The assessment of methylated *BASP1* and *SRD5A2* levels in the detection of early hepatocellular carcinoma

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Abstract. We previously identified *BASP1* and *SRD5A2* as novel hepatocellular carcinoma (HCC) methylation markers from among more than 10,000 screened genes. The present study aimed to improve the diagnostic potential of these genes. We compared the methylation status at distinct regions of the *BASP1* and *SRD5A2* genes using quantitative methylation-specific PCR, in 46 sets of HCC and corresponding non-tumor liver tissues. We also examined how their epigenetic status affected transcript levels in tissues and several hepatoma cell lines. We found that *BASP1* and *SRD5A2* loci were methylated in greater than 50% of the HCC tissues. Inverse correlations were identified between the methylation status and transcript levels in the tissues. Assessment of CpG island methylation rate of *BASP1* and *SRD5A2* resulted in different diagnostic powers for discriminating HCC even in the same CpG island. A combination analysis of *BASP1* and *SRD5A2* resulted in the optimum diagnostic performance (84.8% sensitivity and 91.3% specificity) with a maximal area under the receiver operating characteristic curve of 0.878. Even in patients with early HCC (well-differentiated, TNM stage I and small in diameter) and those negative for serum α-fetoprotein, combination analysis enabled an accurate diagnosis of HCC. *In vitro* analysis also showed that *BASP1* and *SRD5A2* transcripts were epigenetically regulated by methylation and acetylation. These results suggest that combined analysis of methylated *BASP1* and *SRD5A2* may prove useful in the accurate diagnosis of HCC, especially early HCC.

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Introduction

Hepatocellular carcinoma (HCC) may develop from chronic liver diseases caused by hepatitis C virus (HCV) or hepatitis B virus (HBV) infection, and represents a major international health problem due to its increasing incidence in many countries (1,2). HCC is also one of the most common fatal cancers identified worldwide, as the vast majority of cases are first diagnosed at an advanced stage since a reliable diagnostic test for the detection of HCC, among at-risk individuals with chronic hepatitis and liver cirrhosis, is currently unavailable (1,2). To date, α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) have served as the predominant markers used in the screening for HCC (3). However, these tumor markers exhibit numerous limitations in the early detection of HCC. The identification of robust biomarkers for early diagnosis of HCC is urgently required.

Several investigators have hypothesized that not only genetic mutation, but also epigenetic alterations, including aberrant methylation on CpG islands, is a fundamental contributor to carcinogenesis and tumor progression (3-5). Numerous studies have documented aberrant methylation of CpG islands in various cancers including HCC (6-11), supporting the hypothesis that detection of methylation on specific genes derived from cancer cells may be useful for accurate cancer diagnosis.

Our recent genome-wide study identified DNA methylation at the Brain abundant, membrane attached signal protein 1 (*BASP1*) and Steroid-5-α-reductase, alpha 2 (*SRD5A2*) loci, and suggested that these genes may prove useful as biomarkers for the early diagnosis of HCC (12). We also identified that the decreased levels of *BASP1* and *SRD5A2* mRNA in HCC tissue was due to methylation status in the promoter regions. Of clinical importance, we found that methylation analysis using pyrosequencing was successful in diagnosing early-stage HCC, well-differentiated HCC or small HCC that were less than 2 cm in diameter. However, a shortcoming of this study was the small sample size (n=9) subjected to the quantitative methylation-specific PCR (qMSP) analyses used to examine the correlation between methylation status examined

by sequencing and qMSP. Such comparison is critical for the accurate application of qMSP analyses of epigenetic status in the daily surveillance of this disease. Thus, re-evaluation of a larger cohort is required. Moreover, it has been suggested that differences in the DNA region amplified by PCR may affect the assessment of methylation status in the genes tested (13). To address these issues, we performed qMSP analyses at 2 distinct regions of each CpG island in a large cohort of HCC patients, and examined whether decreased levels of *BASP1* and *SRD5A2* mRNA were related to mechanisms other than DNA methylation, such as histone acetylation, in panels of hepatoma cell lines.

