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<td>Author(s)</td>
<td>Honda, Shohei; Minato, Masashi; Suzuki, Hiromu; Fujiyoshi, Masato; Miyagi, Hisayuki; Haruta, Masayuki; Kaneko, Yasuhiko; Hatanaka, Kanako C.; Hiyama, Eiso; Kamijo, Takehiko; Okada, Tadao; Taketomi, Akinobu</td>
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**File Information**

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Clinical prognostic value of DNA methylation in hepatoblastoma: Four novel tumor suppressor candidates

Shohei Honda,1 Masashi Minato,1 Hiromu Suzuki,2 Masato Fujiyoshi,1 Hisayuki Miyagi,1 Masayuki Haruta,3 Yasuhiro Kaneko,3 Kanako C. Hatanaka,4 Eiso Hiyama,3 Takehiko Kamijo,3 Tadao Okada6 and Akinobu Taketomi1

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Key words
Hepatoblastoma, methylation, prognostic marker, survival, tumor suppressor

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Hepatoblastoma (HB) is very rare but the most common malignant neoplasm of the liver occurring in children. Despite improvements in therapy, outcomes for patients with advanced HB that is refractory to standard preoperative chemotherapy remain unsatisfactory. To improve the survival rate among this group, identification of novel prognostic markers and therapeutic targets is needed. We have previously reported that altered DNA methylation patterns are of biological and clinical importance in HB. In the present study, using genome-wide methylation analysis and bisulfite pyrosequencing with specimens from HB tumors, we detected nine methylated genes. We then focused on four of those genes, GPR180, MST1R, OCIAD2, and PARP6, because they likely encode tumor suppressors and their increase of methylation was associated with a poor prognosis. The methylation status of the four genes was also associated with age at diagnosis, and significant association with the presence of metastatic tumors was seen in three of the four genes. Multivariate analysis revealed that the presence of metastatic tumors and increase of methylation of GPR180 were independent prognostic factors affecting event-free survival. These findings indicate that the four novel tumor suppressor candidates are potentially useful molecular markers predictive of a poor outcome in HB patients, which may serve as the basis for improved therapeutic strategies when clinical trials are carried out.
and prognostic assessment of HB, and serve as the basis for improved therapeutic strategies.

Materials and Methods

Patients. This study consists of two parts: (i) screening for candidate genes by genome-wide assays in FFPE specimens obtained from two HB patients; and (ii) methylation analysis of the candidate genes using bisulfite pyrosequencing in fresh-frozen samples obtained from 74 HB tumors.

The FFPE specimens were obtained from two patients referred to our institution for surgical treatment in 2009 and 2010. Both patients were female, aged 25 and 18 months. DNA samples extracted from a fresh-frozen HB tumor specimen from each of the 74 patients and normal liver specimens from 4 patients were supplied by the JPLT. All patients were treated within the context of the JPLT-2 study, in which the protocols include pre- and postoperative chemotherapy with cisplatin and 4′-O-tetrahydropyranyl-adiamycin. Sixty-four patients underwent preoperative chemotherapy, and complete disappearance or at least a 50% reduction in tumor size was obtained in 51 patients (80.0%). The extent of disease was determined at the time of initial biopsy or resection using the classification defined in the PRETEXT staging system.

Metastatic tumors were found in 15% of the patients (Table S1). The median follow-up of survivors was 63 months (range, 9–148 months). The 5-year OS and EFS rates were 86.7% and 73.4%, respectively.

The ethics committee at our institution approved the study protocol. In all cases, informed signed consent was obtained by local physicians at the participating institutions.

Genome-wide analysis of methylation. Tissue FFPE samples that included fetal tumor cells, embryonal tumor cells, or normal liver cells were collected from tumors resected from two patients. After dissecting the samples under a light microscope, which enabled us to avoid contamination by normal tissues or mesenchymal components, we extracted two sets of DNA samples from each fetal tumor, embryonal tumor, and normal liver specimen. To extract the DNA, we used a QIAamp DNA FFPE Tissue Kit Qiagen (Valencia, CA, USA) according to the manufacturer’s instructions. We carried out a quality check of the DNA samples using RT-PCR, following the Infinium HD FFPE QC Assay protocol Illumina (San Diego, CA, USA), and we confirmed that all the samples were appropriate for the methylation assay. We next carried out genome-wide methylation analyses using an Infinium HumanMethylation450 BeadChip (Illumina) and the six DNA samples, following the Illumina Infinium HD Methylation protocol. This array includes 485 577 cytosine positions in the human genome (482 421 CpG sites [99.4%], 3091 non-CpG sites, and 65 random single nucleotide polymorphisms). We linked the UCSC Genome Browser annotation (version hg19 of the human reference genome available at https://genome.ucsc.edu/) to each of the CpG sites on the array. Based on the UCSC chromosome annotation, we filtered out DNA methylation from the X and Y chromosomes. We next excluded the probes whose β-values in normal liver specimens were more than 0.2. We then screened for probes that showed more than a twofold difference in their β-value when comparing between fetal and/or embryonal HB tumors and normal liver tissues.

