



Title	A novel risk stratification model based on the Children's Hepatic Tumours International Collaboration-Hepatoblastoma Stratification and deoxyribonucleic acid methylation analysis for hepatoblastoma
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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
3
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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

57 **Introduction**

58 Hepatoblastoma (HB) is the most common liver tumor in children, mostly occurring in those <3 years
59 old. Its annual incidence is 1.5 cases per million [1]. HB treatment comprises stratification based on
60 clinicopathological factors, surgery for complete resection, and cisplatin-based chemotherapy [2]. To
61 date, four study groups, namely, the Children’s Oncology Group, International Childhood Liver
62 Tumors Strategy Group, Society for Pediatric Oncology and Hematology, and Japanese Pediatric Liver
63 Tumors Group (JPLT), have played a central role in conducting clinical studies according to individual
64 stratification based on clinicopathological factors, such as the PRETreatment EXTent of disease
65 (PRETEXT) group, metastatic disease, serum alpha-fetoprotein (AFP) levels, and treatment regimens
66 [3–6]. In these studies, the overall survival (OS) was approximately 80%. However, the prognosis of
67 high-grade malignant cases, such as metastatic cases, remains poor, and long-term toxicity associated
68 with chemotherapy remains a serious challenge [2,7]. Therefore, the importance of providing
69 treatment without excesses and deficiencies for properly stratified patients is increasing. Thus, the
70 Children’s Hepatic Tumors International Collaboration-Hepatoblastoma Stratification (CHIC-HS), a
71 new international stratification system that integrates various clinicopathological factors, has been
72 recently established based on a large-scale CHIC database (N=1,605), and the Pediatric Hepatic
73 International Tumor Trial using CHIC-HS is currently underway [8].

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

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

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 characteristics 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	

<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

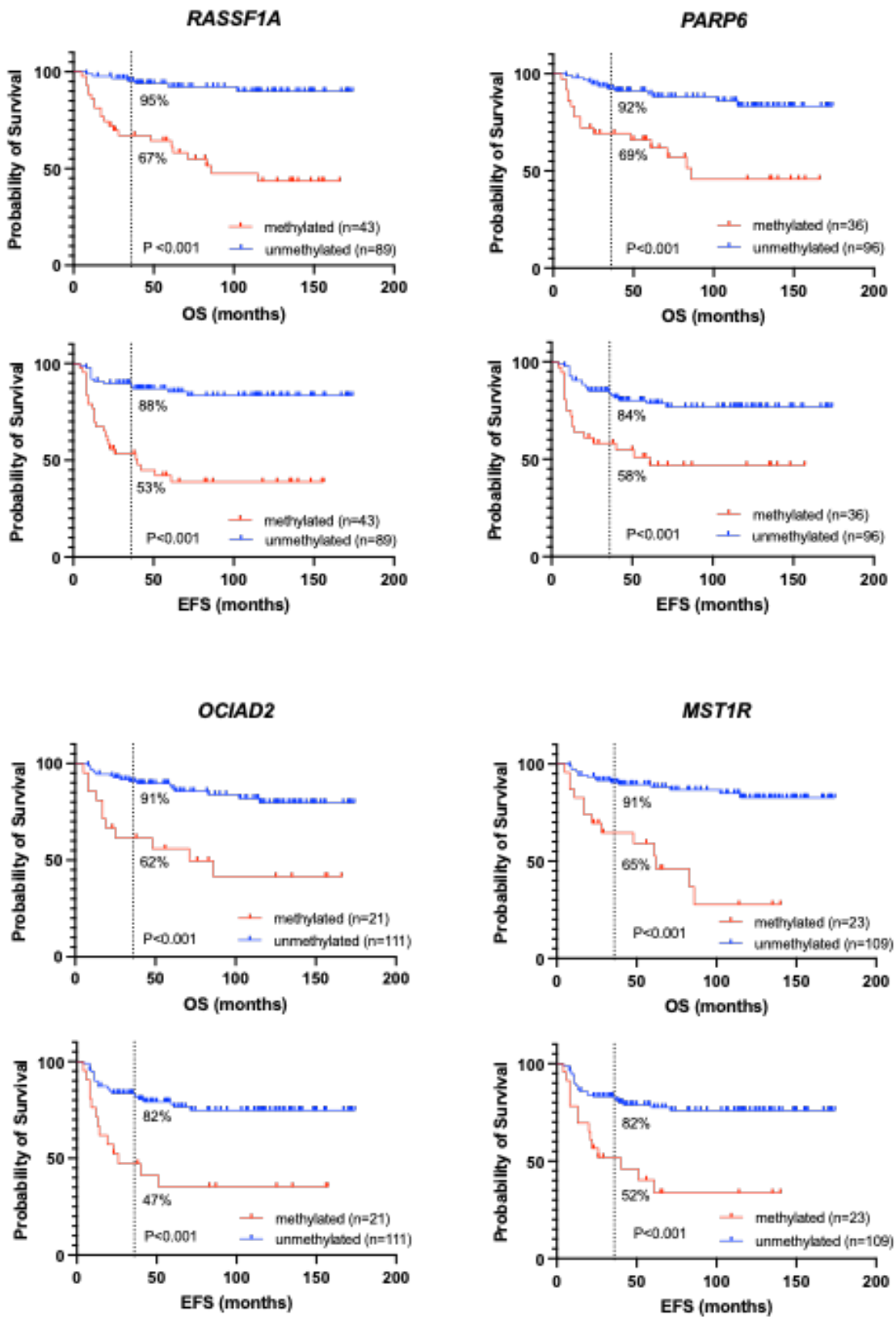
132 The patients were classified into risk groups according to CHIC-HS, with 17, 57, 29, and 29 in the
133 very low-risk, low-risk, intermediate-risk, and high-risk groups, respectively (Table 1). The 3-year OS
134 and EFS rates of each risk group were 100%, 94.7%, 81.8%, and 65.0% ($P=0.001$) and 93.8%,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