Materials and methods

Patient information. HCC and non-HCC liver tissue was obtained from 46 patients who underwent curative hepatectomy for HCC at our Institute between July 2000 and October 2006. Informed, written consent was obtained from each patient prior to their participation in this study. The study protocol was approved in advance by the Institutional Review Board for Human Use at Yamaguchi University Graduate School of Medicine. Clinicopathologic features of the 46 HCC patients entered into this study are summarized in Table I. Of the 46 samples observed, 33 samples were used for both the methylation and expression analyses.

HCC cell lines. The human hepatoma cell lines Hep G2, Hep 3B, HLE, HuH-6, HuH-7 and SK-HEP-1 were used in this study, and were purchased from the Health Science Research Resources Bank (Osaka, Japan) and the American Type Culture Collection (Rockville, MD, USA). Cell lines were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Tokyo, Japan), penicillin (100 units/ml), streptomycin (100 μg/ml) and sodium bicarbonate (1.5 g/l) at 37˚C in 5% CO_2 in air.

Methylation-specific PCR (MSP). We quantified methylation status based on the Methylight method (14) with some minor modifications. Genomic DNA was extracted using the Ultra Clean Tissue DNA kit (Mo Bio Laboratories, Carlsbad, CA) followed by bisulfite treatment using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA). We designed novel primers and probes (see Table II) directed against the transcription start site of *BASP1* and *SRD5A2*, in addition to those used in our previous study (12). Real-time PCR amplification was performed using LightCycler 480 Probe Master (Roche Diagnostics, Tokyo, Japan), primer (10 pmol), hydrolysis probe (2 pmol) and genomic DNA treated with bisulfite in a LightCycler System Version 3 (Roche Diagnostics). Amplification was performed according to a 2-step cycle procedure consisting of 55 cycles of denaturation at 95˚C for 10 sec and annealing/elongation at 60˚C for 30 sec. We measured methylation status semiquantitatively using the delta-delta-quantification cycle (ΔΔCq) method. Ribonuclease P RNA component H1 (*RPPH1*) was used as the reference gene. The values are expressed as relative to 100% of the methylated control DNA (EpiTech Control DNA, Qiagen, Tokyo, Japan).

Table I. Clinicopathological features of 46 patients used in this study.

	HCC patients n=46
Sex	
Male	35
Female	11
Age (years)	65.9±13.3
Virus infection	
HCV positive	26
HBV positive	12
HCV/HBV negative	8
Tissues surrounding HCC	
Normal liver	2
Chronic hepatitis	27
Liver cirrhosis	17
AFP (ng/ml)	958±2670
Primary lesion	
Single tumor	31
Multiple tumor	15
Tumor size (cm)	4.1±2.4
Stage[a]	
I	14
II	29
IIIA-IV	3
Histological grade	
G1 (well-differentiated)	15
G2 (moderate-differentiated)	27
G3 (poor-differentiated)	4

[a]The tumor-node-metastasis (TNM) stage of UICC (2002).

Administration of demethylating agent and HDAC inhibitor. Ten μM of the demethylating agent 5-aza-2'-deoxycitidine (5-aza-dC) or 10 mM of the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) were added to the medium. HuH-6 cells were treated with 2 mM NaB, but not 10 mM. Following 48 h incubation, cells were collected and subjected to semiquantitative real-time PCR analysis.

Semiquantitative real-time PCR. Semiquantitative real-time PCR was performed as described previously (15) with minor modifications. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen) and purified with the Pure Link Micro-to-Midi Total RNA Purification System (Invitrogen). The extracted RNA was then reverse-transcribed using the Prime Script RT reagent kit (Takara Bio, Shiga, Japan). Real-time PCR amplification was performed using LightCycler 480 Probe Master (Roche Diagnostics), primer (10 pmol), probe (2 pmol) (see Table II) and cDNA corresponding to a concentration of 10 ng of the initial RNA in a LightCycler System version 3 (Roche Diagnostics). Amplification was performed

Table II. Primers and probes used in this study.

