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**Author(s):** 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

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

Shohei Honda, Masashi Minato, Hiromu Suzuki, Masato Fujiyoshi, Hisayuki Miyagi, Masayuki Haruta, Yasuhiro Kaneko, Kanako C. Hatanaka, Eiso Hiyama, Takehiko Kamijo, Tadao Okada and Akinobu Taketomi

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 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-adriamycin. 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 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 $\beta$-values in normal liver specimens were more than 0.2. We then screened for probes that showed more than a twofold difference in their $\beta$-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 $\mu$M 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 RNeasy 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 bisulfite-treated 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 mmol/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, or 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, or 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 $\beta$-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 95 genes, we selected 19 determined to be aberrantly hypermethylated in various types of cancer, or to be associated with cancer development, based the findings of a PubMed search using the search terms “cancer” [All Fields] OR “methylation” [All Fields] (Table 2).

**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′-deoxycitidine (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).

### 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
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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

| Gene symbol | Full name | Gene location | Function | Methylation level, %, mean ± SD | Cut-off value, % (AUC†) | Number of tumors with methylated gene§, ‡, n (%) |
|-------------|-----------|---------------|----------|---------------------------------|-------------------------|---------------------------------------------|
| CADM2       | Cell adhesion molecule 2 | 3p12 | Cell adhesion | 8.78 ± 8.91 (74) 6.87 ± 2.87 | 5.26 (0.669) | – |
| CAMTA1      | Calmodulin binding transcription activator 1 | 1p36 | Transcriptional factor | 5.22 ± 1.27 (50) 6.42 ± 0.12 | 5.20 (0.710) | – § |
| CCDC8       | CCDC8 coiled-coil domain containing 8 | 19q13 | Apoptosis | 27.18 ± 22.38 (74) 8.19 ± 1.20 | 34.8 (0.592) | 27 (35.5) |
| CRB3        | Crumbs homolog 3 | 19p13 | Cell adhesion | 4.51 ± 1.30 (74) 4.22 ± 0.75 | 3.93 (0.576) | – |
| EHD1        | Echinoderm microtubule associated protein like 1 | 14q32 | Microtubule | 5.28 ± 1.27 (74) 5.49 ± 0.98 | 4.11 (0.510) | – § |
| FZD8        | Frizzled family receptor 8 | 10p11 | Wnt signaling | 4.87 ± 1.94 (48) 6.02 ± 0.57 | 6.01 (0.525) | – § |
| GPR180      | G protein-coupled receptor 180 | 13q32 | Signal transduction | 5.28 ± 11.72 (74) 0.00 ± 0.00 | 4.11 (0.796) | 19 (25.7) |
| MDU1        | Mannose-P-dolichol utilization defect 1 | 17p13 | Glucosylation | 1.49 ± 1.24 (74) 0.62 ± 0.71 | 1.33 (0.580) | – |
| MST1R       | Macrophage stimulating 1 receptor | 3p21 | Tyrosine kinase | 14.53 ± 14.89 (74) 5.42 ± 2.21 | 20.8 (0.690) | 16 (21.6) |
| NEFH        | Neurofilament, heavy polypeptide | 22q12 | Neurofilament | 11.11 ± 4.00 (74) 12.02 ± 0.95 | 20.0 (0.425) | – § |
| NNR1        | Neurtin 1 | 6p25 | Neuritogenesis | 9.31 ± 7.83 (74) 6.07 ± 1.66 | 44.4 (0.425) | 2 (2.7) |
| OCIAD2      | OCIA domain containing 2 | 4p11 | Unknown | 15.15 ± 16.57 (74) 10.62 ± 5.97 | 34.3 (0.736) | 13 (17.6) |
| PARP6       | Poly (ADP-ribose) polymerase family, member 6 | 15q23 | ADP-ribose transferase | 12.89 ± 14.19 (74) 2.72 ± 3.60 | 8.09 (0.786) | 23 (31.1) |
| PON3        | Paraoxonase 3 | 7q21 | Lipoprotein metabolism | 6.34 ± 11.28 (74) 5.90 ± 4.62 | 4.25 (0.515) | – |
| RAPGEF3     | Rap guanine nucleotide exchange factor (GEF) 3 | 12q13 | Inhibition of MAPK | 3.46 ± 1.45 (50) 1.46 ± 1.77 | 3.21 (0.674) | – |
| VIM         | Vimentin | 10p13 | Cell adhesion | 12.72 ± 14.32 (74) 5.01 ± 1.01 | 9.12 (0.588) | 27 (36.5) |
| ZAR1        | Zygote arrest 1 | 4p11 | Unknown | 12.99 ± 10.39 (74) 11.19 ± 0.37 | 25.2 (0.426) | 13 (17.6) |
| ZC3H13      | Zinc finger CCCH-type containing 13 | 13q14 | Unknown | 15.40 ± 18.43 (74) 3.42 ± 0.68 | 13.2 (0.453) | 25 (33.8) |
| ZMYND10     | Zinc finger, MYND-type containing 10 | 3p21 | Unknown | 4.39 ± 5.11 (74) 0.91 ± 1.41 | 1.99 (0.683) | – |

†Area under the receiver-operator curve analysis of overall survival establishing the cut-off value for each gene. ‡Aberant 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).