Gene expression in HB cell lines treated with a demethylating agent. To assess restoration of expression, cells from the HuH6 and HepG2 HB lines were treated with 1.0 μM 5-aza-dC (Sigma, St. Louis, MO, USA) for 72 h, replacing the drug and medium every 24 h. Total RNA was then extracted using an RNaseq kit (Qiagen), and sample amplification and labeling were done using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies, Santa Clara, CA, USA), both according to the manufacturer’s instructions. Samples labeled with Cy3 were hybridized and processed on a 4x44K Whole Human Genome Oligo Microarray. Scanning was done with an Agilent G2565BA microarray scanner using the settings recommended by Agilent Technologies. All raw data were normalized and analyzed using GeneSpring GX 10.0 (Agilent Technologies). We screened for genes whose expression was increased more than twofold by 5-aza-dC treatment in HuH6 or HepG2 cells.

Bisulfite pyrosequencing. We used bisulfite pyrosequencing to examine the methylation status of 19 selected genes in the 74 tumor samples and four samples of normal liver tissue. The primer sequences and locations used for the methylation analysis are shown in Table S2 and Figure S1. This enabled us to determine the level of methylation at each CpG site in a sample after bisulfite treatment. Genomic DNA (500 ng) was modified with sodium bisulfite using an Epitect bisulfite kit (Qiagen), after which bisulfite pyrosequencing was carried out as described previously. Following PCR, the biotinylated product was purified, made single-stranded, and used as a template in the pyrosequencing reaction. Briefly, the PCR product was bound to streptavidin Sepharose beads HP (Amersham Biosciences, Amersham, UK), after which beads containing the immobilized product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotage, Uppsala, Sweden) and Pyro Q-CpG software (Biotage). The methylation levels at different CpG sites, as measured by pyrosequencing, were averaged to represent the degree of methylation in each sample for each gene.

Statistics. Statistical analysis and data visualization were carried out using R software version 3.0.2 (www.r-project.org) and JMP version 11.0 (www.jmp.com) for Windows. Survival curves were constructed according to the methods of Kaplan and Meier, and were compared using the log–rank test. Overall survival was defined as the time interval from the date of diagnosis to the date of death (as a result of any cause) or the date of the last follow-up. Event-free survival was defined as the time interval from the date of diagnosis to the date of progression, the date of relapse, the date of death, the date of diagnosis of a second malignant neoplasm, or the date of the last follow-up. Event-free survival was defined as the time interval from the date of diagnosis to the date of progression, the date of relapse, the date of death, the date of diagnosis of a second malignant neoplasm, or the date of the last follow-up, whichever occurred first. Correlations between the methylation status and clinicopathological factors were analyzed using Fisher’s exact test. Univariate analysis of variables was also undertaken, after which selected variables were analyzed using the Cox proportional hazard model for multivariate analysis. P-values < 0.05 were considered statistically significant.

Results

Selection of candidate tumor suppressor genes. When we used a genome-wide methylation assay to screen for genes showing more than a twofold difference in their β-values between HB tumors and normal liver tissue, 3451 and 4553
Prognostic value of DNA methylation in HB

Fig. 1. After screening using genome-wide assays, the Venn diagram shows the relationship between genes showing increase of methylation in fetal and embryonal hepatoblastoma cells and genes whose expression was upregulated by treatment with 5-aza-2′-deoxycytidine (5-aza-dC).