BASP1 for mRNA levels	
Primers	5'-GCCCCAGCTTCAGACTCA-3' and 5'-GGGGTCTCCTTGGAAGAGG-3'
Probe	Universal Probe Library #75 (Roche Diagnostics)
BASP1 for methylation rates (region 1)	
Methylated [position: -1144 to -1049, the same design as previous study (12)]	
Primers	5'-TGTTCGTTTTTTTAGGGTATTC-3' and 5'-AATTAACCGAAACAACCCG-3'
Probe	5'-FAM-acgctactacttacgaacgctcgaa-BHQ-3'
Unmethylated (position: -1144 to -1049)	
Primers	5'-TGTTTGTTTTTTTAGGGTATTT-3' and 5'-AATTAACCAAAACAACCCA-3'
Probe	5'-FAM-acactactacttacaaacactcaaa-BHQ-3'
BASP1 for methylation rates (region 2)	
Methylated (position: -42 to +117)	
Primers	5'-AATGTAGAGGTTGTAGCGGC-3' and 5'-GAAATCGCTAACGTCGTCTAA-3'
Probe	5'-FAM-tttcgtttcgggtttcgtcgtcgagt-BHQ-3'
Unmethylated (position: -45 to +120)	
Primers	5'-AGAAATGTAGAGGTTGTAGTGGT-3' and 5'-ACCAAAATCACTAACATCATCTAA-3'
Probe	5'-FAM-ttttgttttgggttttgttgttgagt-BHQ-3'
SRD5A2 for mRNA levels	
Primers	5'-CAGCTACAGGATTCCACAAGG-3' and 5'-TCAATGATCTCACCGAGGAA-3'
Probe	Universal Probe Library #50 (Roche Diagnostics)
SRD5A2 for methylation rates (region 1)	
Methylated [position: -92 to +1, the same design as previous study (12)]	
Primers	5'-AATCGCGTTAGGGTTGGACGC-3' and 5'-AACGCCAAACGCCACCCG-3'
Probe	5'-FAM-ctcgaccttaactcccgcccct-BHQ-3'
Unmethylated (position: -92 to +1)	
Primers	5'-AATTGTGTTAGGGTTGGATGT-3' and 5'-AACACCAAACACCACCCA-3'
Probe	5'-FAM-ctcaaccttaactcccacccct-BHQ-3'
SRD5A2 for methylation rates (region 2)	
Methylated (position: +36 to +178)	
Primers	5'-GGGAGGGTAGCGGTTATC-3' and 5'-TACTTCCCGTAACCGAAAAA-3'
Probe	5'-FAM-cggcgcgatgtaggtttagtgttagt-BHQ-3'
Unmethylated (position: +33 to +179)	
Primers	5'-TTTGGGAGGGTAGTGGTTATT-3' and 5'-ATACTTCCCATAACCAAAAAACT-3'
Probe	5'-FAM-tggtgtgatgtaggtttagtgttagt-BHQ-3'
GAPDH for mRNA levels (control)	
Primers	5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'
Probe	Universal Probe Library #60 (Roche Diagnostics)
RPPH1 for methylation rates (control)	
Primers	5'-GGGTGGAGGGAAGTTTATTAGTG-3' and 5'-CTCACCTCCCCAAAACTCAA-3'
Probe	5'-FAM-gggaaggtttgagattagggttagaggtgg-BHQ-3'

according to a 2-step cycle procedure consisting of 45 cycles of denaturation at 95˚C for 10 sec, and annealing/elongation at 60˚C for 30 sec. We measured mRNA levels semiquantitatively using the ΔΔCq method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. The values are expressed as relative to the appropriate control.