147

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

149 (lower panel) of 132 patients with hepatoblastoma classified by the methylation status of four genes
 150 (*RASSF1A*, *PARP6*, *OCIAD2*, and *MSTIR*). 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)

		<i>RASSF1</i>			<i>MSTIR</i>			<i>OCIAD2</i>			<i>PARP6</i>			
		<i>A</i>		<i>P-</i>			<i>P-</i>			<i>P-</i>			<i>P-</i>	
		M	U	valu	M	U	valu	M	U	valu	M	U	valu	
		n=	n=	e	n=	n=	e	n=	n=	e	n=	n=	e	
		43	89		23	109		21	111		36	96		
Age (years)	group	0-2	20	85		8	97		10	95		17	88	
					<0.0			<0.0			<0.0			
		3-7	16	3	01	9	10	01	9	10	01	14	5	01
		≥8	7	1		6	2		2	6		5	3	
Sex		M	22	54	0.34	9	67	0.06	12	64		16	60	0.08
		F	21	35	9	14	42	4	9	47	1	20	36	5
Serum AFP ng/mL		Y	7	15		3	19		1	21		3	19	
					0.80			0.56			0.11			0.07
		N	36	63	8	20	79	5	20	79	8	33	66	6
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 extension	Y	2	0	0.10	0	2		0	2		1	1	0.47
	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 diagnosis	Y	14	6	<0.0	5	15	0.34	7	13	0.01	8	12	0.18
	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).