probes were identified as differentially methylated in fetal and embryonal HB, respectively. Among the methylated probes in fetal HB, 686 probes in TSS1500, 838 probes in TSS200, 429 probes in 5′-UTR, 476 probes in 1stExon, 957 probes in Body, and 65 probes in 3′-UTR were included. In embryonal HB, however, 956 probes in TSS1500, 1042 probes in TSS200, 570 probes in 5′-UTR, 622 probes in 1stExon, 1277 probes in Body, and 86 probes in 3′-UTR were detected as differentially methylated. Consequently, we found 1683 and 2019 unique methylated genes in fetal and embryonal HB, respectively. In addition, expression of 905 genes was increased more than twofold by 5-aza-dC treatment in HuH6 and HepG2 HB lines. Using a Venn diagram, we then selected 95 candidate tumor suppressor genes that were hypermethylated in fetal and/or embryonal HB and whose expression was increased twofold by 5-aza-dC (Fig. 1). Datasets obtained from the genome-wide methylation analysis and the gene expression analysis are shown in Tables S3 and S4. From among those 95 genes, we selected 19 determined to be aberrantly hypermethylated in some of those genes (Table 2). However, the methylation status did not correlate with the histological type or PRETEXT classification.

After selecting variables from among the six clinical and tumor-associated factors listed in Table S5, taking into account the methylation status of GPR180, MST1R, OCIAD2, and PARP6, multivariate analysis showed that only the presence of metastatic tumors was independently correlated with a poor OS (Table S6). In addition, in a multivariate analysis of tumor recurrence, increase of methylation of GPR180 and the presence of metastatic tumors were found to be independent prognostic factors affecting EFS (Table 3, Fig. S5).

Discussion

We used genome-wide assays to identify 95 candidate genes whose increase of methylation may be involved in HB progression by examining different types of tumor cells. From among them, we used pyrosequencing analysis to ultimately select nine genes showing increase of methylation in HB tumors. We then evaluated the association between the methylation status of those nine genes and prognosis, which revealed that the methylation status of four genes, GPR180, MST1R, OCIAD2, and PARP6, was significantly associated with several clinical parameters, including the age at diagnosis and the presence of metastatic disease or hepatic vein invasion, as well as a poor outcome. However, screening of only two sets of samples using HM450 has been carried out, limiting the possible discoveries of this study. We expect that genome-wide screening of large and well-annotated patient cohorts will lead us to identifying more powerful prognostic biomarkers in the future.

Originally identified by Strausberg et al.,(17) OCIAD2 was previously shown to be a marker for a subtype of lung adenocarcinoma mixed subtype with bronchioloalveolar adenocarcinoma that showed a favorable prognosis, which suggests it may function as a tumor suppressor.(18) Poly(ADP-ribose) polymerase is an enzyme that catalyzes post-translational protein modification, and PARP6 belongs to the mono(ADP-ribose) transferase class. PARP6 reportedly acts as a tumor suppressor in colorectal cancer through its role in cell cycle control.(19) To date, however, epigenetic dysregulation of these genes has not been described. Our present finding that methylation of these two genes is associated with poor outcomes might be consistent with those earlier reports, if increase of
methyla-
tion of the regions we examined has negative correla-
tion with expression.

In contrast to **OCIAD2** and **PARP6**, **MST1R** expression is associated with poor outcomes in several cancers, although Hodgkin’s lymphoma is an exception, in which its expression is associated with better survival. At first glance it appears contradictory that **MST1R** expression was associated with poor outcomes in patients with various cancers, while methylation of the **MST1R** promoter, presumably silencing the gene, was also associated with poor outcomes in HB patients. But hypermethylation of the RON (**MST1R**) proximal promoter is associated with a deficiency in full-length RON and with transcription of oncogenic short-form RON driven by an internal promoter. Short-form RON has been shown to drive small-cell and non-small-cell lung cancer cell proliferation. This suggests hypermethylation of the **MST1R** promoter contributes to tumor progression regulated by two promoters coex-
inling in the same gene.

