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1   A novel risk stratification model based on the Children's Hepatic Tumors International Collaboration-

2   Hepatoblastoma Stratification and deoxyribonucleic acid methylation analysis for hepatoblastoma

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29

33   **Abstract**

34   **Introduction:** Hepatoblastoma (HB) is the most common pediatric liver tumor, and epigenetic

35   aberrations may be important in HB development. Recently, the Children's Hepatic Tumors

36   International Collaboration-Hepatoblastoma Stratification (CHIC-HS) developed risk stratification

37   based on clinicopathological factors. This study aimed to construct a more accurate model by

38   integrating CHIC-HS with molecular factors based on DNA methylation.

39   **Methods:** HB tumor specimens (N=132) from patients treated with the Japanese Pediatric Liver

40   Tumors Group-2 protocol were collected and subjected to methylation analysis by bisulfite

41   pyrosequencing. Associations between methylation status and clinicopathological factors, overall

42   survival (OS), and event-free survival (EFS) were retrospectively analyzed. We investigated the

43   effectiveness of the evaluation of methylation status in each CHIC-HS risk group and generated a new

44   risk stratification model.

45   **Results:** Most specimens (82%) were from post-chemotherapy tissue. Hypermethylation in ≥ 2 of the

46   four genes (*RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R*) was significantly associated with poorer OS

47   and EFS. Multivariate analysis indicated that ≥ 2 methylated genes was an independent prognostic

48   factor (hazard ratios of 6.014 and 3.684 for OS and EFS, respectively). Two or more methylated genes

49   was also associated with poorer OS in the CHIC-very low (VL)-/low (L)-risk and CHIC-intermediate

50   (I) risk groups (3-year OS rates were 83% vs. 98% and 50% vs. 95%, respectively). The 3-year OS

51   rates of the VL/L, I, and high-risk groups in the new stratification model were 98%, 90%, and 62%

52   (vs. CHIC-HS [96%, 82%, and 65%, respectively]), optimizing CHIC-HS.

53   **Conclusions:** Our proposed stratification system considers individual risk in HB and may improve

54   patient clinical management.

55

56   **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

4

82   analysis in a large Japanese cohort.

83

84   **Material and Methods**

85   **Patients and samples**

86   Genomic DNA was extracted from freshly frozen HB tumor tissues from 132 patients provided by the

87   JPLT. All patients underwent hepatectomy and pre- and/or postoperative chemotherapy in the JPLT

88   institutions between 1999 and 2012 according to the treatment regimens of the JPLT-2 [6]. The

89   specimens obtained from resection after preoperative chemotherapy were used for DNA extraction in

90   all cases except those that were resectable at diagnosis. Clinicopathological factors, such as age at

91   diagnosis, sex, AFP levels at diagnosis, PRETEXT, annotation factors, histology, and survival

92   information, were obtained from the JPLT database retrospectively. Annotation factors were evaluated

93   according to the criteria at the time of registration. The study protocol was approved by the ethics

94   committee of our institution. Informed consent was obtained from all patients by local physicians at

95   the participating institutions.

96

97   **Bisulfite pyrosequencing**

98   Methylation status was examined using bisulfite pyrosequencing as described previously [18]. The

99   polymerase chain reaction and sequencing primers have been described previously [16,17]. Genomic

100   DNA (500 ng) was modified with sodium bisulfite using an EpiTect® Bisulfite Kit (Qiagen, Hilden,

101   Germany). Reactions were performed on a PSQ96MA system (Biotage, Uppsala, Sweden). The

102   methylation rate (%) of each gene was defined as the average value of methylation levels at each CpG

103   site included in the sequences analyzed by Pyro Q-CpG software (Biotage, Uppsala, Sweden).