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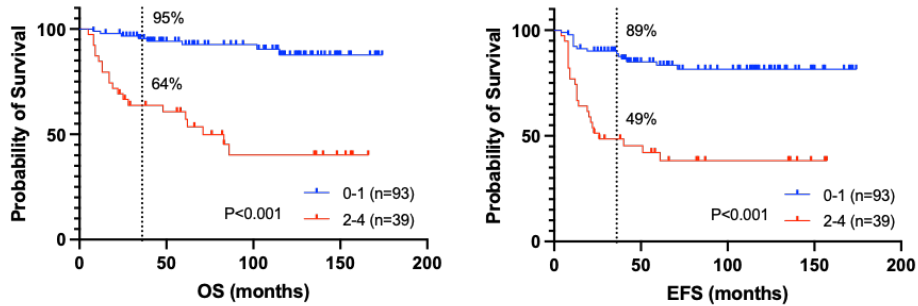
170 **Usefulness of assessing the number of methylated genes for predicting prognosis**

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).

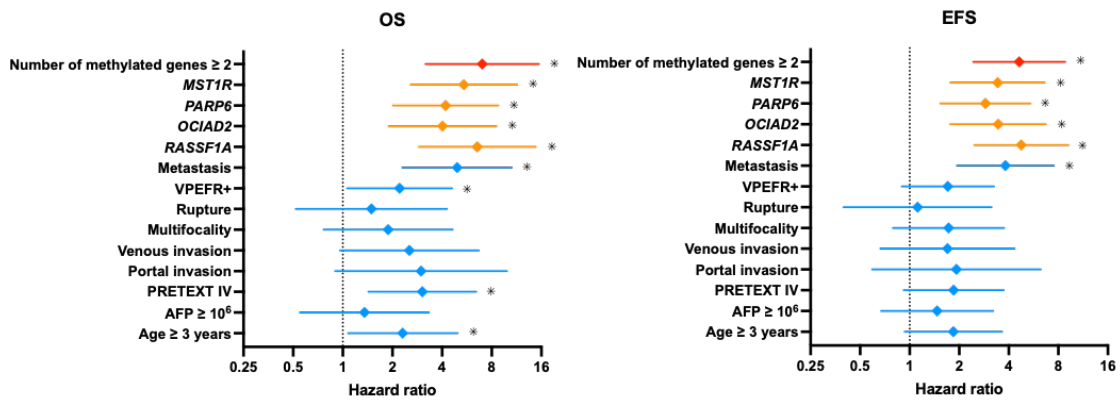
175

A

The number of methylated genes



B



176

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

186

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

188 methylated genes had the highest hazard ratio for OS (mean, 7.005; range, 3.177–15.45; $P < 0.001$)
 189 among the existing clinicopathological factors and methylation assessment of every single gene (Fig.
 190 2B). Multivariate Cox proportional hazards regression analysis revealed that the number of methylated
 191 genes ≥ 2 is a significant independent prognostic factor for OS and EFS (Table 3).

192

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

OS	HR	95% CI	<i>P</i> -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	0.124
Number of methylated genes ≥ 2	6.014	2.367–15.28	< 0.001

