A novel risk stratification model based on the Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification and deoxyribonucleic acid methylation analysis for hepatoblastoma

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Abstract

Introduction: Hepatoblastoma (HB) is the most common pediatric liver tumor, and epigenetic aberrations may be important in HB development. Recently, the Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification (CHIC-HS) developed risk stratification based on clinicopathological factors. This study aimed to construct a more accurate model by integrating CHIC-HS with molecular factors based on DNA methylation.

Methods: HB tumor specimens (N=132) from patients treated with the Japanese Pediatric Liver Tumors Group-2 protocol were collected and subjected to methylation analysis by bisulfite pyrosequencing. Associations between methylation status and clinicopathological factors, overall survival (OS), and event-free survival (EFS) were retrospectively analyzed. We investigated the effectiveness of the evaluation of methylation status in each CHIC-HS risk group and generated a new risk stratification model.

Results: Most specimens (82%) were from post-chemotherapy tissue. Hypermethylation in ≥2 of the four genes (*RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R*) was significantly associated with poorer OS and EFS. Multivariate analysis indicated that ≥2 methylated genes was an independent prognostic factor (hazard ratios of 6.014 and 3.684 for OS and EFS, respectively). Two or more methylated genes was also associated with poorer OS in the CHIC-very low (VL)-/low (L)-risk and CHIC-intermediate (I) risk groups (3-year OS rates were 83% vs. 98% and 50% vs. 95%, respectively). The 3-year OS rates of the VL/L, I, and high-risk groups in the new stratification model were 98%, 90%, and 62% (vs. CHIC-HS [96%, 82%, and 65%, respectively]), optimizing CHIC-HS.

Conclusions: Our proposed stratification system considers individual risk in HB and may improve patient clinical management.

Keywords: Hepatoblastoma, CHIC, DNA methylation, Biomarker, Risk stratification

Introduction

Hepatoblastoma (HB) is the most common liver tumor in children, mostly occurring in those <3 years old. Its annual incidence is 1.5 cases per million [1]. HB treatment comprises stratification based on clinicopathological factors, surgery for complete resection, and cisplatin-based chemotherapy [2]. To date, four study groups, namely, the Children's Oncology Group, International Childhood Liver Tumors Strategy Group, Society for Pediatric Oncology and Hematology, and Japanese Pediatric Liver Tumors Group (JPLT), have played a central role in conducting clinical studies according to individual stratification based on clinicopathological factors, such as the PRETreatment EXTent of disease (PRETEXT) group, metastatic disease, serum alpha-fetoprotein (AFP) levels, and treatment regimens [3–6]. In these studies, the overall survival (OS) was approximately 80%. However, the prognosis of high-grade malignant cases, such as metastatic cases, remains poor, and long-term toxicity associated with chemotherapy remains a serious challenge [2,7]. Therefore, the importance of providing treatment without excesses and deficiencies for properly stratified patients is increasing. Thus, the Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification (CHIC-HS), a new international stratification system that integrates various clinicopathological factors, has been recently established based on a large-scale CHIC database (N=1,605), and the Pediatric Hepatic International Tumor Trial using CHIC-HS is currently underway [8].

The stratification of high-grade malignant cases by molecular markers has been conducted through comprehensive expression analysis [9–13]. HB is a tumor with a few mutations (2.9–3.9 mutations/tumor) [11,14]; hence, epigenetic alterations play an important role in HB development. We have particularly focused on aberrant DNA methylation and speculated that the silencing of tumor suppressor genes due to DNA hypermethylation increases the malignancy of HB. Previously, we have revealed that the methylation status of *RASSF1A*, *PARP6*, *OCIAD2*, *MST1R*, and *GPR180* is useful for the prognostic stratification of HB [15–17]. This study aimed to establish a more accurate stratification model by integrating CHIC-HS with molecular factors based on DNA methylation

analysis in a large Japanese cohort.

Material and Methods

Patients and samples

Genomic DNA was extracted from freshly frozen HB tumor tissues from 132 patients provided by the JPLT. All patients underwent hepatectomy and pre- and/or postoperative chemotherapy in the JPLT institutions between 1999 and 2012 according to the treatment regimens of the JPLT-2 [6]. The specimens obtained from resection after preoperative chemotherapy were used for DNA extraction in all cases except those that were resectable at diagnosis. Clinicopathological factors, such as age at diagnosis, sex, AFP levels at diagnosis, PRETEXT, annotation factors, histology, and survival information, were obtained from the JPLT database retrospectively. Annotation factors were evaluated according to the criteria at the time of registration. The study protocol was approved by the ethics committee of our institution. Informed consent was obtained from all patients by local physicians at the participating institutions.

Bisulfite pyrosequencing

Methylation status was examined using bisulfite pyrosequencing as described previously [18]. The polymerase chain reaction and sequencing primers have been described previously [16,17]. Genomic DNA (500 ng) was modified with sodium bisulfite using an EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany). Reactions were performed on a PSQ96MA system (Biotage, Uppsala, Sweden). The methylation rate (%) of each gene was defined as the average value of methylation levels at each CpG site included in the sequences analyzed by Pyro Q-CpG software (Biotage, Uppsala, Sweden).