104

105   **Statistical analysis**

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

5

107 Software, San Diego, CA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama,

108 Japan), a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria) [19].

109 Receiver operating characteristic (ROC) analysis was used to determine adequate cutoff values for the

110 methylation rate to predict patient survival. OS was defined as the time from the date of diagnosis to

111 the date of death or last follow-up. Event-free survival (EFS) was defined as the time from the date of

112 diagnosis to the date of first relapse, death, diagnosis of secondary cancer, or the last follow-up,

113 whichever occurred first. The Kaplan–Meier method was used to construct the OS and EFS curves

114 and determine the estimated 3-year OS and EFS. The log-rank test was used to compare the OS and

115 EFS curves. The association between methylation status and clinicopathological factors was analyzed

116 using Fisher's exact test. Multivariate analysis of the association between methylation status and

117 clinicopathological factors found to be significant in the univariate analysis and survival time was

118 performed using Cox proportional hazards model. $P<0.05$ was considered statistically significant.

119

120 **Results**

121 **Patient characteristics**

122 A total of 132 patients were included in this study, with a median age of 18 months (range, 0–177)

123 (Table 1). These patients represented 37% of cases enrolled in JPLT-2. There were no significant

124 differences in patient charactersitics between the cohort of this study and overall JPLT-2 except for

125 venous invasion and portal invasion [7].

126

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

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

6

<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%)

128 AFP, alpha-fetoprotein; NA, non-available; CHIC-HS, Children's Hepatic Tumors International

129 Collaboration-Hepatoblastoma Stratification (VL, very low; L, low; I, intermediate; H, high risk);

130 PRETEXT, PRETreatment EXTent of disease.

131

132 The patients were classified into risk groups according to CHIC-HS, with 17, 57, 29, and 29 in the

133 very low-risk, low-risk, intermediate-risk, and high-risk groups, respectively (Table 1). The 3-year OS

134 and EFS rates of each risk group were 100%, 94.7%, 81.8%, and 65.0% (*P*=0.001) and 93.8%, 83.8%,

135 78.6%, and 51.7% (*P*=0.004), respectively.

136

137 **Association between clinicopathological factors and methylation status**

138 Bisulfite pyrosequencing results revealed the following median methylation rates of *RASSF1A*, *PARP6*,

139 *OCIAD2*, *MST1R*, and *GPR180*: 13.42% (range, 1.97–84.35%), 7.01% (0–65.38%), 5.91% (0–

140 82.16%), 7.73% (0–81.62%), and 1.61% (0–64.37%), respectively (Supplementary Fig. 1). ROC

141 analysis (Supplementary Fig. 2) determined the following cutoff values of *RASSF1A*, *PARP6*, *OCIAD2,*

142 and *MST1R*: 31.77, 9.93, 34.33, and 32.94, respectively. The cutoff value of *GPR180* was 2.34, which

143 was significantly low that it was within the error range of the pyrosequencer [20]. Thus, *GPR180* was

144 excluded from this study. According to the log-rank test, hypermethylation of *RASSF1A*, *PARP6*,

145 *OCIAD2*, and *MST1R* was a significant poor prognostic factor for OS and EFS (Fig. 1).