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

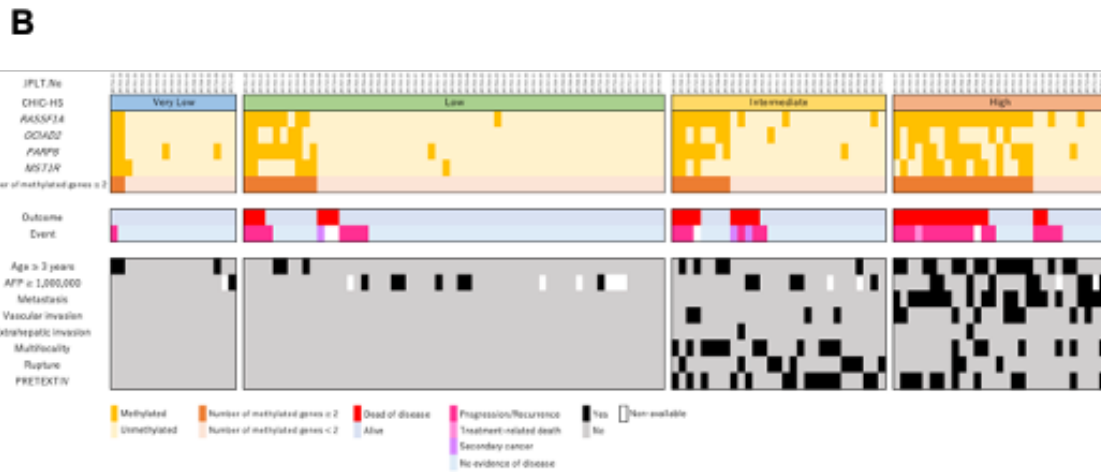
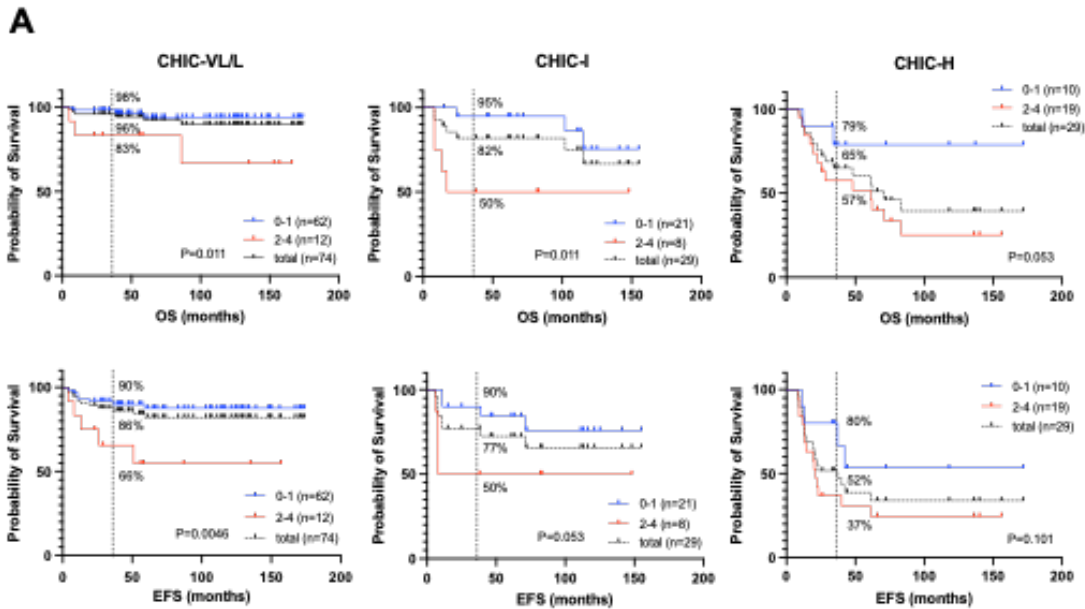
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196 HR, hazard ratio; CI, confidence interval; PRETEXT, PRETreatment EXTent of disease; VPEFR+, at
 197 least one of the PRETEXT annotation factors (involvement of hepatic vein, involvement of portal vein,
 198 extrahepatic tumor extension, multifocal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

199

200 **Integration of CHIC-HS and DNA methylation analysis**

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



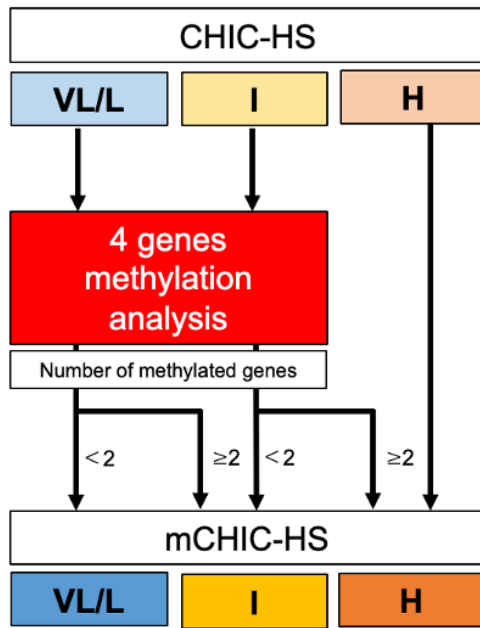
206 Fig. 3 A) Kaplan–Meier curves for overall survival (OS) (upper panel) and event-free survival (EFS)
 207 (lower panel) of 132 patients with hepatoblastoma (HB) classified by the number of the methylated
 208 genes in each Children’s Hepatic Tumors International Collaboration-Hepatoblastoma Stratification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

