples, genes that were found to be highly methylated in normal liver tissues from healthy individuals were excluded as well. Methylation profiles of the remaining genes were further investigated by pyro-sequencing (17), a quantitative sequencing method using the Pyrosequencer PSQ96MA and the Pyro Gold Reagents (Biotag AG, Uppsala, Sweden), in the 20 tumor and the corresponding non-tumor liver tissues from other 20 HCC patients (second round methylation profiling group; see Table I), liver tissues from 2 HCV carriers with chronic hepatitis and liver tissues from 3 healthy individuals. The pyrosequencing results are shown as a Pyrogram (Fig. 1). The methylation rate (%) at each CpG position is given by the formula, peak height of Cytosine/(peak height of Cytosine + peak height of thymine) x 100. Furthermore, the average of methylation rates at all the examined CpG positions on candidate genes for each sample was calculated. Genes that had higher average methylation rates in tumor tissues of HCC patients than in both non-tumor liver tissues of HCC patients and liver tissues of HCV carriers, were finally judged as HCC-specific methylation genes. The selected genes were only slightly methylated in liver tissues of healthy individuals as well as in those of HCV carriers.

PCR amplification of CpG island regions. For sequencing analysis, the region containing CpG islands of each gene was amplified by PCR with a primer set listed in Table II. The PCR reaction solution was composed of 26.7 ng of BIS-treated DNA, 2 units of rTaq DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) which was pretreated with the equal volume of TaqStart™ Antibody (Clontech Laboratories, Inc., Mountain View, CA) for 5 min at room temperature, 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 0.01% Tween-20, 200 μM dNTPs, each 1 μM of a primer pair and 1.5 or 3 mM magnesium chloride in a final volume of 100 μl. DNA amplification was performed by using the GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA) as a PCR amplification instrument by initial denaturation at 95˚C for 2 min followed by 5 cycles of denaturation at 95˚C for 25 sec, annealing at 70˚C for 45 sec, extension at 72˚C for 45 sec and followed by 50 cycles of denaturation at 95˚C for 25 sec, annealing at 55˚C for 50 sec, extension at 72˚C for 45 sec.

Table I. Patient characteristics in this study.

Patient	Gene expression database[a] HCC (n=76)	1st round methylation profiling[b] HCC (n=20)	2nd round methylation profiling[c] HCC (n=20)	HCV carrier (n=2)
Gender				
Male/Female	54/22	15/5	13/7	0/2
Age (yrs)	62.1±9.9	65.2±8.7	67.3±7.1	61.5±3.5
Viral infection				
HCV positive	50	19	16	2
HBV positive	15	1	3	0
HCV/HBV negative	11	0	3	0
Primary lesion				
Single tumor	35	14	15	-
Multiple tumors	41	16	15	-
Tumor size (cm)	5.0±4.2	2.7±0.6	3.9±3.3	-
Stage				
I/II	56	20	11	-
IIIA/IVA/IVB	20	0	9	-
Histological grade				
Gl (well-differentiated)	9	5	10	-
G2 (moderate-differentiated)	53	14	9	-
G3 (poor-differentiated)	14	1	1	-

[a]These samples were used for the construction of the gene expression database for selection of HCC-specific methylation candidate genes, which were down-regulated specifically in HCC tissues. [b]These samples were used for 1st round methylation profiling and aberrantly methylated genes in early-stage HCC of TNM stages I and II were selected by the analysis. [c]These samples were used for 2nd round methylation profiling and specifically methylated genes in HCC tissues but not non-tumor liver tissues and liver tissues of the HCV carrier were selected by the analysis.

The PCR products (351-495 bp) were concentrated using the SUPREC™-02 (Takara Bio Inc., Otsu, Shiga, Japan) and subjected to electrophoresis on 2% agarose gel. The target bands of amplified products were excised from gel and isolated using the QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany).