146

RASSF1A

PARP6

OCIAD2

MST1R
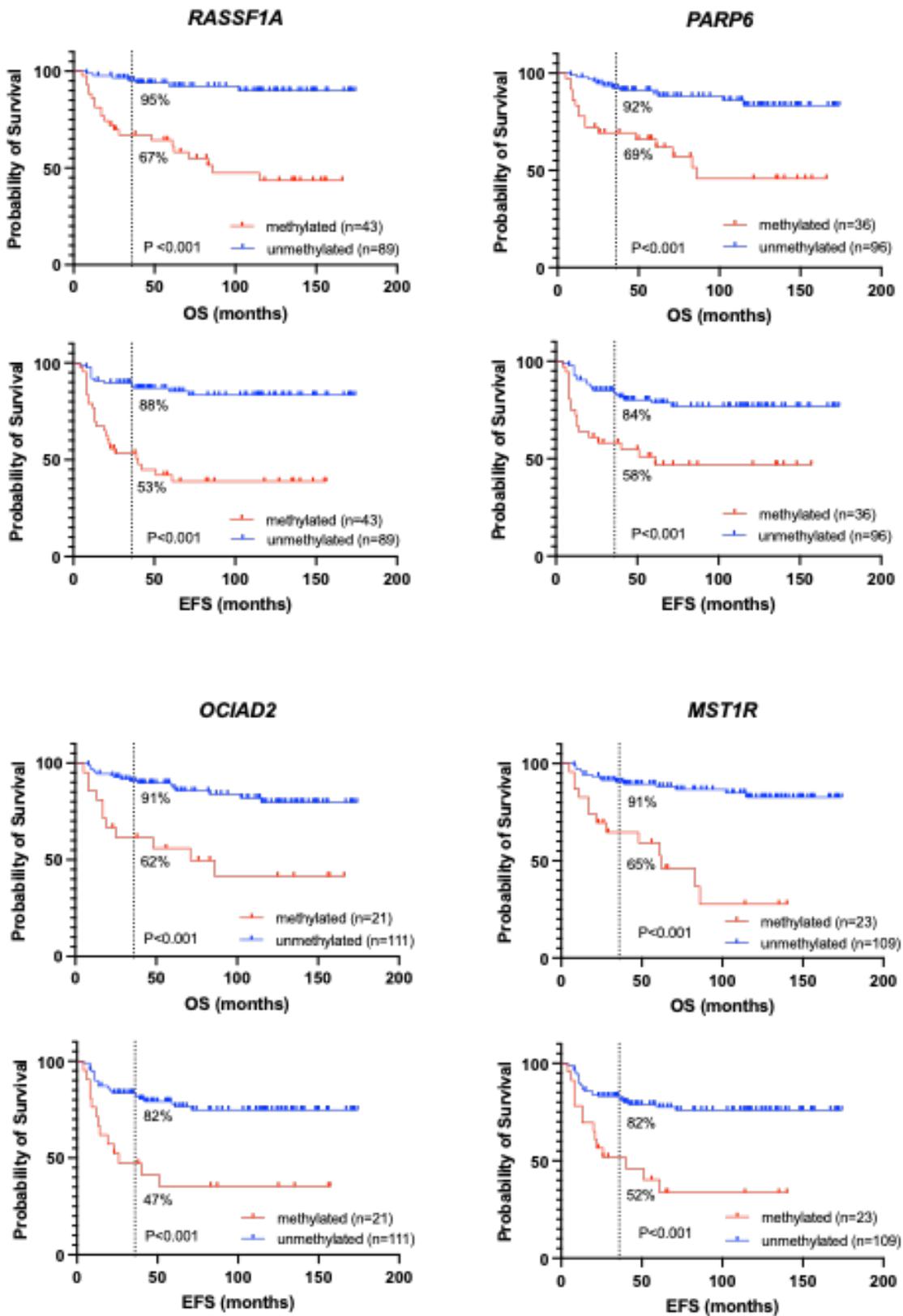
147

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

9

149 (lower panel) of 132 patients with hepatoblastoma classified by the methylation status of four genes

150 (*RASSF1A*, *PARP6*, *OCIAD2*, and *MST1R*). The blue line indicates the unmethylated group, and the

151 red line indicates the methylated group. The 3-year (dashed line) OS and EFS are shown on the side

152 of the survival curve. The log-rank test was performed to compare the OS and EFS curves.

153

154   The association between the methylated or unmethylated groups of the four genes and the

155 clinicopathological factors is shown in Table 2.