Statistical analysis

Statistical analysis and data visualization were performed using GraphPad Prism 9 (GraphPad

Software, San Diego, CA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria) [19]. Receiver operating characteristic (ROC) analysis was used to determine adequate cutoff values for the methylation rate to predict patient survival. OS was defined as the time from the date of diagnosis to the date of death or last follow-up. Event-free survival (EFS) was defined as the time from the date of diagnosis to the date of first relapse, death, diagnosis of secondary cancer, or the last follow-up, whichever occurred first. The Kaplan–Meier method was used to construct the OS and EFS curves and determine the estimated 3-year OS and EFS. The log-rank test was used to compare the OS and EFS curves. The association between methylation status and clinicopathological factors was analyzed using Fisher's exact test. Multivariate analysis of the association between methylation status and clinicopathological factors found to be significant in the univariate analysis and survival time was performed using Cox proportional hazards model. $P<0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 132 patients were included in this study, with a median age of 18 months (range, 0–177) (Table 1). These patients represented 37% of cases enrolled in JPLT-2. There were no significant differences in patient charactersitics between the cohort of this study and overall JPLT-2 except for venous invasion and portal invasion [7].

Table 1. Clinicopathological factors in patients with hepatoblastoma (N=132).

	Total (n=132)
Age, months (median, [range])	18 [0–177]
Age group	

<3 years	105
3–7 years	19
≥8 years	8
Sex (male/female)	76/56
Serum AFP, ng/mL (median, [range])	266,000 [110–7,653,000]
Serum AFP group	
≤100 ng/mL	0
101–1,000 ng/mL	5
>1,000 ng/mL	116
NA	11
Clinical classification: CHIC-HS (VL/L/I/H)	17/57/29/29
Preoperative chemotherapy (Y/N, %)	108/24 (82%)
Tumor characteristics	
PRETEXT stage (I/II/III/IV)	13/44/50/25
Venous invasion (Y/N, %)	11/121 (9%)
Portal invasion (Y/N, %)	6/126 (5%)
Extrahepatic extension (Y/N, %)	2/130 (2%)
Multifocality (Y/N, %)	19/113 (14%)
Rupture (Y/N, %)	12/120 (9%)
Metastasis at diagnosis (Y/N, %)	20/112 (15%)
Histology	
Fetal/embryonal	23/11
Mixed epithelial/mixed epithelial and mesenchymal/NA	43/16/39

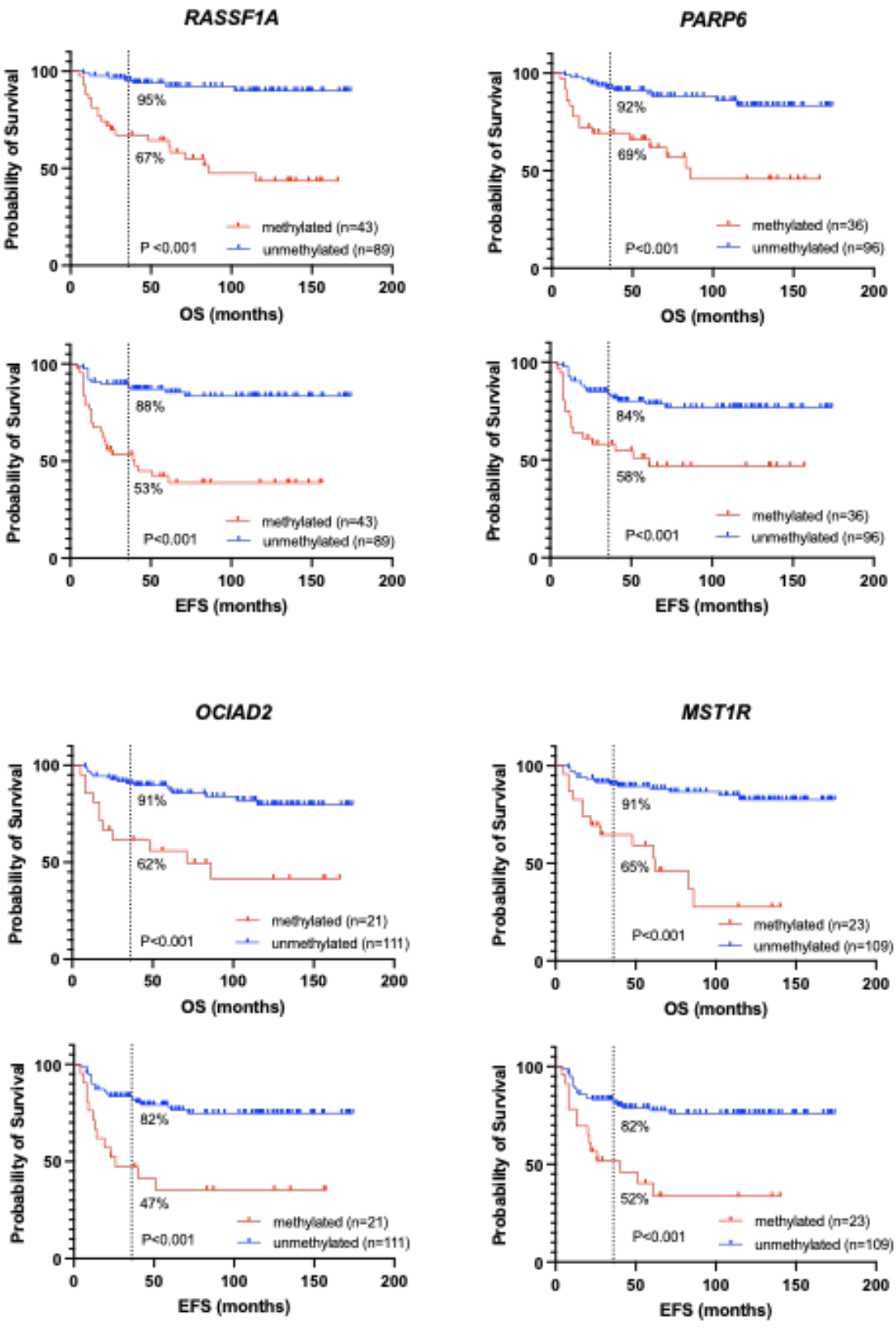
Follow-up, months (median, [range])	68 [0–174]
Outcome	
Deaths (Y/N, %)	29/103 (22%)
Cancer-related deaths or tumor recurrence (Y/N, %)	39/93 (30%)