Methylation-specific PCR. Methylation patterns of HCC-specific methylation genes were also confirmed by quantitative real-time methylation-specific PCR (MSP). Briefly, PCR amplification using a primer pair specific to the nucleotide sequence containing methylated cytosines at CpG positions was performed using the LightCycler® Taq Man master kit for an amplification reagent and the LightCycler® II (Roche Diagnostics GmbH, Mannheim, Germany) for a nucleic acid amplification instrument. For the *BASP1* gene, the PCR reaction mixture was composed of 5 μl of diluted BIS-treated DNA (1 ng), 0.25 μM of a primer pair (Forward: 5'-TGTTCGTTTTTTTAGGGTATTC-3', Reverse: 5'-AATT AACCGAAACAACCCG-3') and 0.1 μM of Taq Man probe (5'-FAM-ACGCTACTACTTACGAACGCTCGAA-TAMRA-3') in a final volume of 20 μl of 1x Master mix. PCR amplification was carried out by initial denaturation at 95˚C for 10 min followed by 50 cycles of 95˚C for 10 sec, 63˚C for 45 sec and 72˚C for 5 sec followed by warming of 40˚C for 30 sec. Fluorescent signal was detected after extension reaction at 72˚C for each cycle. Amplification of the target gene was monitored by F1/F3 analysis mode in LightCycler software and the amount of methylated DNA in 75 μl of BIS-treated DNA solution was quantified using a standard curve made with simultaneously measured standards (dilution series of artificially methylated DNA: at 1000, 200, 40 and 4 pg/μl). As for the *SRD5A2* gene, the PCR reaction mixture was composed of 5 μl of diluted BIS-treated DNA (1 ng), 0.2 μl of 2 M potassium acetate (pH 7.5), 0.04 μl of Aptamer 48, 50 mM tricine (pH 8.3), 3 mM magnesium acetate, 375 μM dNTPs, 2.5% glycerol, 0.15 unit ZO5 (thermostable DNA polymerase), 0.4 μM of a primer pair (Forward: 5'-AATCGCGTTAGGGTTGGACGC-3', Reverse: 5'-AACGCCAAACGCCACCCG-3') and 0.05 μM of Taq Man probe (5'-FAM-ACTCGACCTTAACTCCCGCCCCT-TAMRA-3') in a final volume of 20 μl. PCR amplification was carried out by initial denaturation at 95˚C for 2 min, followed by 50 cycles of 95˚C for 15 sec, 66˚C for 45 sec and 72˚C for 5 sec, followed by warming of 40˚C for 30 sec. After amplification, the amount of methylated DNA in 75 μl of BIS-treated DNA solution was quantified using a standard curve that was made with simultaneously measured standards (dilution series of artificially methylated DNA: at 1000, 200, 50 and 8 pg/μl).