156

157 Table 2. Association between the methylation status of four genes and clinicopathological factors in

158 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	0.11	3	19	0.076
	N	36	63		20	79		20	79		33	66	
Preoperative	Y	37	71	0.47	17	91	0.37	18	90	0.76	28	80	0.45

chemotherapy	N	6	18	4	6	18	0	3	21	4	8	16	7
Tumor characteristics:													
PRETEXT IV	Y	14	11	0.00	7	18	0.14	4	21		10	15	0.13
(Y/N)	N	29	78	9	16	91	5	17	90	1	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		4	2	0.04
	N	39	87	8	20	106	5	20	106	1	32	94	7
Extrahepatic	Y	2	0	0.10	0	2		0	2		1	1	0.47
extension	N	41	89	4	23	107	1	21	109	1	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	Y	14	6	<0.0	5	15	0.34	7	13	0.01	8	12	0.18
diagnosis	N	29	83	01	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	

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

160 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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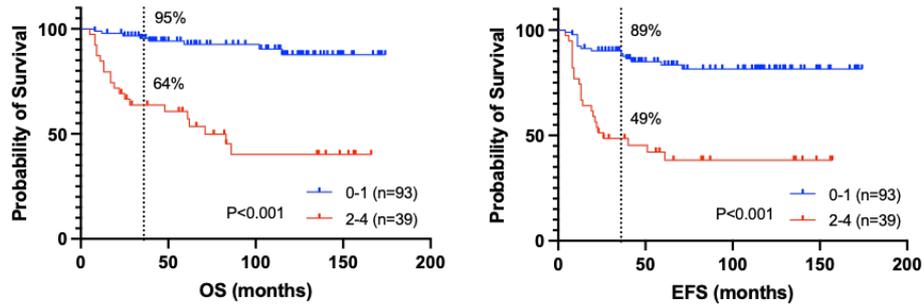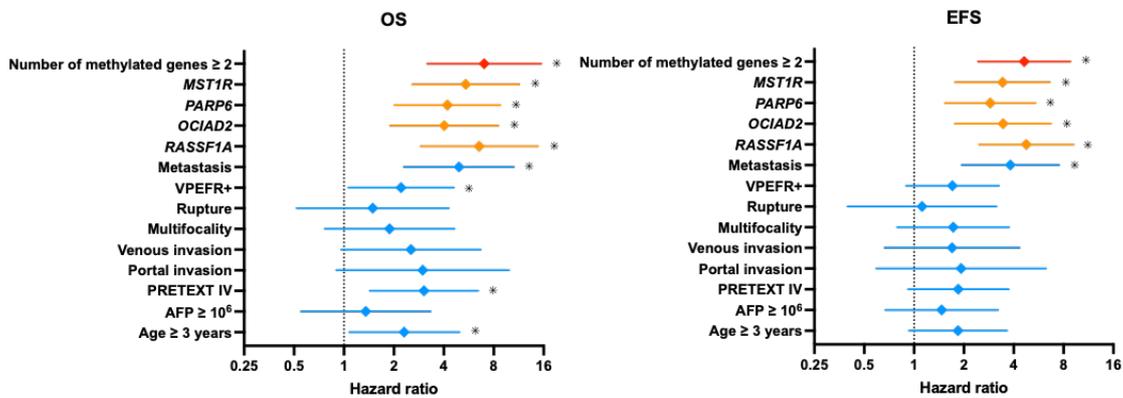
Fig. 2 A) Kaplan–Meier curves for overall survival (OS) (left panel) and event-free survival (EFS) (right panel) of 132 patients with hepatoblastoma (HB) classified by the number of the methylated genes. The blue line is the group with <2 methylated genes, and the red line is the group with ≥2 methylated 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).

