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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.

Hepatoblastoma is very rare but the most common malignant neoplasm of the liver occurring in children. Within this patient population, over 90% of those considered to be at standard risk achieve long-term survival. In contrast, among those considered to be at high risk due to the presence of extrahepatic tumors, macroscopic invasion of large vessels, or distant or lymph node metastasis, the mortality rate ranges from 35% to 50%. Complete surgical resection of the tumor or liver transplantation and mainstream treatment with cytotoxic drugs are essential for achieving a favorable long-term outcome. To reduce the mortality rate among those with refractory HB, increasing the complete resection rate and more effective preoperative chemotherapy is mandatory. In addition, identification of novel molecular-genetic markers predictive of treatment outcome is needed for better therapy planning.

Factors currently known to be predictive of outcome in HB patients include age at diagnosis, histology, local pattern of tumor growth, presence of metastasis, and the level of α-feto protein. In addition, chromosomal gains on 2q, 8q, and 20 and strong expression of telomerase reverse transcriptase or polo-like kinase 1 are known to be predictive of a poor outcome. CTNNB1 mutations are seen in the majority of HB tumors, but there have been few reports on genetic alterations of other oncogenes or tumor suppressor genes. We previously reported that RASSF1A methylation is independently correlated with a poor outcome and suggested that RASSF1A may be a promising molecular-genetic marker predictive of treatment outcome in HB patients. Moreover, disruption of imprinting status, mainly due to aberrant DNA methylation, has been implicated in the pathogenesis of HB. This suggested to us that epigenetic aberrations may be an important mechanism involved in the pathogenesis of HB. However, the precise role of DNA methylation in the development, progression, and classification of HB remains unknown.

Available technology now makes genome-wide analysis of DNA methylation possible. Furthermore, because methylation of particular genes is known to be associated with patient outcome and sensitivity to chemotherapeutic drugs, the presence of aberrant DNA methylation in tissue specimens could be a clinically useful biomarker. In the present study, therefore, we used genome-wide analysis to investigate the incidence of aberrant methylation in HB, and evaluated their association with the clinicopathological characteristics of the disease and its prognosis. Here, we report for the first time that the methylation status of four genes, GPR180, MST1R, OCIAD2, and PARP6, could be clinically useful for diagnostic...
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. The 74 patients, with a median age of 18 months, underwent tumor resection and partial hepatectomy between December 1999 and December 2003 at the institutions of 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-tetrahydropropyl-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, after which bisulfite pyrosequencing was carried out.

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, 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
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 fetal and embryonal hepatoblastoma cells and genes whose expression was upregulated by treatment with 5-aza-2′-deoxycytidine (5-aza-dC).

**Bisulfite pyrosequencing to examine methylation of candidate genes.** We next used bisulfite pyrosequencing to assess the methylation status of 19 selected genes in the 74 HB tumor specimens and four normal liver specimens obtained from 74 patients. Cut-off values for classification as either methylated or unmethylated were calculated individually for each gene using receiver–operator characteristic analysis of OS (Fig. S2), and 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 difference in the methylation level between the tumor and normal liver tissues. As shown in Table 1, we found 9 of the 19 genes to show an increase of methylation in HB tumors.

In the methylation assay, 51 tumors (68.9%) were classified as having at least one methylated gene among the nine genes examined, and there was a positive correlation between the number of the methylated genes and age at diagnosis (Fig. S3). Notably, Kaplan–Meier curves for OS and EFS showed that tumors in which GPR180, MST1R, OCIAD2, and PARP6 were methylated were significantly associated with poorer OS (Fig. 2) and poorer EFS (Fig. S4). Moreover, the percentage of patients who died increased stepwise as the number of genes identified as methylated increased (Fig. 3a). As the Kaplan–Meier OS curve in Figure 3(b) shows, patients who had tumors in which more than four of the nine genes were methylated had a significantly poorer prognosis than those who had tumors with fewer methylated genes.