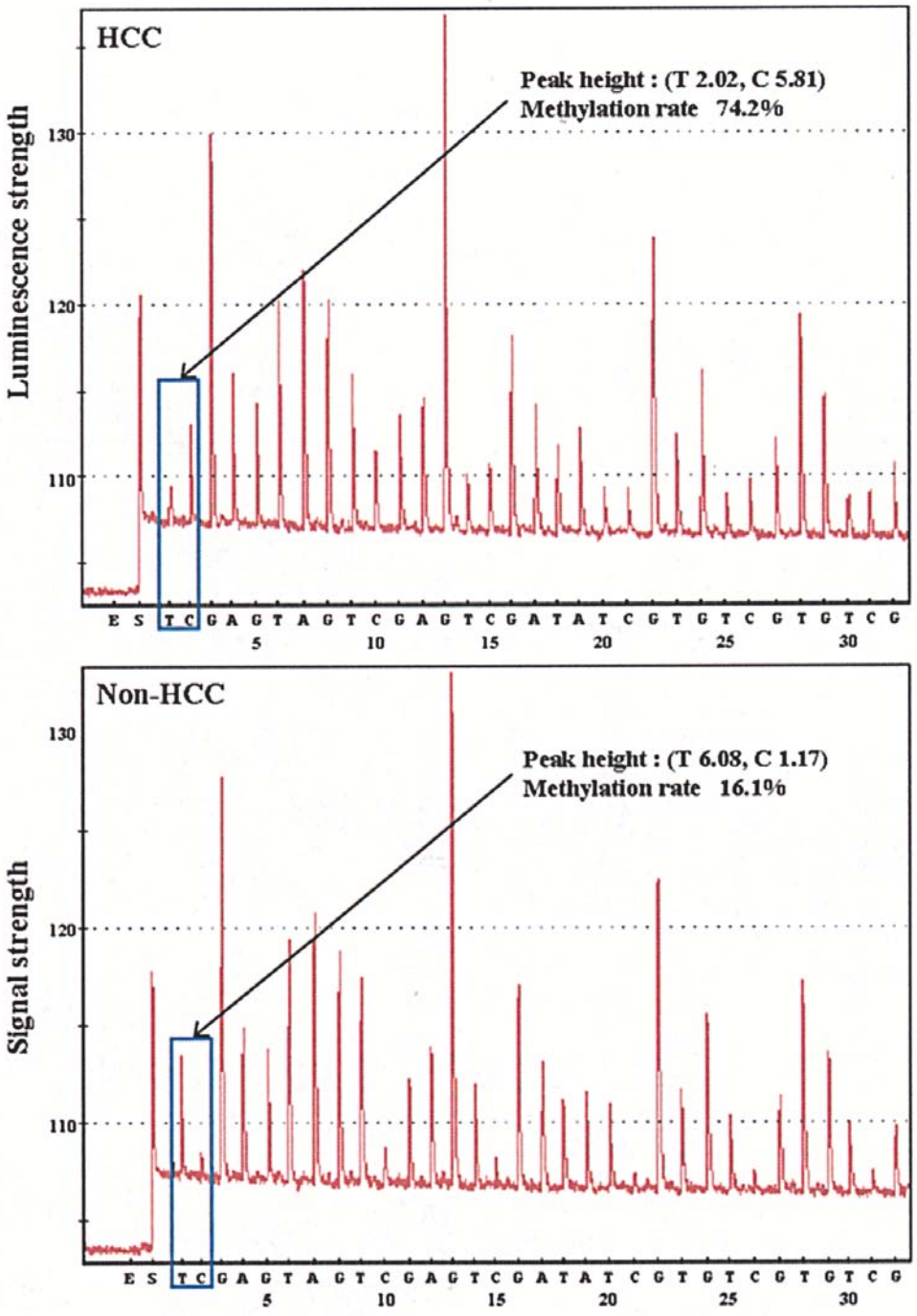

Figure 1. Representative pyrogram for methylation analysis. HCC and non-HCC tissues from an HCC patient were analyzed for *SRD5A2* gene. The *y*- and *x*-axes show luminescence strength and dispensation order (time), respectively. To discriminate methylated cytosine and unmethylated cytosine at the CpG position, T and C were dispensed continuously. Peak height was calculated by subtracting the baseline from luminescence strength.

Statistical analysis. Continuous variables were analyzed by the Student's t-test, Mann-Whitney's U test and Welch's t-test. Correlations among data were evaluated by calculating Pearson's correlation coefficient. A value of $p<0.05$ was considered to be statistically significant. Analyses were conducted with Statcel Ver.2 software.

Results

Novel HCC-specific methylation genes, BASP1 and SRD5A2. Of the ~12,600 genes on the Affymetrix chip, only 101 (0.8%) were differentially expressed between HCC tissues and non-tumor liver tissues. Out of these 101 genes, 23 (0.18%) carrying CpG islands in the regions containing promoter and exon 1 were chosen *in silico* by CpG mapping analysis (Table II). These genes were then subjected to the methylation profiling step. Among the 23 HCC-specific methylation gene candidates, 6 genes which were identified as aberrantly methylated genes in HCC, *ABCC9* (ATP-binding cassette, sub-family C, member 9), *APOF* (apolipoprotein F), *BASP1* (brain abundant, membrane attached signal protein 1), *GLS2* (glutaminase 2), *HAL* (histidine ammonia-lyase) and *SRD5A2*