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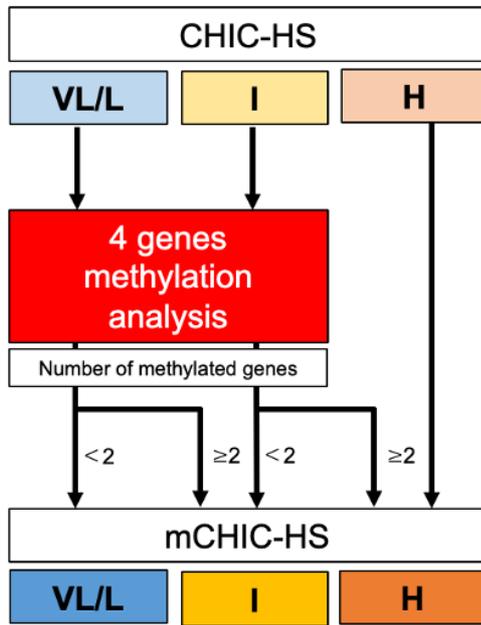

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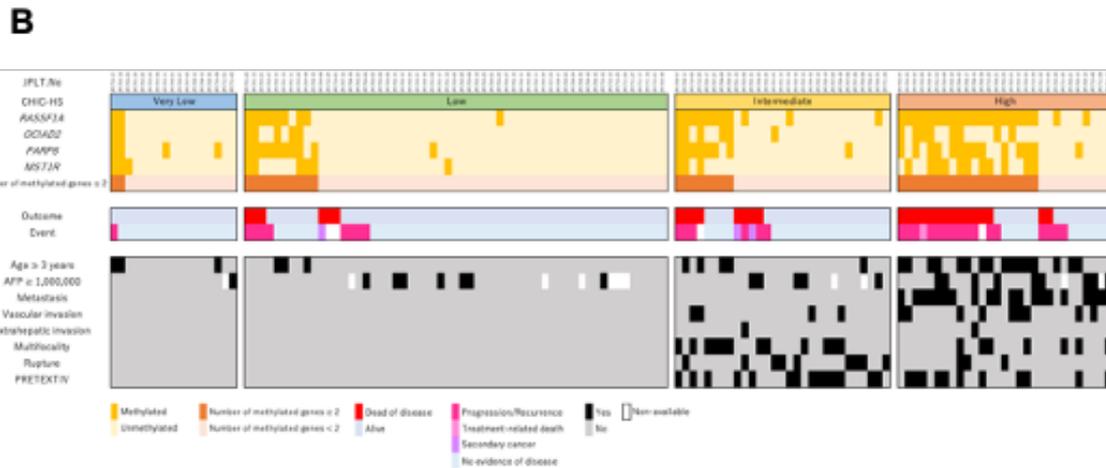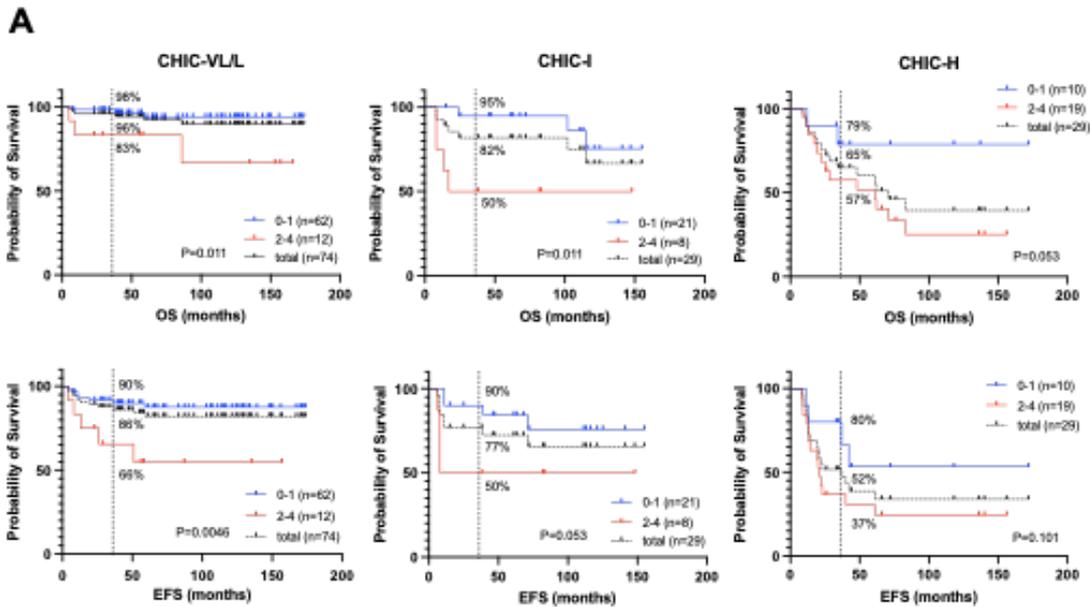
Fig. 3 A) Kaplan–Meier curves for overall survival (OS) (upper panel) and event-free survival (EFS) (lower panel) of 132 patients with hepatoblastoma (HB) classified by the number of the methylated genes in each Children's Hepatic Tumors International Collaboration-Hepatoblastoma Stratification risk 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.