**GPR180** is known to be a G protein-coupled receptor pro-
duced predominantly in vascular smooth muscle cells and to play an important role in the regulation of vascular remodeling. **GPR180** was identified as being highly overexpressed in colorectal cancer cells, and its knockdown using RNAi sig-
nificantly reduced cell viability. These findings also seem contradictory to the observation in HB that **GPR180** methyla-
tion is associated with a poor outcome. Identification of the

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**Table 1. Nineteen genes that were further selected from 95 identified in genome-wide assays, whose increase of methylation may be involved in hepatoblastoma progression**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Full name</th>
<th>Gene location</th>
<th>Function</th>
<th>Methylation level, %, mean ± SD</th>
<th>Cut-off value, % (AUC)†</th>
<th>Number of tumors with methylated gene/s,‡ n (%)</th>
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</thead>
<tbody>
<tr>
<td>CADM2</td>
<td>Cell adhesion molecule 2</td>
<td>3p12</td>
<td>Cell adhesion</td>
<td>8.78 ± 8.91 (74)</td>
<td>6.87 ± 2.87</td>
<td>5.26 (0.669)</td>
</tr>
<tr>
<td>CAMTA1</td>
<td>Calmodulin binding transcription activator 1</td>
<td>1p36</td>
<td>Transcriptional factor</td>
<td>5.22 ± 1.27 (50)</td>
<td>6.42 ± 0.12</td>
<td>5.20 (0.710)</td>
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<tr>
<td>CCDC8</td>
<td>CCDC8 coiled-coil domain containing 8</td>
<td>1q13</td>
<td>Apoptosis</td>
<td>27.18 ± 22.38 (74)</td>
<td>8.19 ± 1.20</td>
<td>34.8 (0.592)</td>
</tr>
<tr>
<td>CRB3</td>
<td>Crumbs homolog 3</td>
<td>1q13</td>
<td>Cell adhesion</td>
<td>4.51 ± 1.30 (74)</td>
<td>4.22 ± 0.75</td>
<td>3.93 (0.576)</td>
</tr>
<tr>
<td>EML1</td>
<td>Echinoderm microtubule associated protein like 1</td>
<td>14q32</td>
<td>Microtubule</td>
<td>5.28 ± 1.27 (74)</td>
<td>5.49 ± 0.98</td>
<td>4.11 (0.510)</td>
</tr>
<tr>
<td>FZD8</td>
<td>Frizzled family receptor 8</td>
<td>10p11</td>
<td>Wnt signaling</td>
<td>4.87 ± 1.94 (48)</td>
<td>6.02 ± 0.57</td>
<td>6.01 (0.525)</td>
</tr>
<tr>
<td>GPR180</td>
<td>G protein-coupled receptor 180</td>
<td>13q32</td>
<td>Signal transduction</td>
<td>5.28 ± 11.72 (74)</td>
<td>0.00 ± 0.00</td>
<td>4.11 (0.796)</td>
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<td>MPDU1</td>
<td>Mannose-P-dolichol utilization defect 1</td>
<td>17p13</td>
<td>Glucosylation</td>
<td>1.49 ± 1.24 (74)</td>
<td>0.62 ± 0.71</td>
<td>1.33 (0.580)</td>
</tr>
<tr>
<td>MST1R</td>
<td>Macrophage stimulating 1 receptor</td>
<td>3p21</td>
<td>Tyrosine kinase</td>
<td>14.53 ± 14.89 (74)</td>
<td>5.42 ± 2.21</td>
<td>20.8 (0.690)</td>
</tr>
<tr>
<td>NEFH</td>
<td>Neurofilament, heavy polypeptide</td>
<td>22q12</td>
<td>Neurofilament</td>
<td>11.11 ± 4.00 (74)</td>
<td>12.02 ± 0.95</td>
<td>20.0 (0.425)</td>
</tr>
<tr>
<td>NRN1</td>
<td>Neuritin 1</td>
<td>6p25</td>
<td>Neuritogenesis</td>
<td>9.31 ± 7.83 (74)</td>
<td>6.07 ± 1.66</td>
<td>44.4 (0.425)</td>
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<td>OC1AD2</td>
<td>OC1A domain containing 2</td>
<td>12q13</td>
<td>Inhibition of MAPK</td>
<td>12.72 ± 14.32 (74)</td>
<td>5.01 ± 1.01</td>
<td>9.12 (0.588)</td>
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<tr>
<td>PARP6</td>
<td>Poly (ADP-ribose) polymerase family, member 6</td>
<td>15q23</td>
<td>ADP-ribose transferase</td>
<td>12.89 ± 14.19 (74)</td>
<td>2.72 ± 3.60</td>
<td>8.09 (0.786)</td>
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<tr>
<td>PON3</td>
<td>Paraoxonase 3</td>
<td>7q21</td>
<td>Lipoprotein metabolism</td>
<td>6.34 ± 11.28 (74)</td>
<td>5.90 ± 4.62</td>
<td>4.25 (0.515)</td>
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<tr>
<td>RAPGEF3</td>
<td>Rap guanine nucleotide exchange factor (GEF) 3</td>
<td>12q13</td>
<td>Inhibition of MAPK</td>
<td>3.46 ± 1.45 (50)</td>
<td>1.46 ± 1.77</td>
<td>3.21 (0.674)</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
<td>10p13</td>
<td>Cell adhesion</td>
<td>12.72 ± 14.32 (74)</td>
<td>5.01 ± 1.01</td>
<td>9.12 (0.588)</td>
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<tr>
<td>ZAR1</td>
<td>Zygote arrest 1</td>
<td>4p11</td>
<td>Unknown</td>
<td>12.99 ± 10.39 (74)</td>
<td>11.19 ± 0.37</td>
<td>25.2 (0.426)</td>
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<tr>
<td>ZC3H13</td>
<td>Zinc finger CCCH-type containing 13</td>
<td>13q14</td>
<td>Unknown</td>
<td>15.40 ± 18.43 (74)</td>
<td>3.42 ± 0.68</td>
<td>13.2 (0.453)</td>
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<tr>
<td>ZMYND10</td>
<td>Zinc finger, MYND-type containing 10</td>
<td>3p21</td>
<td>Unknown</td>
<td>4.39 ± 5.11 (74)</td>
<td>0.91 ± 1.41</td>
<td>1.99 (0.683)</td>
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†Area under the receiver-operator curve analysis of overall survival establishing the cut-off value for each gene. †A aberrant hypermethylation was deemed to be present (+) when at least one sample showed a methylation level > cut-off value. The genes whose cut-off values were below the methylation level of (mean ± SD) in normal liver tissues were deemed not to be aberrantly hypermethylated, as there was no significant dif-
ference in the methylation level between the tumor and normal liver tissues. §**CAMTA1**, **EML1**, **FZD8**, and **NEFH** were determined not to be aber-
rantly hypermethylated because the mean methylation level in normal liver tissues was greater than that in tumor tissues. –, none (zero).
The precise functions of MST1R and GPR180 in cancer development will require further study.