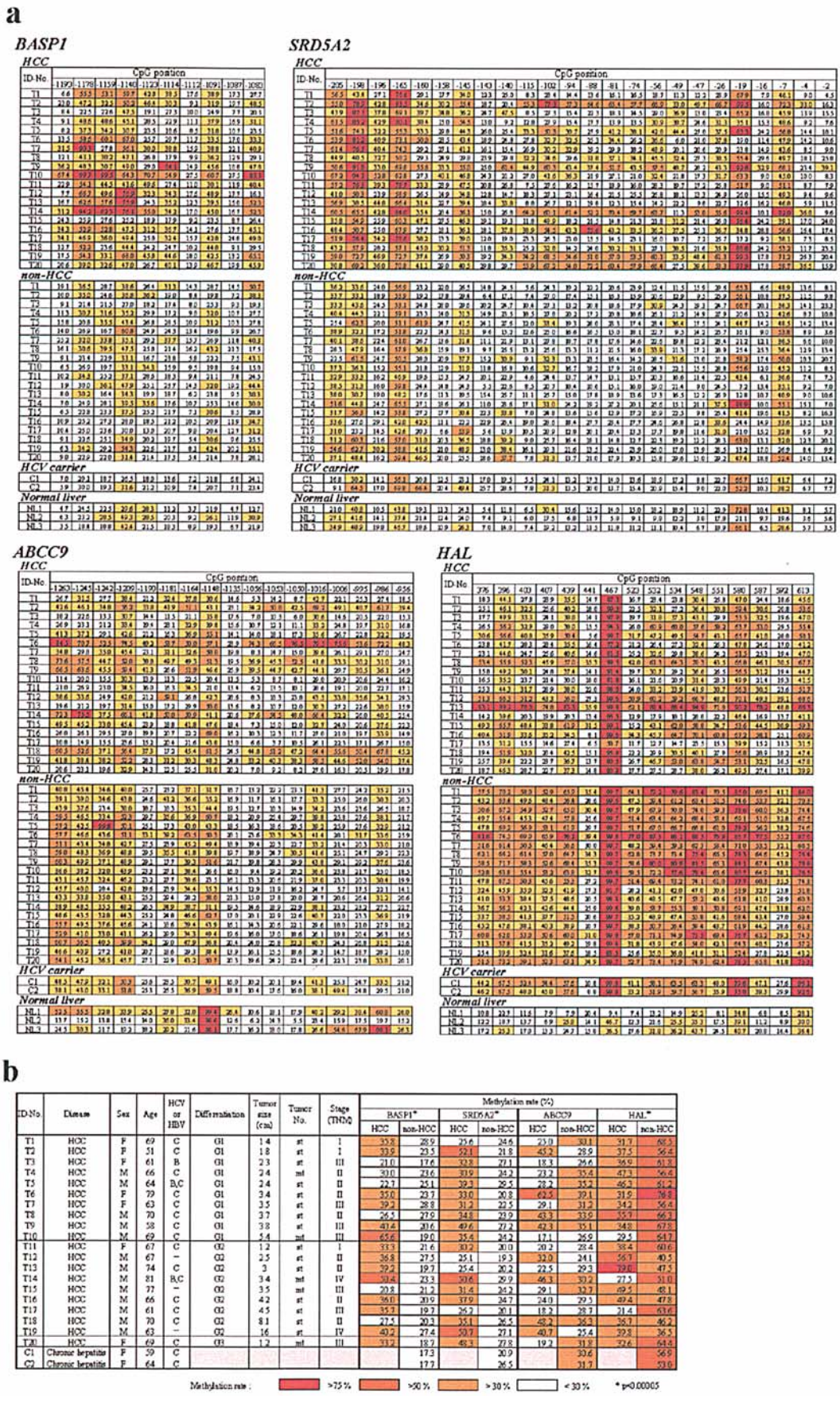

Figure 2. Methylation profiling in HCC and non-HCC tissues for *BASP1*, *SRD5A2*, *ABCC9* and *HAL* genes by pyrosequencing. (a) Methylation patterns for 4 genes with HCC and non-tumor liver tissues in second round methylation analysis. Twenty HCC and the corresponding non-HCC tissues from 20 HCC patients (T1-T20) and liver tissues from 2 HCV carriers with chronic hepatitis (C1,C2) in second round methylation analysis (see Table I) were employed for the analysis. As a reference, normal liver tissues from 3 healthy individuals (NL1-NL3) were also analyzed. Methylation rates are shown in different colors (red >75%, orange 75-50%, pale orange 50-30% and white <30%). Position of bisulfite sequencing region was given relative to exon 1 start site. (b) Comparison of average of methylation rates among 4 genes. Average values of methylation rates of all the CpG positions in (a) were calculated for each patient. Methylation rates are shown in different colors as in (a).