15

213

214   Patients who had ≥2 methylated genes in the intermediate-risk group had a significantly worse OS

215   (3-year OS: 95% vs. 50%, $P=0.011$) and tended to have a worse EFS (3-year EFS: 90% vs. 50%,

216      $P=0.053$) (Fig. 3A). In the CHIC-high-risk group, patients who had ≥2 methylated genes tended to

217   have a worse OS (3-year OS: 79% vs. 57%, $P=0.053$) and EFS (3-year OS: 80% vs. 37%, $P=0.101$);

218   however, the differences were not statistically significant (Fig. 3A). These findings suggest that the

219   evaluation of the number of methylated genes in the four genes could optimize the stratification by

220   CHIC-HS. The distribution of the methylation status of the four genes and clinicopathological factors

221   are shown in Fig. 3B. This indicates that the evaluation of the methylation status of the four genes

222   enabled us to select the patients with poor prognosis, whose prognosis was not appropriately predicted

223   by the clinicopathological factors used to define the risk stratification in CHIC-HS. For example, the

224   four patients from the leftmost column of the CHIC-low group had poor prognoses, even though they

225   were stratified into the good prognosis group. However, in the methylation analysis, they were

226   classified in the poor prognosis group with ≥2 methylated genes (Fig. 3B). Based on the new

227   stratification system that integrates CHIC-HS and DNA methylation analysis data (mCHIC-HS),

228   patients in the CHIC-very low-/low- and CHIC-intermediate-risk groups were reclassified according

229   to the presence or absence of ≥2 methylated genes (Fig. 4A).