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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 \textit{MST1R} and \textit{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.\textsuperscript{(26)} The degree of aberrant methylation in normal-appearing tissues (epigenetic field defect) correlates with the risk of cancer development.\textsuperscript{(26)} Given that most HB patients are diagnosed before the age of 2 years, it seems unlikely that such accumulation contributes greatly to
RASSF1A methylation correlates with different histological 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. This suggests hypermethylation of some critical genes may drive changes in the phenotype of HB cells, resulting in acquisition of aggressive characteristics. Cairo et al. (31) 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. (32) In this study, RASSF1A 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 region of RASSF1A showed more than 3.7-fold differences in their methylation levels between tumors and normal liver tissues, proving that genome-wide methylation assay can be a reliable tool for screening. 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 intervention.

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. This suggests hypermethylation of some critical genes may drive changes in the phenotype of HB cells, resulting in acquisition of aggressive characteristics. Cairo et al. (31) 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.

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

| Gene     | Methylation status | M (n = 19) | U (n = 55) | P-value† | M (n = 16) | U (n = 58) | P-value† | M (n = 13) | U (n = 61) | P-value† | M (n = 23) | U (n = 51) | P-value† |
|----------|--------------------|-----------|-----------|----------|-----------|-----------|----------|-----------|-----------|----------|-----------|-----------|----------|
| GPR180   | M                  | 11        | 34        | 0.790    | 8         | 37        | 0.389    | 8         | 37        | 1.000    | 11        | 34        | 0.198    |
|          | U                  | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| MST1R    | M                  | 8         | 21        | 0.008    | 8         | 21        | 0.002    | 13         | 19        | 0.287    | 2         | 3         | 0.782    |
|          | U                  | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| OCIAD2   | M                  | 18        | 34        | 0.005    | 11         | 52        | 0.052    | 7         | 56        | 0.003    | 16        | 47        | 0.029    |
|          | U                  | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| PARP6    | M                  | 12        | 51        | 0.005    | 11         | 52        | 0.052    | 7         | 56        | 0.003    | 16        | 47        | 0.029    |
|          | U                  | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| Rupture  | No                 | 7         | 4         | 6         | 6         | 7         | 56       | 6         | 5         | 7         | 4         |          |
|          | Yes                | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| Metastasis| No                | 17        | 52        | 0.598    | 15         | 54        | 1.000    | 12         | 57        | 1.000    | 22        | 47        | 1.000    |
|          | Yes                | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| Hepatic vein invasion| No               | 16        | 54        | 0.050    | 15         | 55        | 1.000    | 12         | 58        | 0.547    | 20        | 50        | 0.086    |
|          | Yes                | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
| Histological type| Fetal              | 5         | 23        | 0.299    | 8         | 20        | 0.146    | 4         | 24        | 0.216    | 9         | 19        | 0.236    |
|          | Combined fetal/  | (n = 19)  | (n = 55)  |          | (n = 16)  | (n = 58)  |          | (n = 13)  | (n = 61)  |          | (n = 23)  | (n = 51)  |          |
|          | embryonal         | 12        | 28        | 0.133    | 7         | 33        | 0.077    | 8         | 32        | 0.216    | 13        | 27        |          |

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

Table 3. Multivariate analysis of values that are predictive of event-free survival in 74 hepatoblastoma patients

|                          | P-value | Hazard ratio (95% CI) |
|--------------------------|---------|----------------------|
| GPR180 methylation level | ≥2.6%   | 0.0224               |
| MST1R methylation level  | ≥20.5%  | 0.6050               |
| OCIAD2 methylation level | ≥34.3%  | 0.4534               |
| PARP6 methylation level  | ≥8.0%   | 0.2589               |
| Age at diagnosis         | >1 year | 0.7370               |
| Metastatic disease       | Present | 0.0099               |

Cl, confidence interval.
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.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

5-aza-dc: 5-aza-2'-deoxycytidine
CIMP: CpG island methylator phenotype
DFS: disease-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

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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′-deoxycitidine (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′-deoxycitidine (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.