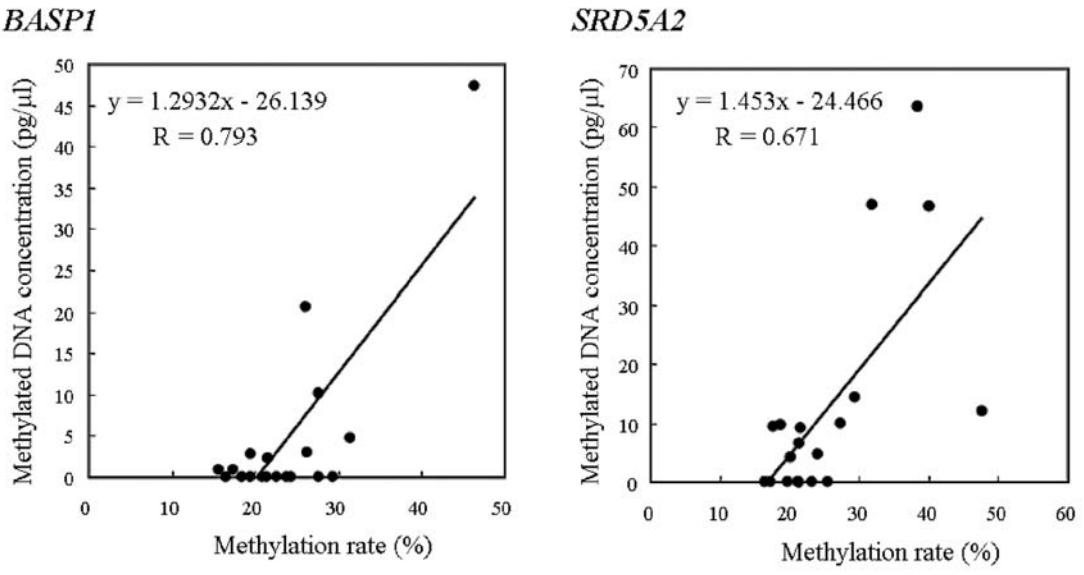

Figure 3. Correlation between the methylated DNA concentration by real-time MSP and the methylation rate by pyrosequencing for *BASP1* and *SRD5A2*. The *y*- and *x*-axes are the methylated DNA concentration (pg/μl) and the methylation rate (%), respectively.

(steroid-5-α-reductase, α polypeptide 2), were selected in the first round methylation profiling performed using methylation analysis of CpG islands on genomic DNAs in tumor tissues from 20 patients with HCC of TNM stages I and II. The six genes were further examined in liver tissues from 3 healthy individuals. From this analysis, *APOF* and *GLS2* were excluded due to hypermethylation in normal liver tissues (data not shown). Then, in the second round methylation profiling by comparing methylation profiles of 20 tumor and the corresponding non-tumor liver tissues (12 liver cirrhosis, 7 chronic hepatitis and 1 normal liver) from other 20 HCC patients, liver tissues from 2 HCV carriers with chronic hepatitis and normal liver tissues from healthy individuals, *ABCC9* was eliminated because the methylation patterns of tumor tissues of HCC patients were indistinguishable from those of non-tumor liver tissues of HCC patients and the liver tissues of HCV carriers, as shown in Fig. 2a and b. Furthermore, *HAL* was also excluded due to a much higher methylation rate ($p<0.00005$) (Fig. 2b) in non-tumor liver tissues relative to tumor tissues of HCC patients, although this gene was at least more frequently methylated in HCC tissues, than in normal liver tissues from healthy individuals (Fig. 2a). The remaining two genes, *BASP1* and *SRD5A2*, fulfilled the criterion of the HCC-specific methylation gene, because of the definite difference in the extent of methylation of the genes between tumor and non-tumor liver tissues from HCC patients ($p<0.00005$) (Fig. 2a and b). These 2 genes were also not extensively methylated in liver tissues of HCV carrier and healthy individuals, with only slight methylation observed on a small part of the CpG positions (Fig. 2a). In addition, the aberrant methylation of *BASP1* or *SRD5A2* was positive in 11 (100%) of 11 HCCs of TNM stages I and II, 10 (100%) of 10 well-differentiated HCCs and 4 (100%) of 4 small HCCs of <2 cm in maximum diameter, when a cut-off value of 30% in methylation rate was used in a new cohort of 20 HCC patients (Fig. 2b). By contrast, the aberrant methylation of the 2 genes was not detected in any of the corresponding non-tumor liver tissues (Fig. 2b). There were no significant associations among HCC methylation rates for the 4 genes (*BASP1*, *SRD5A2*, *ABCC9* and *HAL*) and gender, age, tumor differentiation, tumor size, tumor number or TNM stages (data not shown).