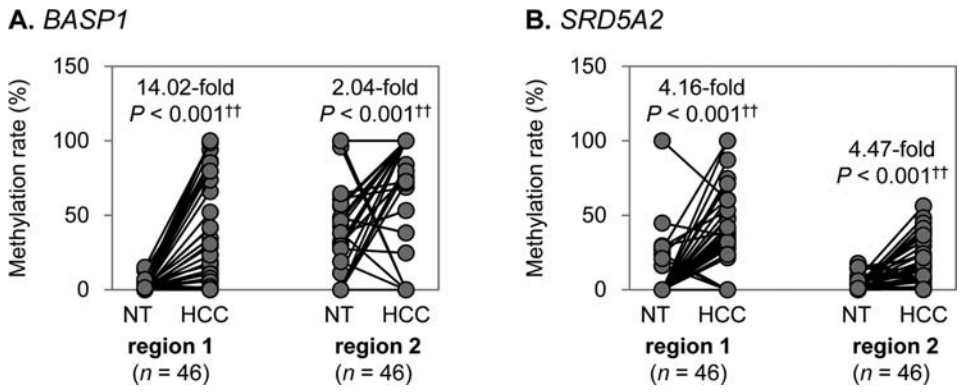

Figure 1. The methylation rate at *BASP1* and *SRD5A2* loci in non-tumor and HCC liver samples. The methylation rates at each region were examined by MSP. Levels of the *BASP1* and *SRD5A2* methylation rate in HCC were significantly higher than in corresponding non-tumor liver tissue samples. NT, non-tumor liver tissue; ††Wilcoxon signed-rank test.

Statistical analysis. Data are presented as mean ± standard deviation. Significant differences between paired groups were evaluated using the Wilcoxon signed-rank test, and significant differences between the 4 groups were evaluated using the Dunnett's test. Receiver operating characteristic (ROC) analysis was conducted to determine the area under the curve (AUC), sensitivity and specificity. Calculations were performed using the SPSS Statics 17.0 software (SPSS, Tokyo, Japan), and P<0.05 was considered statistically significant.

Results

The methylation rates of BASP1 and SRD5A2 in HCC and corresponding non-tumor liver tissues. We determined the methylation rate of the 5'-flanking regions of the *BASP1* and *SRD5A2* genes in 46 paired HCC and non-tumor liver samples using qMSP. This study compared the diagnostic power of 2 distinct regions, termed region 1 and region 2, of *BASP1* and *SRD5A2* in order to obtain a diagnostic tool with high accuracy. The methylation rates at the 2 regions of *BASP1* and *SRD5A2* in HCC were significantly higher than those in the non-tumor liver tissues (Fig. 1). In most non-tumor liver tissues, the methylation status of *BASP1* region 1 and *SRD5A2* region 2 were found to be low, while those of *BASP1* region 2 and *SRD5A2* region 1 showed a broad range of methylation rate, respectively. *BASP1* and *SRD5A2* mRNA levels in 33 HCC samples were 23.2-fold (P<0.001) and 12.9-fold (P<0.001) lower than those in the non-tumor liver tissues, respectively. In addition, there was a significant inverse correlation between the mRNA levels and methylation rates of *BASP1* and *SRD5A2* (*BASP1* mRNA levels vs.methylation rates of *BASP1* region 1 and 2: r=-0.393 and -0.311, respectively, *SRD5A2* mRNA levels vs. methylation rates of *SRD5A2* region 1 and 2: r=-0.264 and -0.312, respectively).

Diagnostic performance of the DNA methylation rates of BASP1 and SRD5A2. ROC analysis revealed that the methylation rate of each gene allowed for the accurate determination of HCC and non-tumor liver tissue (Fig. 2). In particular, methylation analysis of the 2 genes combined increased the discrimination of tissue samples when compared to the analysis of a single gene. A combination analysis of *BASP1*