Interestingly, we found a clear positive correlation between the number of genes showing increase of methylation and age at diagnosis (Fig. S3). This suggests the number of methylated genes may be age-dependent. It is well known that both aging and chronic inflammation contribute to aberrant DNA methylation, which is particularly prominent in chronic inflammation-associated cancers, such as gastric cancer, hepatocellular carcinoma, and colitic cancer. The degree of aberrant methylation in normal-appearing tissues (epigenetic field defect) correlates with the risk of cancer development. Given that most HB patients are diagnosed before the age of 2 years, it seems unlikely that such accumulation contributes greatly to...
methylation correlates with different histological progression of HB remain unknown. We previously reported that methylation assay of a large number of samples. Further investigations using genome-wide methylation assays of a large number of samples. Resulted in the identification of a gene signature that discriminates between childhood hepatic tumors having a fairly well-differentiated histology and favorable prognosis and those with a poorly differentiated histology and dismal prognosis. Thus, the gene signatures that underlie the phenotypes may enable molecular classification of HB tumors after thorough clinical testing.

The epigenetic alterations contributing to the malignant progression of HB may be a source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis. DNA methylation profiles represent a chemically and biologically stable source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis.

In addition to their pathogenic implications, DNA methylation profiles may be a source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis. DNA methylation profiles represent a chemically and biologically stable source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis.

Table 2. Correlation between the methylation status of four identified genes and clinicopathological factors in 74 hepatoblastoma tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>M</th>
<th>U</th>
<th>P-value†</th>
<th>M</th>
<th>U</th>
<th>P-value†</th>
<th>M</th>
<th>U</th>
<th>P-value†</th>
<th>M</th>
<th>U</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR180</td>
<td>M</td>
<td>U</td>
<td>0.790</td>
<td>M</td>
<td>U</td>
<td>0.389</td>
<td>M</td>
<td>U</td>
<td>1.000</td>
<td>M</td>
<td>U</td>
<td>0.198</td>
</tr>
<tr>
<td>MST1R</td>
<td>M</td>
<td>U</td>
<td>0.008</td>
<td>M</td>
<td>U</td>
<td>0.002</td>
<td>M</td>
<td>U</td>
<td>0.008</td>
<td>M</td>
<td>U</td>
<td>0.012</td>
</tr>
<tr>
<td>OCIAD2</td>
<td>M</td>
<td>U</td>
<td>0.005</td>
<td>M</td>
<td>U</td>
<td>0.052</td>
<td>M</td>
<td>U</td>
<td>0.003</td>
<td>M</td>
<td>U</td>
<td>0.029</td>
</tr>
<tr>
<td>PARP6</td>
<td>M</td>
<td>U</td>
<td>0.005</td>
<td>M</td>
<td>U</td>
<td>0.052</td>
<td>M</td>
<td>U</td>
<td>0.003</td>
<td>M</td>
<td>U</td>
<td>0.029</td>
</tr>
</tbody>
</table>