AFP, alpha-fetoprotein; NA, non-available; CHIC-HS, Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification (VL, very low; L, low; I, intermediate; H, high risk); PRETEXT, PRETreatment EXTent of disease.

The patients were classified into risk groups according to CHIC-HS, with 17, 57, 29, and 29 in the very low-risk, low-risk, intermediate-risk, and high-risk groups, respectively (Table 1). The 3-year OS and EFS rates of each risk group were 100%, 94.7%, 81.8%, and 65.0% (P=0.001) and 93.8%, 83.8%, 78.6%, and 51.7% (P=0.004), respectively.

Association between clinicopathological factors and methylation status

Bisulfite pyrosequencing results revealed the following median methylation rates of *RASSF1A*, *PARP6*, *OCIAD2*, *MST1R*, and *GPR180*: 13.42% (range, 1.97–84.35%), 7.01% (0–65.38%), 5.91% (0–82.16%), 7.73% (0–81.62%), and 1.61% (0–64.37%), respectively (Supplementary Fig. 1). ROC analysis (Supplementary Fig. 2) determined the following cutoff values of *RASSF1A*, *PARP6*, *OCIAD2,* and *MST1R*: 31.77, 9.93, 34.33, and 32.94, respectively. The cutoff value of *GPR180* was 2.34, which was significantly low that it was within the error range of the pyrosequencer [20]. Thus, *GPR180* was excluded from this study. According to the log-rank test, hypermethylation of *RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R* was a significant poor prognostic factor for OS and EFS (Fig. 1).



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148 Fig. 1 Kaplan–Meier curves for overall survival (OS) (upper panel) and event-free survival (EFS)

(lower panel) of 132 patients with hepatoblastoma classified by the methylation status of four genes (*RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R*). The blue line indicates the unmethylated group, and the red line indicates the methylated group. The 3-year (dashed line) OS and EFS are shown on the side of the survival curve. The log-rank test was performed to compare the OS and EFS curves.

The association between the methylated or unmethylated groups of the four genes and the clinicopathological factors is shown in Table 2.

Table 2. Association between the methylation status of four genes and clinicopathological factors in patients with hepatoblastoma (N=132)

		RASSF1A		*P*-value	***MST1R***		*P*-value	***OCIAD2***		*P*-value	***PARP6***		*P*-value
		M n=43	U n=89		M n=23	U n=109		M n=21	U n=111		M n=36	U n=96	
Age group (years)	0-2	20	85	<0.001	8	97	<0.001	10	95	<0.001	17	88	<0.001
	3-7	16	3		9	10		9	10		14	5	
	≥8	7	1		6	2		2	6		5	3	
Sex	M	22	54	0.349	9	67	0.064	12	64	1	16	60	0.085
	F	21	35		14	42		9	47		20	36	
Serum AFP ≥1,000,000 ng/mL	Y	7	15	0.808	3	19	0.565	1	21	0.118	3	19	0.076
	N	36	63		20	79		20	79		33	66	
Preoperative	Y	37	71	0.470	17	91	0.370	18	90	0.768	28	80	0.450