213

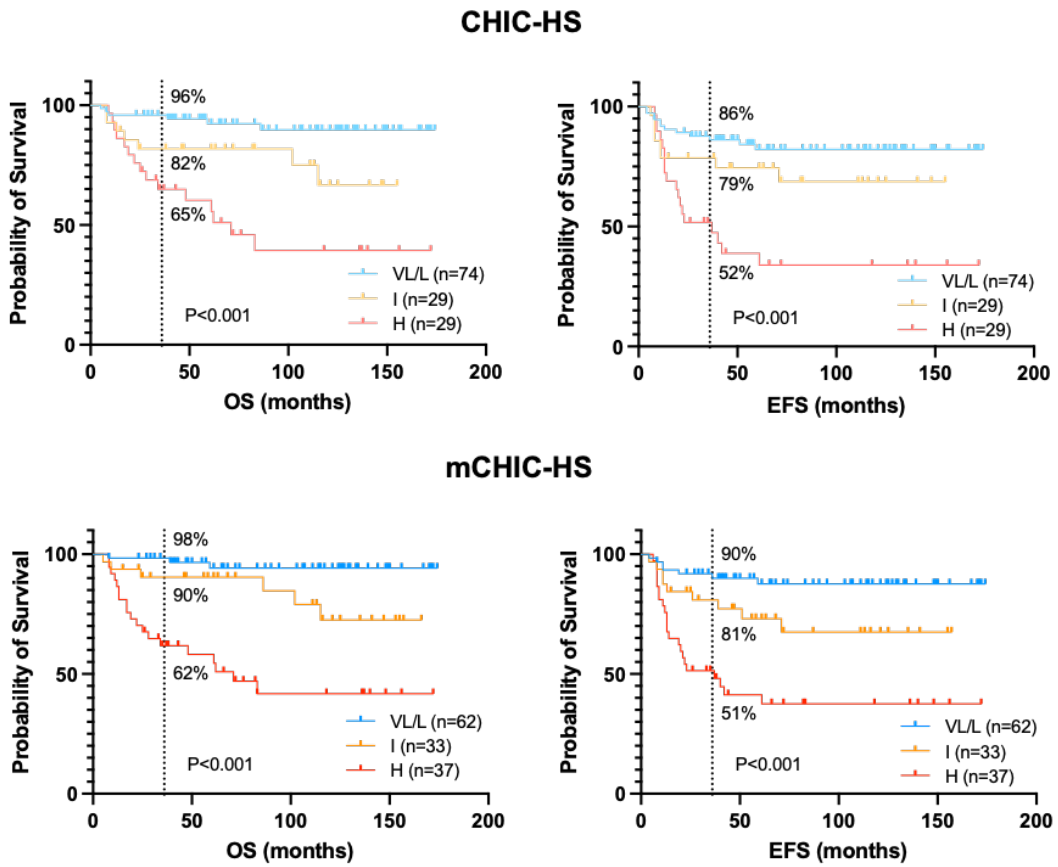
214 Patients who had ≥ 2 methylated genes in the intermediate-risk group had a significantly worse OS
215 (3-year OS: 95% vs. 50%, $P=0.011$) and tended to have a worse EFS (3-year EFS: 90% vs. 50%,
216 $P=0.053$) (Fig. 3A). In the CHIC-high-risk group, patients who had ≥ 2 methylated genes tended to
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

A



B



231

232 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 $>180^\circ$ 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

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334

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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 interest.