†Fisher's exact test. M, methylated; PRETEXT, Pretreatment Extent of Disease; U, unmethylated.

The epigenetic alterations contributing to the malignant progression of HB may be a source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis. DNA methylation profiles represent a chemically and biologically stable source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis.

In addition to their pathogenic implications, DNA methylation profiles may be a source of molecular diagnostic information. Recent technology enables genome-wide screening for altered DNA methylation profiles, which may then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis.
management of this disease. That said, our findings need to be validated in a long-term study that includes a larger number of patients to establish prognostic markers for clinical usage.

In conclusion, the methylation status of four genes, GPR180, MST1R, OCIAD2, and PARP6, was found to be a potentially useful molecular marker predictive of a poor outcome in HB patients. By further investigating the epigenetic aberrations in HB, we expect to establish molecular-genetic markers of treatment outcome in HB patients that could enable efficient stratification of patients and development of better therapeutic strategies.

Acknowledgments

We would like to thank all the staff at the institutes participating in the JPLT for enrolling their patients in this study. We are also grateful to the JPLT steering committee members (Drs. T. Hishiki; K. Ida; and K. Inoue), and the data administrator for JPLT (Dr. K. Hiyama) for data management and clinicopathological review of the patients. The methylation analysis using bisulfite pyrosequencing was supported by a Scientific Support Programs for Cancer Research Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

- 5-aza-dC: 5-aza-2′-deoxycytidine
- CIMP: CpG island methylator phenotype
- EFS: event-free survival
- FFPE: formalin-fixed, paraffin-embedded
- GPR180: G protein-coupled receptor 180
- HB: hepatoblastoma
- JPLT: Japanese Study Group for Pediatric Liver Tumors
- MST1R: macrophage stimulating 1 receptor
- OCIAD2: OCIA domain containing 2
- OS: overall survival
- PARP: poly(ADP-ribose) polymerase
- PRETEXT: Pretreatment extent of DISEASE

References

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Locations of the fragments analyzed using bisulfite pyrosequencing are shown as horizontal arrows. The translational start site of each gene is shown as a bent arrow.

**Fig. S2.** Receiver–operator curve (ROC) analysis of overall survival establishing the cut-off value for each gene. Numbers in parenthesis show the area under the ROC curve.

**Fig. S3.** Correlation between the number of genes showing increase of methylation and the age at diagnosis. Spearman’s correlation analysis was used to evaluate the association.

**Fig. S4.** Kaplan–Meier curves for event-free survival for the nine genes showing increase of methylation in 74 hepatoblastoma tumors. Blue line, unmethylated group (U); red line, methylated group (M).

**Fig. S5.** Receiver–operator curve (ROC) analysis of event-free survival and Kaplan–Meier curves of tumor recurrence rate in 74 hepatoblastoma tumors. Numbers in parenthesis show the area under the ROC curve.

**Table S1.** Clinical characteristics of 74 hepatoblastoma tumors at diagnosis.

**Table S2.** Primer sequences and PCR product sizes used in this study.

**Table S3.** Genes upregulated by 5-aza-2'-deoxycytidine (5-aza-dC), showing more than a twofold difference in their β-values between embryonal hepatoblastoma tumors and normal liver tissue.

**Table S4.** Genes upregulated by 5-aza-2'-deoxycytidine (5-aza-dC), showing more than a twofold difference in their β-values between fetal hepatoblastoma tumors and normal liver tissue.

**Table S5.** Univariate analysis of predictive values for overall survival and event-free survival in 74 hepatoblastoma patients.

**Table S6.** Multivariate analysis of values that are predictive of overall survival in 74 hepatoblastoma patients.