chemotherapy	N	6	18	4	6	18	0	3	21	4	8	16	7
Tumor characteristics:													
PRETEXT IV (Y/N)	Y	14	11	0.00	7	18	0.14	4	21	1	10	15	0.13
	N	29	78	9	16	91	5	17	90		26	81	6
Venous invasion	Y	8	3	0.00	4	7	0.10	3	8	0.38	7	4	0.00
	N	35	86	5	19	102	0	18	103	1	29	92	9
Portal invasion	Y	4	2	0.08	3	3	0.06	1	5	1	4	2	0.04
	N	39	87	8	20	106	5	20	106		32	94	7
Extrahepatic extension	Y	2	0	0.10	0	2	1	0	2	1	1	1	0.47
	N	41	89	4	23	107		21	109		35	95	3
Multifocality	Y	10	9	0.06	5	14	0.32	4	15	0.50	9	10	0.04
	N	33	80	3	18	95	5	17	96	4	27	86	9
Rupture	Y	5	7	0.52	4	8	0.22	3	9	0.40	5	7	0.30
	N	38	82	5	19	101	2	18	102	5	31	89	7
Metastasis at diagnosis	Y	14	6	<0.01	5	15	0.34	7	13	0.01	8	12	0.18
	N	29	83		18	94	3	14	98	9	28	84	0
HB histology:													
Fetal		6	17		5	18		1	22		7	16	
Embryonal		4	7		2	9		2	9		4	7	
Mixed epithelial		16	27	0.51	9	34	0.59	8	35	0.42	12	31	0.77
Mixed epithelial and mesenchymal		3	13	6	16	15	7	28	14	5	3	13	7
NA		14	25		0	33		0	31		10	29	

P-values were calculated using Fisher's exact test.

AFP, alpha-fetoprotein; Y, yes; N, no; PRETEXT, PRETreatment EXTent of disease; NA, non-

161 available; M, methylated; U, unmethylated.

162

163 Methylation of the four genes was found in patients aged >3 years. *RASSF1A* methylation was
164 significantly higher in patients with PRETEXT IV, venous invasion, and metastasis at diagnosis.
165 Patients with methylated *PARP6* were predominantly found to have portal and venous invasion and
166 multifocality. Methylated *OCIAD2* was more frequently found in patients with metastasis at diagnosis
167 than in those who did not. The methylation status of any gene was not significantly associated with
168 pathological subtype (Table 2).

169

170 **Usefulness of assessing the number of methylated genes for predicting prognosis**

171 As the number of methylated genes in the four genes (*RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R*)
172 increased, both OS and EFS gradually worsened (Supplementary Fig. 3). We determined a cutoff value
173 of 2 from ROC analysis, and the patients who had ≥2 methylated genes showed a significantly poorer
174 prognosis in OS and EFS (Fig. 2A).

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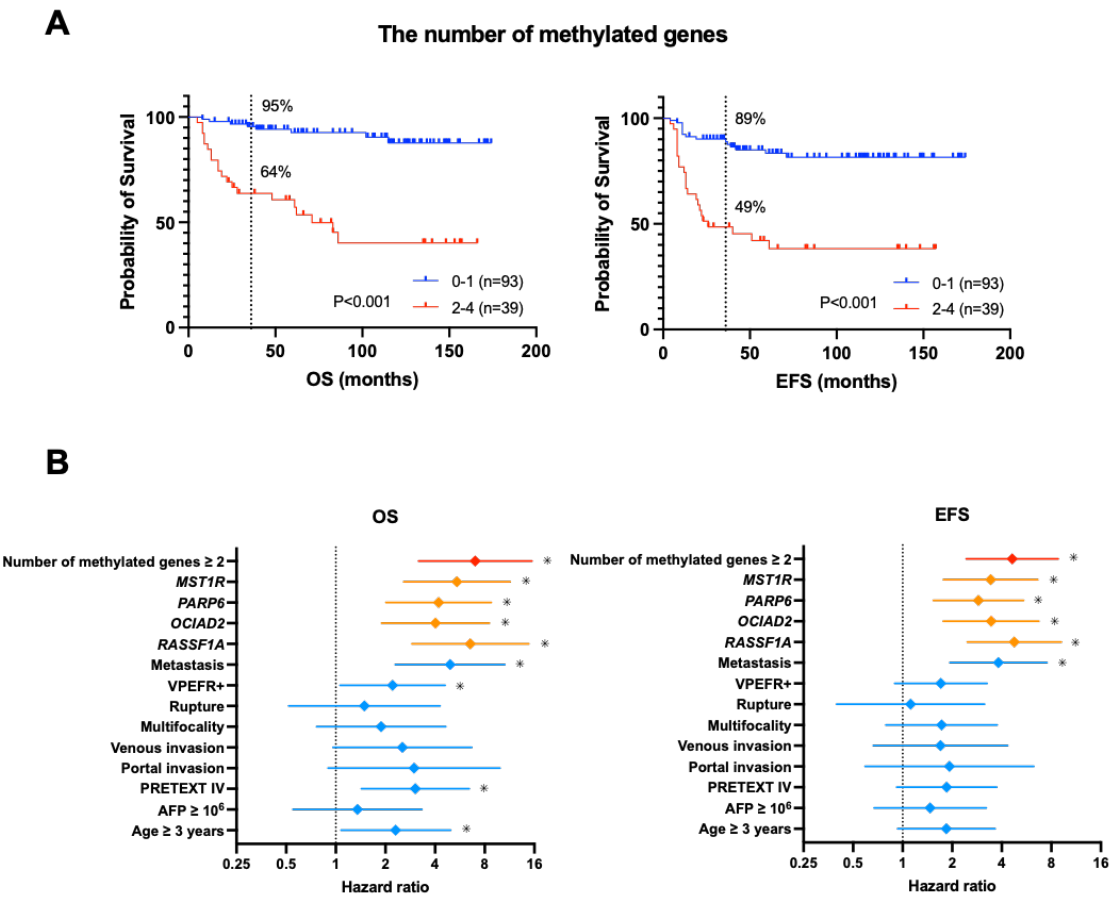