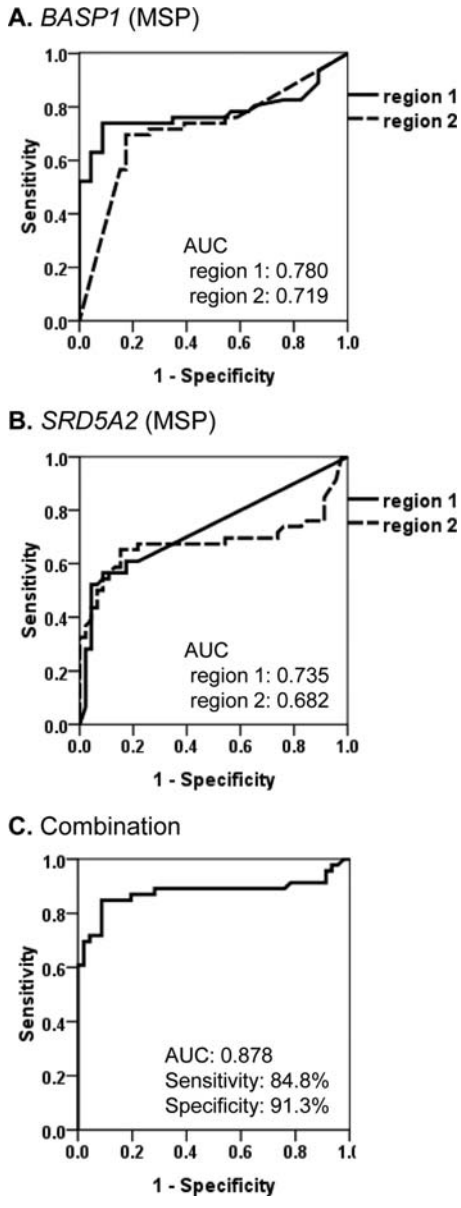

Figure 2. ROC analysis for discriminating HCC from non-tumor liver tissue. (A and B) Each region of *BASP1* and *SRD5A2* could be analyzed to define HCC from non-tumor liver tissue. (C) A combination of the methylation rates at *BASP1* region 1 and *SRD5A2* region 2 resulted in the optimal performance (AUC of 0.878, 84.8% sensitivity and 91.3% specificity).