230

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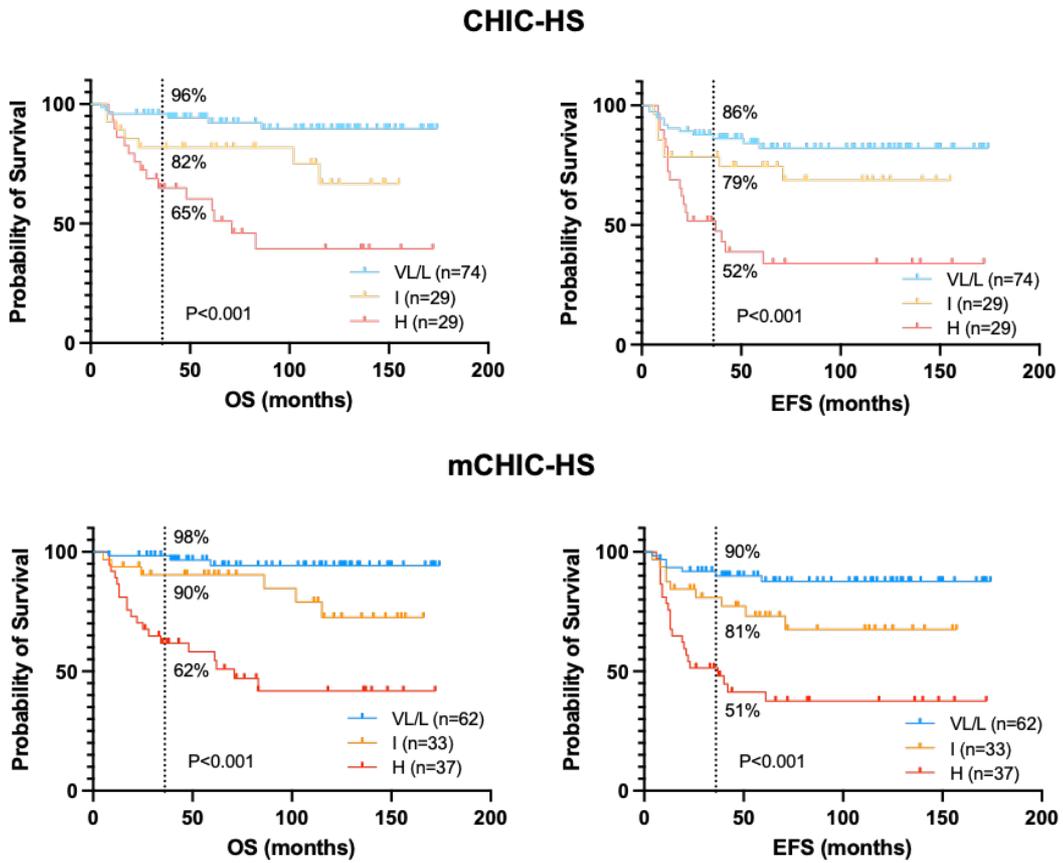
Fig. 4 A) Risk stratification algorithms of methylation-based Children's Hepatic Tumors International

233   Collaboration-Hepatoblastoma Stratification (mCHIC-HS). Methylation analysis of four genes is

234   performed in the CHIC-very low-/low- and intermediate-risk groups and those groups are reclassified

235   according to the number of methylated genes. VL, very low; L, low; I, intermediate; H, high. B)

236   Kaplan–Meier curves for overall survival (OS) (left panel) and event-free survival (EFS) (right panel)

237   of 132 patients with hepatoblastoma stratified by CHIC-HS (upper panel) and mCHIC-HS (lower

238   panel). The blue line is the very low-/low-risk group, the orange line is the intermediate-risk group,

239   and the red line is the high-risk group. The 3-year (dashed line) OS and EFS are shown on the side of

240   the survival curve. The log-rank test was performed to compare the OS and EFS curves.

241

242   The 3-year OS in the mCHIC-very low-/low-risk group increased from 96% to 98%, the number of

243   patients decreased, and the population was redefined with a better prognosis (Fig. 4B). In contrast, the

244   3-year OS in the mCHIC-high-risk group decreased from 65% to 62%, the number of patients

245   increased, and the population had a worse prognosis (Fig. 4B). The area under the ROC curve (AUC)

246   values of mCHIC-HS for 3-year OS and EFS were 0.817 (95% CI: 0.725−0.908) and 0.731 (95% CI:

247   0.626−0.836), which were higher than those of CHIC-HS (AUC: 0.762 [95% CI: 0.649−0.876] and

248   0.687 [95% CI: 0.569–0.804], P=0.087 and 0.128, respectively); however, there was no significant

249   difference.

250

251   **Discussion**

252   This study reconfirmed the usefulness of the methylation-based molecular prognostic markers we have

253   previously identified in a large Japanese cohort and established a more precise stratification model by

254   combining CHIC-HS with methylation analysis. Interestingly, the patients who had more methylated

255   genes out of the four genes had poorer prognoses, and having ≥2 methylated genes was a significant

256   poor prognostic factor identified in the multivariate Cox proportional hazard regression model.