Fig. 2 A) Kaplan–Meier curves for overall survival (OS) (left panel) and event-free survival (EFS) (right panel) of 132 patients with hepatoblastoma (HB) classified by the number of the methylated genes. The blue line is the group with <2 methylated genes, and the red line is the group with ≥2 methylated genes. The 3-year (dashed line) OS and EFS are shown on the side of the survival curve. The log-rank test was performed to compare the OS and EFS curves. B) Forest plots of the hazard ratios of clinicopathological factors (blue) and molecular factors (red and orange) based on methylation analysis for OS (left panel) and EFS (right panel) according to univariate Cox hazard regression analysis. The diamonds represent hazard ratios, and the lines represent 95% confidence intervals. $^{*}P<0.05$.

According to univariate Cox proportional hazards regression analysis, the presence of ≥2

methylated genes had the highest hazard ratio for OS (mean, 7.005; range, 3.177–15.45; $P<0.001$) among the existing clinicopathological factors and methylation assessment of every single gene (Fig. 2B). Multivariate Cox proportional hazards regression analysis revealed that the number of methylated genes ≥2 is a significant independent prognostic factor for OS and EFS (Table 3).

Table 3. Multivariate Cox proportional hazards regression analysis for overall survival (OS) and event-free survival (EFS)

OS	HR	95% CI	*P*-value
Age ≥ 3 years	0.574	0.219–1.505	0.259
Metastasis	2.351	1.029–5.372	0.043
VPEFR+	1.249	0.504–3.098	0.631
PRETEXT IV	1.923	0.837–4.419	0124
Number of methylated genes ≥ 2	6.014	2.367–15.28	<0.001

EFS	HR	95% CI	*P*-value
Metastasis	2.212	1.070–4.574	0.032
Number of methylated genes ≥2	3.684	1.847–7.350	<0.001

HR, hazard ratio; CI, confidence interval; PRETEXT, PRETreatment EXTent of disease; VPEFR+, at least one of the PRETEXT annotation factors (involvement of hepatic vein, involvement of portal vein, extrahepatic tumor extension, multifocal liver tumor, and tumor rupture at diagnosis) was present.

Integration of CHIC-HS and DNA methylation analysis

Subgroup analysis revealed that in the CHIC-very low-/low-risk group, the patients who had ≥2 methylated genes had a significantly worse prognosis in OS (3-year OS: 98% vs. 83%, $P=0.011$) and EFS (3-year EFS: 90% vs. 66%, $P=0.0046$) (Fig. 3A).