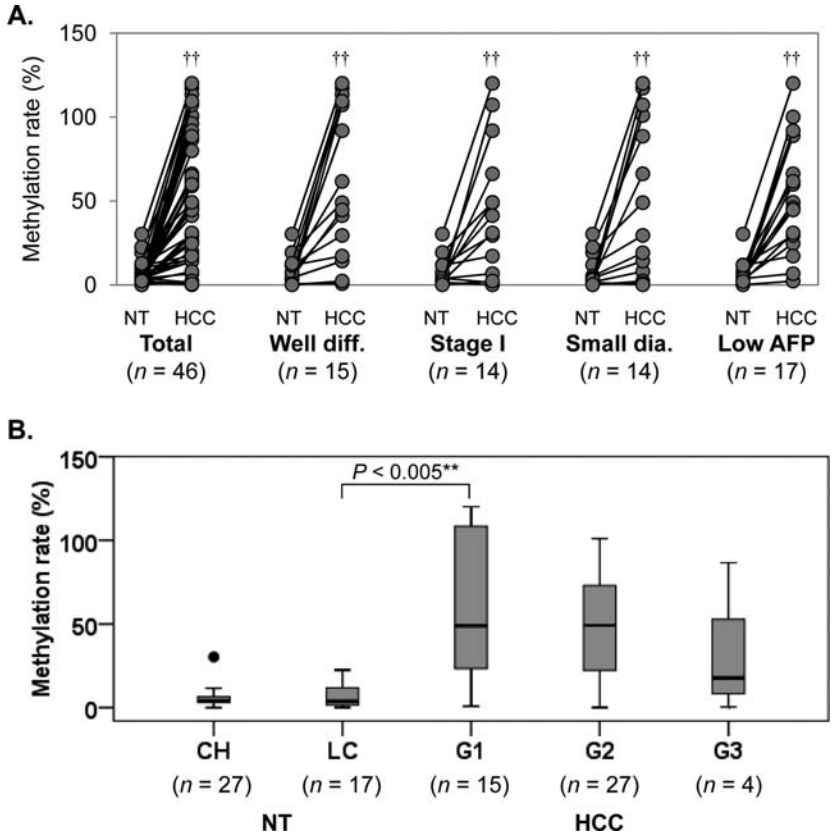

Figure 3. Combined methylation rates in HCC. The combined methylation rates were significantly higher in HCC compared to those in the non-tumor liver tissues. Patients with well-differentiated HCC (n=15), HCC of TNM stage I (n=14), small HCC <3 cm in diameter (n=14) and AFP levels <20 ng/ml (n=17) showed significant differences in the combined methylation rate between HCC and corresponding non-tumor liver samples. There were no significant differences of the combined methylation rates between chronic hepatitis and liver cirrhosis, and also between the grades of tumor differentiation. NT, non-tumor liver tissue; well-diff., well-differentiated HCC; Stage I, the TNM stage I of UICC (2002); small dia., <3 cm in diameter; low AFP, <20 ng/ml; CH, chronic hepatitis; LC, liver cirrhosis; G1, well-differentiated; G2, moderately-differentiated; G3, poorly-differentiated; ††Wilcoxon signed-rank test; **Dunnett's test.

Table III. Induction and methylation status of BASP1 and SRD5A2 in hepatoma cell lines.

	Hep G2	Hep 3B	HLE	HuH-6	HuH-7	SK-HEP-1
Induction of BASP1 mRNA levels (fold)						
5-aza-dC (10 μM)	7.2±2.3[a]	1.9±0.1[a]	1.2±0.1	1.7±0.2[a]	1.0±0.3	1.4±0.2
NaB (10 mM)	25.9±11.0[a]	91.1±8.7[a]	0.8±0.1	2.9±0.4[a]	9.5±2.2[a]	0.4±0.1[a]
Both NaB and 5-aza-dC	420.5±70.4[a]	93.8±8.8[a]	0.7±0.0	2.2±0.3[a]	8.1±0.8[a]	0.5±0.0[a]
Methylation status of BASP1						
Region 1	M	H	U	H	M	U
Region 2	M	H	U	H	M	U
Induction of SRD5A2 mRNA levels (fold)						
5-aza-dC (10 μM)	3.2±0.3[a]	2.8±0.9[a]	1.1±0.4	2.2±0.3[a]	2.6±0.4[a]	1.9±0.4[a]
NaB (10 mM)	8.5±1.5[a]	13.8±1.9[a]	25.2±11.7[a]	3.7±0.7[a]	4.6±0.6[a]	3.2±0.5[a]
Both NaB and 5-aza-dC	9.8±1.1[a]	15.7±2.5[a]	46.0±5.7[a]	3.9±0.6[a]	8.0±0.4[a]	4.4±0.6[a]
Methylation status of SRD5A2						
Region 1	M	M	U	M	H	M
Region 2	M	H	U	M	H	M

The mRNA levels of *BASP1* and *SRD5A2* were measured by real-time PCR. Induction of *BASP1* and *SRD5A2* mRNA levels by 5-aza-dC and NaB were calculated by compared with those levels from non-treatment cells. Represented values are means and standard deviations of more than two independent experiments performed in triplicate. The DNA methylation status was examined by MSP as described in the Materials and methods. [a]P<0.05 by Dunnett's test compared to non-treated cells; M, methylated; U, unmathylated; H, heterogeneous of methylated and unmethylated.

region 1 and *SRD5A2* region 2 resulted in the most accurate diagnostic performance (AUC of 0.878, 84.8% sensitivity and 91.3% specificity, Fig. 2C). In addition, the methylation rate of these 2 regions were significantly higher in the patient subpopulations with well-differentiated HCC (n=15, P<0.05), HCC at TNM stage I (n=14, P<0.05), HCC less than 3 cm in diameter (n=14, P<0.05) and HCC with low AFP levels (<20 ng/ml, n=17, P<0.05) when compared to the corresponding non-tumor liver tissue (Fig. 3A). There were no significant differences in the methylation rate of these regions when comparing between chronic hepatitis and liver cirrhosis, and between the different tumor differentiation grades (Fig. 3B). The results similar to those methylation analyses were obtained for mRNA levels of *BASP1* and *SRD5A2* (data not shown).