Methylation analysis by MSP. We confirmed methylation status by quantitative real-time MSP for 19 samples (9 sets of tumor and the corresponding non-tumor liver tissues and 1 tumor tissue from 10 HCC patients) that were analyzed by pyrosequencing and examined the correlation between the methylation status analyzed by sequencing and MSP. By Pearson's test the methylated DNA concentration in 75 μl of BIS-treated DNA solution measured by MSP was shown to be strongly and significantly correlated with the methylation rate estimated by sequencing analysis ($r=0.793$, $p<0.0001$ and $r=0.671$, $p<0.005$ for *BASP1* and *SRD5A2*, respectively) (Fig. 3).

Discussion

We have successfully identified 2 aberrantly methylated genes, *BASP1* and *SRD5A2*, associated with HCC by genome-wide search using gene expression profiles on the Affymetrix GeneChip. To our knowledge, this is the first study screening comprehensively to identify methylated genes for early diagnosis of HCC. Our strategy is justified by the finding that only 2 of 12,600 genes (0.016%) fulfilled our selection criteria and neither of the 2 genes have been reported as methylated genes in HCC. *BASP1* was originally identified as a membrane bound protein that is abundant in nerve terminals, with several transient phosphorylation sites and PEST [regions rich in proline (P), glutamate (E) and/or aspartate (D) and serine (S) and/or threonine (T)] motifs that typically occur in proteins with high turnover rates (18). It has also been reported to be a transcriptional cosuppressor for Wilms' tumor suppressor protein WT1 (19), however, so far there has been no report

Table II. The 23 genes whose expression level reduced to half or less in HCC tissues against non-tumor liver tissues.

			Gene expression analysis			Methylation analysis on CpG islands		
			Signal strength					
Gene symbol[a]	Gene name	Accession no.[b]	HCC tissue	Non-tumor tissue	Ratio of HCC/ Non-tumor	Bisulfite sequencing region[c]	PCR primer set	Amplified size (bp)
Aberrantly methylated genes in HCC tissues								
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	AF061324	14.0	51.3	0.27	-1289 to -887	5'-TAG TAT TAG GAT TTT TAT TGT TGA AA-3'	
							5'-RAA CTT CTA CCC CTA AAA CTC C-3'	403
APOF	Apolipoprotein F	L27050	164.9	1391.0	0.12	-1097 to -707	5'-TGT TTA TAT ATA GAA GAT TAG ATG TA-3'	
							5'-CAA ATC CAA TAA AAT AAA TTT CAA AAT-3'	391
BASP1	Brain abundant, membrane attached signal protein 1	D45299	409.1	867.6	0.47	-1218 to -761	5'-TTT GTT AAT AGG TAA GTA AAG TGA A-3'	
							5'-TTA AAA TAA ACC CCA ACT ACA AAC-3'	458
GLS2	Glutaminase 2 (liver, mitochondrial)	AF038170	109.0	1250.0	0.09	-559 to -85	5'-TGG TAG AGG GAT ATA AAT TGG AA-3'	
							5'-ATA AAA TTA CCC AAT AAA AAC AAA AC-3'	475
HAL	Histidine ammonia-lyase	D16626	357.3	1027.5	0.35	+239 to +730	5'-AAT GTA TGA ATG AGT AAA GGT GAA-3'	
							5'-ACC TCT ACT CAT CAT TTT CCC T-3'	492
SRD5A2	Steroid-5-α-reductase, polypeptide 2+175	M74047	29.6	147.8	0.20	-290 to -175	5'-TAA GTT ATG GAA GGA TAG TTT AAG-3'	
							5'-AAC AAC TCC TAC AAA AAC CAA AC-3'	465
Sporadically methylated genes in HCC tissues								
BGN	Biglycan	J04599	1664.1	3385.1	0.49	-262 to +213	5'-TGG GTT TTT GTT TGA GTG AGT G-3'	
							5'-TCT AAA TTA CTA AAA AAA CAA CTT AAA-3'	475
CDO1	Cysteine dioxygenase, type I	U80055	793.1	1613.9	0.49	-470 to -32	5'- GGT ATT TTT TGG TTT AGG AGT GG-3'	
							5'-CTT AAA ATC ACT AAA AAT ATA CCA AC-3'	439
IGFBP3	Insulin-like growth factor binding protein 3	M35878	616.2	2135.7	0.29	+133 to +627	5'- YGT TGA TTT TGT TGG TGT TGT TT-3'	
							5'-TCC CCA CAT ATT ATC TCC AAT AA-3'	495
PON3	Paraoxonase 3	L48516	1992.2	5890.7	0.34	-235 to +146	5'-TTG ATT TTT GTT TAA AAA ATA AGG AAT-3'	
							5'-CAA ACA ACC CCT AAC CTC ACT-3'	381
Unmethylated genes in HCC tissues								
ANG	Angiogenin, ribonuclease, RNase A family, 5	M11567	2498.9	6509.7	0.38	-157 to +253	5'-GGG GTT TAG TTG AGG AAG GAG-3'	
							5'-AAA AAA ACA AAA ATA TAA ACC TAC CT-3'	410

Table II. Continued.