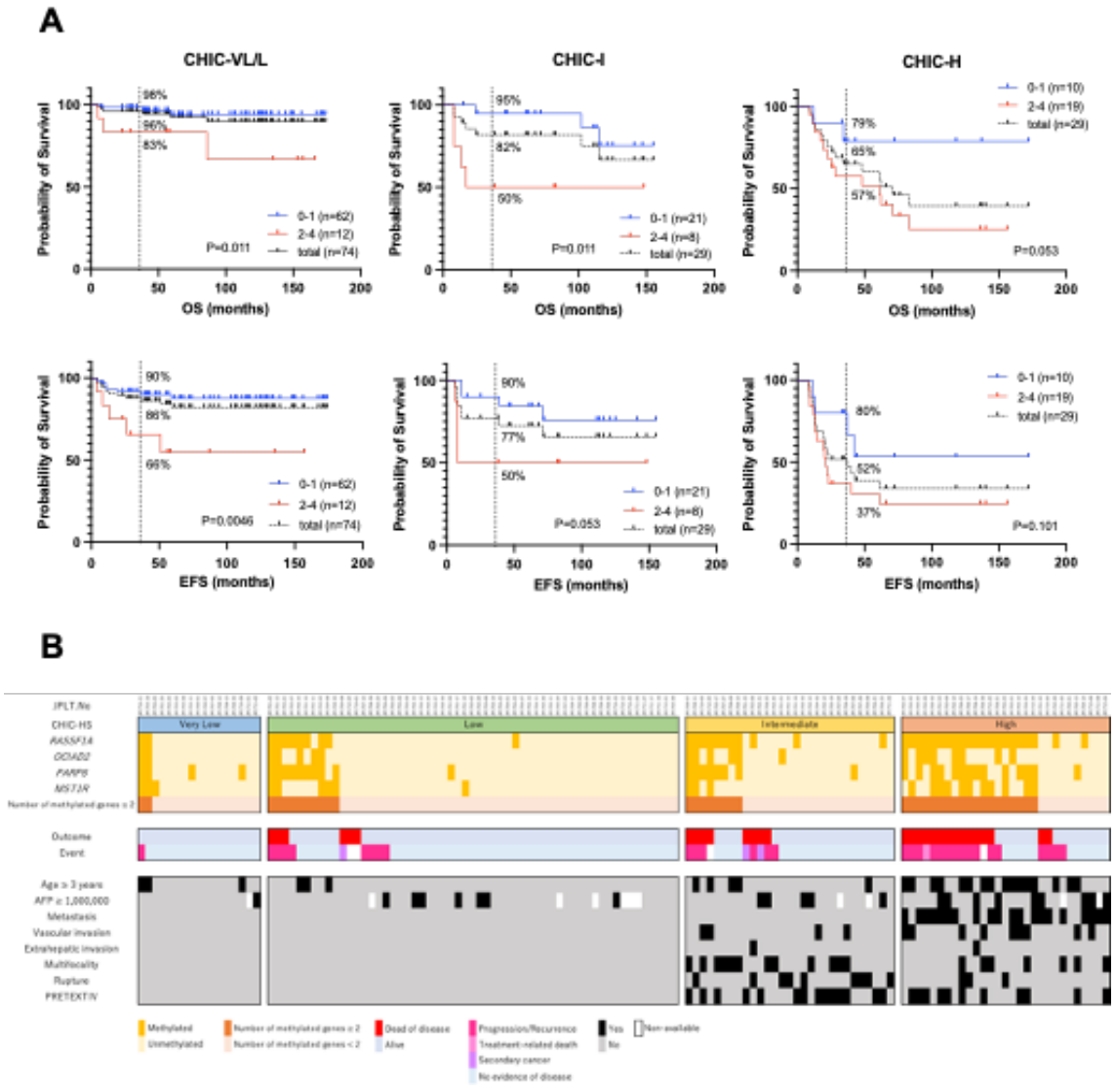
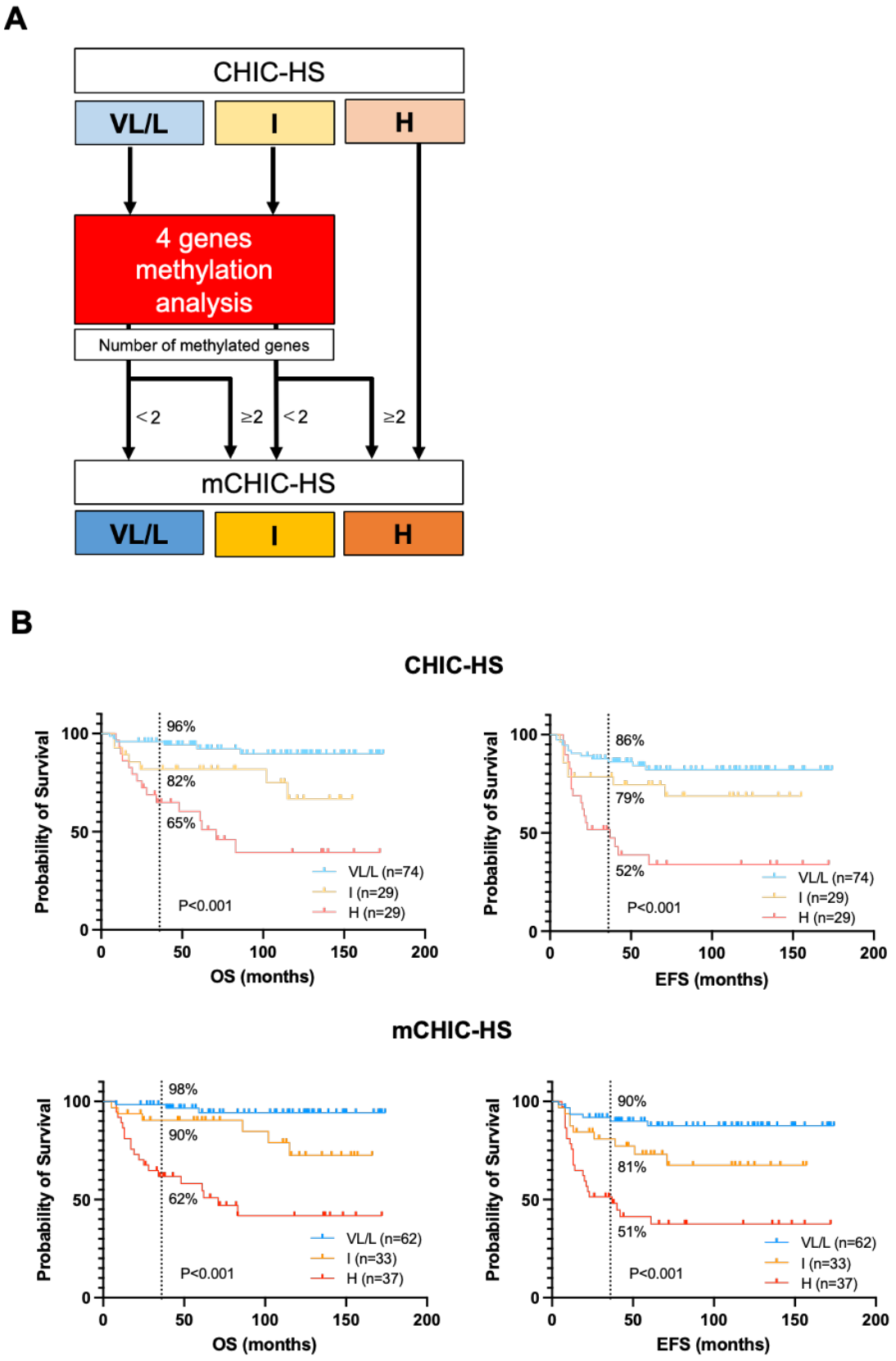