BASP1 and SRD5A2 mRNA levels and epigenetic regulation in hepatoma cell lines. We also measured the *BASP1* and *SRD5A2* mRNA levels in 6 hepatoma cell lines. Given that the mRNA levels of the 2 genes were significantly lower in all 6 cell lines when compared to the non-tumor liver tissues (data not shown), we examined potential epigenetic mechanisms underlying the transcriptional regulation of the *BASP1* and *SRD5A2* genes. We found that *BASP1* levels were induced in 4 of the 6 cell lines, while *SRD5A2* mRNA levels were induced in 5 of the 6 cell lines following administration of the demethylating agent, 5-aza-dC (Table III). In the cell lines demonstrating induction of *BASP1* and *SRD5A2* mRNA levels following 5-aza-dC addition, DNA methylation at the *BASP1* and *SRD5A2* loci was also detected by MSP. We also observed DNA methylation of *BASP1* in HuH-7 cells; however, induction of mRNA expression via demethylation was not identified. Administration of the HDAC inhibitor NaB also increased *BASP1* and *SRD5A2* mRNA expression in the vast majority of cell lines tested. HLE and SK-HEP-1 cells demonstrated comparatively high basal *BASP1* mRNA levels and regulation of expression by the methylation was not identified (data not shown). Epigenetic regulation of *SRD5A2* mRNA expression by methylation or acetylation was observed in all tested cell lines.

Discussion

The *BASP1* and *SRD5A2* genes have been previously demonstrated to be both down-regulated and aberrantly methylated in TNM stage I and II HCC (12). As a result, we hypothesized that these genes may prove to be reliable markers for the diagnosis of early HCC. The fact that we selected these genes mathematically from among more than 10,000 genes without any bias also supports this hypothesis (12). To enable the use of these genes as clinical diagnostic tools, we re-evaluated the performance of *BASP1* and *SRD5A2* in the early diagnosis of HCC in a large independent cohort, and identified the optimal performance combination of *BASP1* and *SRD5A2* genes using qMSP analysis with newly designed primers and probes.

We confirmed that the methylation rates of *BASP1* and *SRD5A2* in HCC were higher than those in non-tumor liver tissues, a result that was inversely correlated with mRNA levels. We then examined the diagnostic power of the methylation rates of *BASP1* and *SRD5A2* in HCC using ROC analysis. The diagnostic powers were found to be only moderate when the methylation rate was determined for a single gene (AUC of 0.682-0.780). Interestingly, calculation of the methylation rate for both *BASP1* and *SRD5A2* genes improved the sensitivity and specificity of HCC diagnosis (AUC of 0.878, 84.8% sensitivity and 91.3% specificity). This finding suggests that the methylation patterns of *BASP1* and *SRD5A2* are independent and mutually complement their HCC diagnostic power. The aberrant methylation and decreases in expression were also observed in the early HCC including HCC of TNM stage I, well-differentiated HCC and those that were small in size. Moreover, patients with low AFP levels also demonstrated an aberrant methylation status in HCC. These results suggest that the methylation status of *BASP1* and *SRD5A2* may allow for the early detection of HCC. Thus, altered epigenetic regulation of *BASP1* and *SRD5A2* may become apparent at the onset of carcinogenesis, rather than the time of viral infection or the development of liver disease (chronic hepatitis and liver cirrhosis). The aberrant methylation may also be maintained throughout tumor progression, as significant differences in methylation status were not observed between the different liver diseases and between the different tumor differentiation grades. In addition, there appeared to be no significant differences between patient background including sex, age and mode of virus infection (data not shown), and thus the analysis of methylation status in both *BASP1* and *SRD5A2* genes enabled the detection of HCC independent of patient background.

The MSP technique has been used in many of the methylation analyses undertaken recently. However, it has been shown that the methylation rates differ greatly depending on the region of the CpG islands analyzed (13). Although the aberrant methylation at *BASP1* region 1 and *SRD5A2* region 2 appeared to be tumor specific, the aberrant methylation at *BASP1* region 2 and *SRD5A2* region 1 were also observed in some of the non-tumor liver tissues. Aberrant methylation was not detected in non-tumor liver tissues in the absence of virus infection or liver disease, although there were only 2 samples analyzed in this study. These findings suggest that some regions of *BASP1* and *SRD5A2* might be methylated during the development of liver disease caused by viral infection, while others may be methylated by carcinogenesis events. It therefore appears important to design the region for MSP analysis carefully and based on results for bisulfite sequencing.