Gene symbol[a]	Gene name	Accession no.[b]	Gene expression analysis: Signal strength HCC tissue	Non-tumor tissue	Ratio of HCC/ Non-tumor	Methylation analysis on CpG islands: Bisulfite sequencing region[c]	PCR primer set	Amplified size (bp)
CTH	Cystathionase (cystathionine γ-lyase)	S52028	226.7	942.7	0.24	-223 to +237	5'-TTA CTC TAA ATC CTA ACC CAC AT-3' 5'-AGG GAT TAA TAT TAT TTG GAT TTA G-3'	460
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	X02612	147.0	302.1	0.49	-755 to -355	5'-AGT TAA GAG GGA TTT TTA AGG GT-3' 5'-TCT TCR TCA TTT TTA CAC CCA CT-3'	401
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	J02843	3222.1	13817.5	0.23	+941 to +1375	5'-TTT TGA GGG GAG GGT TTT TTT TA-3' 5'-TAT TAC TAT TAA TTT ATT ATT TAA CCA T-3'	435
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	U37143	569.7	1187.0	0.48	-147 to +259	5'- GTT TTT AGT TTG GTT TTT TTT GAG A-3' 5'- CCT ACC AAC TAA ACC TCC AAA T-3'	406
ENO3	Enolase 3, (ß, muscle)	X56832	75.6	261.1	0.29	-1425 to -1027	5'-GGA GTT TGG GTT AGG GGT AAG-3' 5'-CTC CCC ACC CCT ACT CCC-3'	399
ETFDH	Electron-transferring-flavoprotein dehydrogenase	S69232	249.3	506.5	0.49	-865 to -508	5'-GGT GGT ATT TTA GTT GTT GGT AT-3' 5'- ACC CTA AAA AAT TAC AAA TTT CTT C-3'	358
GCGR	Glucagon receptor	L20316	98.7	418.9	0.24	-165 to +329	5'- GTT TTT TGY GTA GGG TGG GTA GGA T-3' 5'- CTA AAA CCC CTA AAC CTA CCC-3'	494
GHR	Growth hormone receptor	X06562	41.5	336.7	0.12	-345 to +114	5'- GTT TTT GTA ATT TGG ATT TGG GGG ATT G-3' 5'- TAA AAT CCC AAA ATT CAC CCT CA-3'	459
GNMT	Glycine N-methyltransferase	X62250	317.0	1511.3	0.21	-78 to +360	5'- GTT ATT GGT TAG GTG GGG TTG-3' 5'-TAC TCC TAA CCC TAA TAT CCC A-3'	438
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	D13891	2246.6	4934.7	0.46	-150 to +201	5'- GTA TTT TAT TTA TTA TTT TAG TTG GG-3' 5'- RAA ACT AAA AAC TTT CAT ACT AAC C-3'	351
LY6E	Lymphocyte antigen 6 complex, locus E	U66711	224.3	682.2	0.33	-145 to +236	5'-TTA GTT TTT TTA TTT ATT TTA AGG AGT-3' 5'- CTT ACT CTC TCC CAC CCT CT-3'	381
SORL1	Sortilin-related receptor, L (DLR class) A repeats-containing	U90916	449.2	938.2	0.48	-270 to +112	5'- TAG GGA GAA TAA GGA GGT GTG-3' 5'-CRA CTC CCT CCT ACT ACT CC-3'	382

[a]Gene symbols used are based on the data from LocusLink. [b]Accession number of each gene was obtained from PubMed or the Institute for Genomic Research database. [c]Position of bisulfite sequencing region was given relative to exon 1 start site.