257   Patients who had methylated genes had a significantly poorer prognosis and were older. Aging is one

258   of the causes of inducing aberrant methylation [21]. We compared the methylation rates of the four

259   genes in each age group and found that all genes, except *OCIAD2*, showed higher methylation rates

260   in older patients (Supplementary Fig. 4). Age-related methylation may be the molecular background

261   for age to be a clinically important prognostic factor in HB.

262   Recent studies have proposed prognostic models integrating CHIC-HS and the molecular prognostic

263   factors for HB. Carrillo-Reixach et al. found that a population with overexpressed 14q32 genes of the

264   *DLK1-DIO3* locus and a specific methylation status (Epigenetic-Cluster B: Epi-CB) had a poor

265   prognosis through comprehensive analysis. They proposed molecular risk stratification (MRS-HB), a

266   prognostic prediction model that classifies patients into three groups (MRS-1, MRS-2, and MRS-3)

267   according to the combination of their presence or absence [22]. They combined CHIC-HS with MRS-

268   HB to improve the ability to discriminate between low- and high-risk patients [22]. The Epi-CB group

269   was characterized by the hypermethylation of CpG islands [22]. Since the four genes we examined in

270   this study were also extracted as genes that show hypermethylation of the CpG islands in the promoter

271   region [17], these combinations may reflect such methylation tendencies and function as prognostic

272   factors. Cairo et al. presented a risk classification model based on the combination of CHIC-HS and

273   16-gene signature and reclassified the CHIC-intermediate-risk and CHIC-high-risk groups into

274   intermediate-risk C1 (IR-C1) and high-risk C2 (HR-C2) according to the presence of either the C1 or

275   C2 subtype of the 16-gene signature [13]. This model allows the identification of lower-risk patients

276   from the high-risk group and could reduce unnecessary high-intensity treatment [13].

277   Herein, we propose a novel risk stratification model based on methylation analysis by bisulfite

278   pyrosequencing methods called mCHIC-HS, which could optimize CHIC-HS. According to the

279   CHIC-HS, which is solely based on clinical factors, there were patients in our cohort with poor

280   prognoses who were incorrectly classified in the very low-/low-risk and would be treated insufficiently,

281   despite biologically highly malignant tumors. However, by selecting these cases based on the

282   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

308   not be applicable directly. When applying this model to clinical practice, validation should be

309   performed using specimens that have not been modified by chemotherapy. However, we found no

310   significant difference in the methylation rates of the four genes between those who received

311   preoperative chemotherapy and those who did not in this cohort (Supplementary Fig. 5). Furthermore,

312   we validated whether the evaluation of the methylation status of the four genes could stratify the

313   prognosis, using the results of methylation bead array using biopsy specimens before chemotherapy

314   enrolled in JPLT-2 reported by Nagae et al [12]. The OS was significantly worse in the methylated

315   group (Supplementary Fig. 6A). Using cases included both in this cohort and the study by Nagae et al.

316   [12], we also assessed the correlation between the beta-value obtained from the biopsy specimens

317   before chemotherapy and the methylation rate obtained from the specimens after chemotherapy. A

318   high correlation was found in all four genes (Supplementary Fig. 6B). Therefore, it is expected that

319   our model will also prove useful when validated with biopsy specimens before chemotherapy. Finally,

320   a tailor-made therapy for specific pathways and molecules in each stratified population was not

321   proposed; this would be addressed in the future.

322

323   **Conclusions**

324   We proposed a novel risk stratification model that integrates CHIC-HS with realistically feasible

325   methylation analysis-based molecular prognostic markers to achieve more appropriate risk-adaptive

326   therapy. We aim to conduct a prospective study using this model to verify its effectiveness in future

327   trials.

328

333   providing tissue specimens and the clinical data of the JPLT patients under written informed consent.

334

338

339   **Data Availability Statement**

340   The data that support the findings of this study are available from the corresponding author upon

341   reasonable request.

342

343   **Conflicts of interest**

344   The authors report no conflicts of intrest.

345

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