345

346 **References**

- 347 [1] Spector LG, Birch J. The epidemiology of hepatoblastoma. *Pediatr Blood Cancer* 2012;59:776–9.
348 <https://doi.org/10.1002/pbc.24215>.
- 349 [2] von Schweinitz D. Hepatoblastoma: recent developments in research and treatment. *Semin Pediatr*
350 *Surg* 2012;21:21–30. <https://doi.org/10.1053/j.sempedsurg.2011.10.011>.
- 351 [3] Malogolowkin MH, Katzenstein H, Krailo MD, Chen Z, Bowman L, Reynolds M, et al. Intensified
352 platinum therapy is an ineffective strategy for improving outcome in pediatric patients with
353 advanced hepatoblastoma. *J Clin Oncol* 2006;24:2879–84.
354 <https://doi.org/10.1200/JCO.2005.02.6013>.
- 355 [4] Perilongo G, Maibach R, Shafford E, Brugieres L, Brock P, Morland B, et al. Cisplatin versus
356 cisplatin plus doxorubicin for standard-risk hepatoblastoma. *N Engl J Med* 2009;361:1662–70.
357 <https://doi.org/10.1056/NEJMoa0810613>.
- 358 [5] Haeberle B, Schweinitz DV. Treatment of hepatoblastoma in the German cooperative pediatric
359 liver tumor studies. *Front Biosci (Elite Ed)* 2012;4:493–8. <https://doi.org/10.2741/395>.
- 360 [6] Hishiki T, Matsunaga T, Sasaki F, Yano M, Ida K, Horie H, et al. Outcome of hepatoblastomas
361 treated using the Japanese Study Group for Pediatric Liver Tumor (JPLT) protocol-2: report from
362 the JPLT. *Pediatr Surg Int* 2011;27:1–8. <https://doi.org/10.1007/s00383-010-2708-0>.
- 363 [7] Hiyama E, Hishiki T, Watanabe K, Ida K, Ueda Y, Kurihara S, et al. Outcome and late
364 complications of hepatoblastomas treated using the Japanese Study Group for Pediatric Liver
365 Tumor 2 protocol. *J Clin Oncol* 2020;38:2488–98. <https://doi.org/10.1200/JCO.19.01067>.
- 366 [8] Meyers RL, Maibach R, Hiyama E, Häberle B, Krailo M, Rangaswami A, et al. Risk-stratified
367 staging in paediatric hepatoblastoma: a unified analysis from the Children’s Hepatic tumors
368 International Collaboration. *Lancet Oncol* 2017;18:122–31. [https://doi.org/10.1016/S1470-](https://doi.org/10.1016/S1470-2045(16)30598-8)
369 [2045\(16\)30598-8](https://doi.org/10.1016/S1470-2045(16)30598-8).
- 370 [9] Cairo S, Armengol C, De Reyniès A, Wei Y, Thomas E, Renard CA, et al. Hepatic stem-like