that this protein is correlated with pathogenesis of liver cancer. *SRD5A2* encodes a microsomal protein expressed at high levels in androgen-sensitive tissues such as the prostate. The encoded protein is a membrane-associated enzyme that catalyzes the conversion of testosterone to dihydrotestosterone (DHT), the most potent androgen in the prostate and the polymorphism and expression level has been shown to be functionally associated with prostate cancer (20-22). To date, it was reported that the *SRD5A2* polymorphism could be associated with liver cancer (23), but no study has been done regarding methylation status in liver tissues. Thus, in this study we were the first to be able to show that *BASP1* and *SRD5A2* are exclusively methylated in tumor, but not non-tumor liver tissues from HCC patients and not methylated in any non-tumor liver tissues from HCV carriers. This suggests that *BASP1* and *SRD5A2* could be robust biomarkers for diagnosing correctively hepatic nodules arising in HCV-infected patients to be at-risk for HCC. These 2 genes were originally found to be methylated to a certain extent in liver tissues from healthy individuals (Fig. 2a), as reported for some genes by Lehmann *et al* (11). However, the slightly methylated status was observed to be similar to that of non-tumor and HCV carrier liver tissues in our study. This means *BASP1* and *SRD5A2* may undergo abnormal methylation only after, or concurrently with developing HCC, regardless of inflammation or fibrosis by HCV infection.

It is well known that epigenetic changes, including methylation, are related to aging (24). In our present study, no associations were observed between patient age and the methylation status of *BASP1* and *SRD5A2*. This result might be due in part to the sample size of our present study. To gain insights into our present finding, larger studies are needed.

In our gene expression analysis, expression level of *SRD5A2* in HCC tissues was reduced to one-fifth of that in non-tumor liver tissues (Table II), which should be similar to the level found in normal liver tissues from healthy individuals (25). This entrainment profile between high methylation and suppressed gene expression level suggests that *SRD5A2* gene expression would be entirely epigenetically inactivated by abnormal methylation of CpG islands. *BASP1* was relatively highly expressed in non-tumor liver tissues in our gene expression profiles (Table II), though it has been reported that this gene was not expressed in liver by Western blot analysis (19). Therefore, it could be that mRNA is transcribed, but undergoes only limited translation to protein, possibly due to causes such as fast turnover of mRNA in liver. Expression levels of *BASP1* in tumor liver were reduced to half of those found in non-tumor liver (Table II). This may imply that gene expression is not strongly suppressed by methylation. Indeed, our methylation profiling showed that *BASP1* was not extensively methylated on CpG islands, even in HCC tissues, although it was at least more methylated than in non-tumor liver tissues of HCC patients, liver tissues of HCV carriers and normal liver tissues of healthy individuals (Fig. 2a).

ABCC9 and *HAL* were eliminated through the selection process of HCC-specific methylation genes. Since the 2 genes were found to be strongly methylated in non-HCC tissues and liver tissues of HCV carriers as well as in HCC tissues, although they were not highly methylated in normal liver samples (Fig. 2a and b). For *ABCC9*, the methylation rate in HCC appeared to be slightly higher than in non-HCC, but they were not statistically different from each other. Interestingly, the methylation rate for *HAL* in non-HCC was significantly higher than in HCC as shown in Fig. 2b ($p<0.00005$). It is inferred from these results that *ABCC9* and *HAL* may be methylated with development or progression of chronic liver diseases with HCV infection. Furthermore, *HAL* may undergo demethylation with carcinogenesis.

Most HCCs are initiated as a minute nodule in which cells strongly retain the phenotype of benign hepatocytes. Dysplastic changes, which would mark the transition to a small, well-differentiated HCC, usually occur when the nodules measure between 1 and 2 cm in maximal diameter and herald the ability to proliferate, invade and disseminate (26). At this stage, the tumors are easily detected by imaging techniques such as ultrasonography and computed tomography. However, it is not easy to discriminate pathologically well-differentiated HCC from other benign tumors such as dysplasia. Accordingly, the best way for an accurate early diagnosis of this cancer might be to identify the molecular changes that govern the transition to cancer. In this regard, the efficacy of *BASP1* and *SRD5A2* as tools for early diagnosis of HCC was shown by our present findings that they were aberrantly methylated in all cases of 11 HCCs of TNM stages I and II, of 10 well-differentiated HCCs and of 4 small HCCs <2 cm in maximum diameter. Additionally, our developed MSP has made it possible to easily quantify the methylation status of the 2 genes and may enable an early detection of HCC by measuring methylation levels in cell-free DNA circulating in the peripheral blood, although larger studies are needed to evaluate prospectively the diagnostic accuracy of these biomarkers prior to the blood test.

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