In our present study, mRNA levels and methylation rates of *BASP1* and *SRD5A2* showed a moderate correlation (r=-0.264 to -0.393, P<0.05). Approximately half of the HCC samples did not demonstrate an increase in methylation rate of *BASP1* and *SRD5A2* when compared to the non-tumor liver tissues. In contrast, almost all of the samples showed a decrease in mRNA expression of the 2 genes in the HCC samples when compared to the non-tumor liver tissues. These results suggest that the decrease in *BASP1* and *SRD5A2* mRNA expression in the HCC samples may be due in part to mechanisms other than aberrant methylation. Indeed, mRNA expression of *BASP1* and *SRD5A2* were significantly increased following the addition of the demethylating agent or HDAC inhibitor in several hepatoma cell lines. Thus, epigenetic regulation of *BASP1* and *SRD5A2* expression would appear

to involve both methylation and acetylation processes. Interestingly, the mRNA levels of *BASP1* and *SRD5A2* in hepatoma cell lines were largely induced following combination treatment with 5-aza-dC and NaB when compared to their single administration. Similarly, *T-cadherin* was down-regulated in several hepatoma cell lines and restored following treatment with demethylating agents and HDAC inhibitors (16). Several HCC-related genes including *CCND2*, *p16*, *p21*, *RASFF1A* and *SOCS1* have been shown to be epigenetically regulated by either methylation or acetylation (11,17-26). It has also been reported that combined epigenetic approaches exert more effective anti-tumor activities in hepatoma cells (27). In combination with our results, these studies lead to the hypothesis that *BASP1* and *SRD5A2* may exhibit anti-tumor effects.

BASP1 was originally identified as a membrane bound protein abundant in nerve terminals. *BASP1* contains several transient phosphorylation sites and regions rich in proline (P), glutamate (E) and/or aspartate (D) and serine (S) and/or threonine (T) (PEST) motifs that typically occur in proteins with high turnover rates (28). *BASP1* has also been reported to function as a transcriptional co-suppressor for Wilms' tumor suppressor protein WT1 (29), however, whether this protein is correlated with the pathogenesis of HCC has not been reported to date. *SRD5A2* encodes a microsomal protein that is expressed at high levels in androgen-sensitive tissues such as the prostate. The encoded protein represents a membrane-associated enzyme that catalyzes the conversion of testosterone to dihydrotestosterone. Polymorphisms and alterations in *SRD5A2* expression levels have been associated with the development of prostate cancer (30-32). To the best of our knowledge, an association between *SRD5A2* polymorphism and liver cancer has only been reported in one study (33). In our preliminary study, *BASP1* and *SRD5A2* gene-targeting experiments involving overexpression from expression plasmid vectors or knockdown using small-interfering RNAs resulted in minimal effects on cell growth and cell cycle (data not shown). Although we did not examine additional cancer features such as mobility and invasiveness, it may be that *BASP1* and *SRD5A2* genes exert only a minimal effect on HCC development, or that the aberrant methylation of these two genes might be merely a result of carcinogenesis.

Finally, this study supports the hypothesis that quantification of methylation status of the *BASP1* and *SRD5A2* genes may enable an accurate early diagnosis of HCC. It is also suggests that determination of mRNA levels of both genes may be essential for an accurate diagnosis. However, mRNA analysis requires a tissue specimen to obtain high quality RNA which is an invasive procedure for the patient. It has been reported that increased levels of cell-free circulating DNA is present in many cancer patients including those with HCC (34,35), although cancer-specific DNA was less than 5% of the total circulating DNA (36,37). Aberrant methylation of *p16*, *GSTP1* or *RASSF1A* has been frequently detected in the circulating DNA isolated from HCC patients (38-41). Further experiments may investigate the potential use of our novel MSP for the early diagnosis of HCC using DNA circulating in the bloodstream as a source, thus eliminating the use of invasive preparations.

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