- 371 phenotype and interplay of Wnt/beta-catenin and Myc signaling in aggressive childhood liver
372 cancer. *Canc Cell* 2008;14:471–84. <https://doi.org/10.1016/j.ccr.2008.11.002>.
- 373 [10] Hooks KB, Audoux J, Fazli H, Lesjean S, Ernault T, Dugot-Senant N, et al. New insights into
374 diagnosis and therapeutic options for proliferative hepatoblastoma. *Hepatology* 2018;68:89–102.
375 <https://doi.org/10.1002/hep.29672>.
- 376 [11] Sumazin P, Chen Y, Treviño LR, Sarabia SF, Hampton OA, Patel K, et al. Genomic analysis of
377 hepatoblastoma identifies distinct molecular and prognostic subgroups. *Hepatology*
378 2017;65:104–21. <https://doi.org/10.1002/hep.28888>.
- 379 [12] Nagae G, Yamamoto S, Fujita M, Fujita T, Nonaka A, Umeda T, et al. Genetic and epigenetic
380 basis of hepatoblastoma diversity. *Nat Commun* 2021;12:5423. [https://doi.org/10.1038/s41467-](https://doi.org/10.1038/s41467-021-25430-9)
381 [021-25430-9](https://doi.org/10.1038/s41467-021-25430-9).
- 382 [13] Cairo S, Armengol C, Maibach R, Häberle B, Becker K, Carrillo-Reixach J, et al. A combined
383 clinical and biological risk classification improves prediction of outcome in hepatoblastoma
384 patients. *Eur J Cancer* 2020;141:30–9. <https://doi.org/10.1016/j.ejca.2020.09.026>.
- 385 [14] Eichenmüller M, Trippel F, Kreuder M, Beck A, Schwarzmayr T, Häberle B, et al. The genomic
386 landscape of hepatoblastoma and their progenies with HCC-like features. *J Hepatol*
387 2014;61:1312–20. <https://doi.org/10.1016/j.jhep.2014.08.009>.
- 388 [15] Honda S, Haruta M, Sugawara W, Sasaki F, Ohira M, Matsunaga T, et al. The methylation status
389 of RASSF1A promoter predicts responsiveness to chemotherapy and eventual cure in
390 hepatoblastoma patients. *Int J Cancer* 2008;123:1117–25. <https://doi.org/10.1002/ijc.23613>.
- 391 [16] Honda S, Miyagi H, Suzuki H, Minato M, Haruta M, Kaneko Y, et al. RASSF1A methylation
392 indicates a poor prognosis in hepatoblastoma patients. *Pediatr Surg Int* 2013;29:1147–52.
393 <https://doi.org/10.1007/s00383-013-3371-z>.
- 394 [17] Honda S, Minato M, Suzuki H, Fujiyoshi M, Miyagi H, Haruta M, et al. Clinical prognostic value
395 of DNA methylation in hepatoblastoma: four novel tumor suppressor candidates. *Cancer Sci*

- 396 2016;107:812–9. <https://doi.org/10.1111/cas.12928>.
- 397 [18] Nojima M, Maruyama R, Yasui H, Suzuki H, Maruyama Y, Tarasawa I, et al. Genomic screening
398 for genes silenced by DNA methylation revealed an association between RASD1 inactivation
399 and dexamethasone resistance in multiple myeloma. *Clin Cancer Res* 2009;15:4356–64.
400 <https://doi.org/10.1158/1078-0432.CCR-08-3336>.
- 401 [19] Kanda Y. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics.
402 *Bone Marrow Transplant* 2013;48:452–8. <https://doi.org/10.1038/bmt.2012.244>.
- 403 [20] Murphy SK, Huang Z, Hoyo C. Differentially methylated regions of imprinted genes in prenatal,
404 perinatal and postnatal human tissues. *PLOS ONE* 2012;7:e40924.
405 <https://doi.org/10.1371/journal.pone.0040924>.
- 406 [21] Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from?
407 *Cancer Sci* 2005;96:206–11. <https://doi.org/10.1111/j.1349-7006.2005.00035.x>.
- 408 [22] Carrillo-Reixach J, Torrens L, Simon-Coma M, Royo L, Domingo-Sàbat M, Abril-Fornaguera J,
409 et al. Epigenetic footprint enables molecular risk stratification of hepatoblastoma with clinical
410 implications. *J Hepatol* 2020;73:328–41. <https://doi.org/10.1016/j.jhep.2020.03.025>.
- 411 [23] Towbin AJ, Meyers RL, Woodley H, Miyazaki O, Weldon CB, Morland B, et al. 2017 PRETEXT:
412 radiologic staging system for primary hepatic malignancies of childhood revised for the
413 Paediatric Hepatic International Tumour Trial (PHITT). *Pediatr Radiol* 2018;48:536–54.
414 <https://doi.org/10.1007/s00247-018-4078-z>.