Fig. 3 A) Kaplan–Meier curves for overall survival (OS) (upper panel) and event-free survival (EFS) (lower panel) of 132 patients with hepatoblastoma (HB) classified by the number of the methylated genes in each Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification risk group. The blue line is the group with <2 methylated genes, and the red line is the group with ≥2 methylated genes. The 3-year (dashed line) OS and EFS are shown on the side of the survival curve. The log-rank test was performed to compare the OS and EFS curves. B) Distribution of the methylation status of four genes and clinicopathological factors in 132 patients with HB.

Patients who had ≥2 methylated genes in the intermediate-risk group had a significantly worse OS (3-year OS: 95% vs. 50%, *P*=0.011) and tended to have a worse EFS (3-year EFS: 90% vs. 50%, *P*=0.053) (Fig. 3A). In the CHIC-high-risk group, patients who had ≥2 methylated genes tended to have a worse OS (3-year OS: 79% vs. 57%, *P*=0.053) and EFS (3-year OS: 80% vs. 37%, *P*=0.101); however, the differences were not statistically significant (Fig. 3A). These findings suggest that the evaluation of the number of methylated genes in the four genes could optimize the stratification by CHIC-HS. The distribution of the methylation status of the four genes and clinicopathological factors are shown in Fig. 3B. This indicates that the evaluation of the methylation status of the four genes enabled us to select the patients with poor prognosis, whose prognosis was not appropriately predicted by the clinicopathological factors used to define the risk stratification in CHIC-HS. For example, the four patients from the leftmost column of the CHIC-low group had poor prognoses, even though they were stratified into the good prognosis group. However, in the methylation analysis, they were classified in the poor prognosis group with ≥2 methylated genes (Fig. 3B). Based on the new stratification system that integrates CHIC-HS and DNA methylation analysis data (mCHIC-HS), patients in the CHIC-very low-/low- and CHIC-intermediate-risk groups were reclassified according to the presence or absence of ≥2 methylated genes (Fig. 4A).



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232 Fig. 4 A) Risk stratification algorithms of methylation-based Children's Hepatic Tumors International

Collaboration-Hepatoblastoma Stratification (mCHIC-HS). Methylation analysis of four genes is performed in the CHIC-very low-/low- and intermediate-risk groups and those groups are reclassified according to the number of methylated genes. VL, very low; L, low; I, intermediate; H, high. B) Kaplan–Meier curves for overall survival (OS) (left panel) and event-free survival (EFS) (right panel) of 132 patients with hepatoblastoma stratified by CHIC-HS (upper panel) and mCHIC-HS (lower panel). The blue line is the very low-/low-risk group, the orange line is the intermediate-risk group, and the red line is the high-risk group. The 3-year (dashed line) OS and EFS are shown on the side of the survival curve. The log-rank test was performed to compare the OS and EFS curves.

The 3-year OS in the mCHIC-very low-/low-risk group increased from 96% to 98%, the number of patients decreased, and the population was redefined with a better prognosis (Fig. 4B). In contrast, the 3-year OS in the mCHIC-high-risk group decreased from 65% to 62%, the number of patients increased, and the population had a worse prognosis (Fig. 4B). The area under the ROC curve (AUC) values of mCHIC-HS for 3-year OS and EFS were 0.817 (95% CI: 0.725–0.908) and 0.731 (95% CI: 0.626–0.836), which were higher than those of CHIC-HS (AUC: 0.762 [95% CI: 0.649–0.876] and 0.687 [95% CI: 0.569–0.804], P=0.087 and 0.128, respectively); however, there was no significant difference.

Discussion

This study reconfirmed the usefulness of the methylation-based molecular prognostic markers we have previously identified in a large Japanese cohort and established a more precise stratification model by combining CHIC-HS with methylation analysis. Interestingly, the patients who had more methylated genes out of the four genes had poorer prognoses, and having ≥2 methylated genes was a significant poor prognostic factor identified in the multivariate Cox proportional hazard regression model. Patients who had methylated genes had a significantly poorer prognosis and were older. Aging is one

of the causes of inducing aberrant methylation [21]. We compared the methylation rates of the four genes in each age group and found that all genes, except *OCIAD2,* showed higher methylation rates in older patients (Supplementary Fig. 4). Age-related methylation may be the molecular background for age to be a clinically important prognostic factor in HB.