**Association between clinicopathological factors and methylation status of GPR180, MST1R, OCIAD2, and PARP6.** When clinical and tumor-associated factors were evaluated using univariate analysis, age at diagnosis and the presence of metastatic tumors were found to be significantly associated with the OS and EFS rate (Table S5). When evaluating the association between clinical parameters and the methylation status of GPR180, MST1R, OCIAD2, and PARP6, age at diagnosis and the presence of metastatic tumors or hepatic vein invasion were significantly associated with increase of methylation of 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 bronchioalveolar 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...
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/§, † n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADM2</td>
<td>Cell adhesion molecule</td>
<td>3p12</td>
<td>Cell adhesion</td>
<td>Tumor, n = 74: 8.78 ± 8.91</td>
<td>Normal liver, n = 4: 6.87 ± 2.87</td>
<td>5.26 (0.669)</td>
</tr>
<tr>
<td>CAMTA1</td>
<td>Calmodulin binding</td>
<td>1p36</td>
<td>Transcriptional factor</td>
<td>5.22 ± 1.27 (50)</td>
<td>5.20 (0.710)</td>
<td>–</td>
</tr>
<tr>
<td>CCDC8</td>
<td>CCDC8 coiled-coil</td>
<td>19q13</td>
<td>Apoptosis</td>
<td>27.18 ± 22.38 (74)</td>
<td>8.19 ± 1.20</td>
<td>34.8 (0.592) 27 (35.5)</td>
</tr>
<tr>
<td>CRB3</td>
<td>Crumbs homolog 3</td>
<td>19p13</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</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</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) 19 (25.7)</td>
</tr>
<tr>
<td>MDU1</td>
<td>Mannose-P-dolichol</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) 16 (21.6)</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) 2 (2.7)</td>
</tr>
<tr>
<td>OCIAD2</td>
<td>OCIA domain containing 2</td>
<td>4p11</td>
<td>Unknown</td>
<td>15.15 ± 16.57 (74)</td>
<td>10.62 ± 5.97</td>
<td>34.3 (0.736) 13 (17.6)</td>
</tr>
<tr>
<td>PARP6</td>
<td>Poly (ADP-ribose) polymerase family</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) 23 (31.1)</td>
</tr>
<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>
</tr>
<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) 27 (36.5)</td>
</tr>
<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) 13 (17.6)</td>
</tr>
<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) 25 (33.8)</td>
</tr>
<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>
</tr>
</tbody>
</table>

†Area under the receiver-operator curve analysis of overall survival establishing the cut-off value for each gene. †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 difference in the methylation level between the tumor and normal liver tissues. §CAMTA1, EML1, FZD8, and NEFH were determined not to be aberrantly hypermethylated because the mean methylation level in normal liver tissues was greater than that in tumor tissues. –, none (zero).

methylation of the regions we examined has negative correlation 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 coexisting in the same gene.

GPR180 is known to be a G protein-coupled receptor produced 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 significantly reduced cell viability. These findings also seem contradictory to the observation in HB that GPR180 methylation is associated with a poor outcome. Identification of 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

Fig. 2. Kaplan–Meier curves for overall survival for the nine genes showing increase of methylation in 74 hepatoblastoma tumors. Blue line, unmethylated group (U); red line, methylated group (M).

Fig. 3. (a) Histogram showing that the percentage of patients with hepatoblastoma who died increased stepwise as the number of methylated genes among the identified nine genes increased. The dotted line indicates a cut-off value for classification in (b). (b) Kaplan–Meier curve of overall survival showing the association between tumors in which four or more of the nine genes are methylated and a poor prognosis.
RASSF1A methylation correlates with different histological progression of HB remain unknown. We previously reported that CIMP was not selected as a molecular marker because its expressions in HuH6 and HepG2 cells were restored only by 1.6 and 1.8 times after 5-aza-dC treatment, respectively. In contrast, the genome-wide methylation analysis detected that all four probes located in the promoter of a CIMP, \( n = 13 \), showed more than 3.7-fold differences in their methylation level.

The epigenetic alterations contributing to the malignant progression of HB remain unknown. We previously reported that RASSF1A methylation correlates with different histological phenotypes and may be a promising molecular-genetic marker predictive of treatment outcome in HB patients. It suggests hypermethylation of some critical genes may drive changes in the phenotype of HB cells, resulting in acquisition of aggressive characteristics. Cairo et al. identified a 16-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.

In addition to their pathogenic implications, 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 can then be used to identify new candidate biomarkers for use in making diagnoses and determining prognosis. Moreover, analysis of DNA methylation using pyrosequencing is both highly quantitative and reproducible. It may therefore be possible for hospital laboratories to use this technique as a diagnostic tool and for risk assessment in HB. Pyrosequencing combined with pretreatment biopsies may enable evaluation of the risk of HB progression, and could be of great help for determining the appropriate therapeutic approach.
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; K. Watanebe; S. Kondo; T. Oue; M. Yano; and T. Tajiri), the JPLT pathological committee members (Drs. H. Horie; Y. Tanaka; 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 Program 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

19 Tuncel H, Tanaka S, Oka S et al. PARP6, a mono(ADP-ribosyl) transferase and a negative regulator of cell proliferation, is involved in colorectal cancer development. *Int J Oncol* 2012; 41: 2079–86.
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.