Recent studies have proposed prognostic models integrating CHIC-HS and the molecular prognostic factors for HB. Carrillo-Reixach et al. found that a population with overexpressed 14q32 genes of the *DLK1-DIO3* locus and a specific methylation status (Epigenetic-Cluster B: Epi-CB) had a poor prognosis through comprehensive analysis. They proposed molecular risk stratification (MRS-HB), a prognostic prediction model that classifies patients into three groups (MRS-1, MRS-2, and MRS-3) according to the combination of their presence or absence [22]. They combined CHIC-HS with MRS-HB to improve the ability to discriminate between low- and high-risk patients [22]. The Epi-CB group was characterized by the hypermethylation of CpG islands [22]. Since the four genes we examined in this study were also extracted as genes that show hypermethylation of the CpG islands in the promoter region [17], these combinations may reflect such methylation tendencies and function as prognostic factors. Cairo et al. presented a risk classification model based on the combination of CHIC-HS and 16-gene signature and reclassified the CHIC-intermediate-risk and CHIC-high-risk groups into intermediate-risk C1 (IR-C1) and high-risk C2 (HR-C2) according to the presence of either the C1 or C2 subtype of the 16-gene signature [13]. This model allows the identification of lower-risk patients from the high-risk group and could reduce unnecessary high-intensity treatment [13].

Herein, we propose a novel risk stratification model based on methylation analysis by bisulfite pyrosequencing methods called mCHIC-HS, which could optimize CHIC-HS. According to the CHIC-HS, which is solely based on clinical factors, there were patients in our cohort with poor prognoses who were incorrectly classified in the very low-/low-risk and would be treated insufficiently, despite biologically highly malignant tumors. However, by selecting these cases based on the methylation analysis of the four genes and redefining them as the mCHIC-intermediate-risk groups

283 according to our model, treatment of appropriate intensity can be provided to these patients. Similarly,
284 in the CHIC-intermediate-risk group, patients with a prognosis equivalent to the CHIC-high-risk group
285 could be selected by methylation analysis and redefined as the mCHIC-high-risk group. Compared
286 with previously proposed models, our model exhibits two major differences. First, it was based on the
287 evaluation of methylation rates using a pyrosequencer. Bisulfite pyrosequencing is a highly
288 quantitative and reproducible method; thus, it is reliable for clinical applications. Moreover, when
289 considering the clinical application of the integrated model and collection and analysis of samples at
290 a central facility, the extraction and analysis of DNA from the biopsy samples is advantageous, as
291 DNA is more stable than RNA. It is also feasible that the entry hurdle for introduction is lower than
292 the comprehensive analysis in terms of cost. Although the cost of comprehensive analysis is gradually
293 declining, it remains expensive. For the evaluation of four genes per sample, we estimate that
294 pyrosequencing is about 1 % of the the costs of comprehensive analysis. Therefore, processing large
295 numbers of samples is more economical. In addition, the small number of genes to be evaluated is also
296 an advantage. Second, our model focused on selecting higher-risk patients from the lower-risk group.
297 Cairo et al.'s model [13] did not stratify the CHIC-very low-/low-risk group. Therefore, a more useful
298 model may be obtained by combining the methylation analysis of the four genes with the expression
299 analysis of the 16 genes.
300 This study had some limitations. First, this was a retrospective study. Therefore, there is a difference
301 between the definition of the annotation factors collected by JPLT-2 and those adopted by CHIC [6,23].
302 For example, in the CHIC protocol, blood vessels encircled by tumors by >180° are considered
303 positive for vascular invasion; thus, some cases that were negative for vascular invasion in the JPLT-
304 2 may be considered positive by CHIC. Therefore, in this study, there is a possibility that the low-risk
305 group included cases that were originally in the intermediate-risk group. Second, 82% of the
306 specimens used in this analysis were modified with preoperative chemotherapy. Biopsy specimens
307 unaffected by chemotherapy will be used for analysis in clinical applications; thus, our results may

not be applicable directly. When applying this model to clinical practice, validation should be performed using specimens that have not been modified by chemotherapy. However, we found no significant difference in the methylation rates of the four genes between those who received preoperative chemotherapy and those who did not in this cohort (Supplementary Fig. 5). Furthermore, we validated whether the evaluation of the methylation status of the four genes could stratify the prognosis, using the results of methylation bead array using biopsy specimens before chemotherapy enrolled in JPLT-2 reported by Nagae et al [12]. The OS was significantly worse in the methylated group (Supplementary Fig. 6A). Using cases included both in this cohort and the study by Nagae et al. [12], we also assessed the correlation between the beta-value obtained from the biopsy specimens before chemotherapy and the methylation rate obtained from the specimens after chemotherapy. A high correlation was found in all four genes (Supplementary Fig. 6B). Therefore, it is expected that our model will also prove useful when validated with biopsy specimens before chemotherapy. Finally, a tailor-made therapy for specific pathways and molecules in each stratified population was not proposed; this would be addressed in the future.

Conclusions

We proposed a novel risk stratification model that integrates CHIC-HS with realistically feasible methylation analysis-based molecular prognostic markers to achieve more appropriate risk-adaptive therapy. We aim to conduct a prospective study using this model to verify its effectiveness in future trials.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors report no conflicts of intrest.

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