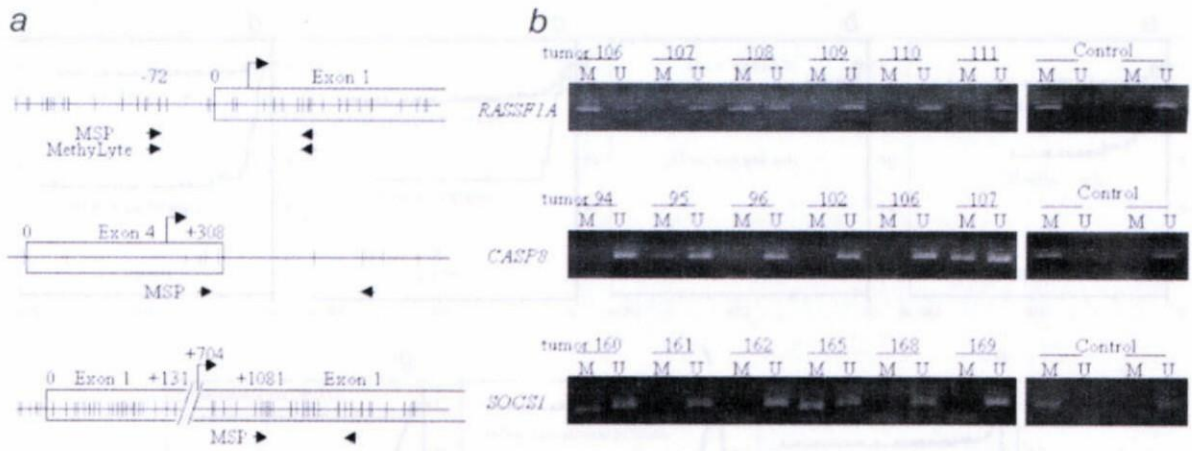

FIGURE 1 – (*a*) The location of the *RASSF1A*, *CASP8* or *SOCS1* fragment analyzed by the conventional or quantitative (MethyLyte) MSP method is shown as horizontal arrows. The transcription start site of each gene is shown as a bent arrow. (*b*) Examples of methylation status using conventional methylation-specific PCR. PCR products of methylated or unmethylated *RASSF1A*, *CASP8* and *SOCS1* from hepatoblastoma tumors are shown. M, methylated products; U, unmethylated products.

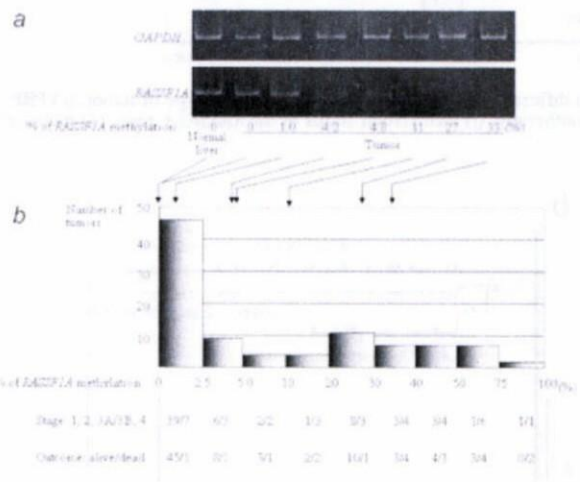

FIGURE 2 – (*a*) Histogram showing the number of tumors categorized by the percentage of *RASSF1A* methylation. The number of tumors classified by the stage of disease and clinical outcome are shown under the columns. (*b*) RT-PCR analysis of *RASSF1A* mRNA in 1 normal liver and 7 tumor samples.

RASSF1A methylation and stage of the disease or clinical outcome (Fig. 2*a*). Patients were classified into 3 groups (0~<5%, 5~<30% and 30~100% of the methylation), and we found that the higher the percentage of the methylation was, the higher the incidence of tumors at advanced stages or with poor outcome was ($p < 0.001$ and $p < 0.001$). On the basis of this cutoff value, 43 (44.3%) tumors were classified as having methylated *RASSF1A* and 54 as having unmethylated *RASSF1A*. In contrast, 30 (30.9%) tumors were classified as having methylated *RASSF1A* by conventional MSP; therefore, 13 (13.4%) tumors classified as the unmethylated group by conventional MSP changed to the methylated group by quantitative MSP. We used this incidence rate of hypermethylation in subsequent analysis of the correlation between *RASSF1A* methylation and clinicopathological characteristics in hepatoblastoma.

Mutation and deletion of the CTNNB1 gene

Of 97 tumors, 19 (19.6%) had a point mutation in *CTNNB1* and 46 (47.4%) had various sizes of *CTNNB1* deletion, ranging from 9 to 1061 bp, always including a region from amino acid 32 to 45, wherein lie 4 serine/threonine residues, which are targeted for phosphorylation. One tumor had both an insertion of 7 bp and a deletion of 19 bp in the same locus.

Incidences of tumors with RASSF1A methylation or CTNNB1 mutation between tumors obtained before or after chemotherapy

CTNNB1 mutation and *RASSF1A* methylation were found in 47 (65.2%) and 33 (47.2%) of 72 tumors preoperatively treated with chemotherapy and in 18 (72.0%) and 10 (40.0%) of 25 preoperatively untreated tumors. There were no differences in the incidences of *CTNNB1* mutation or *RASSF1A* methylation between tumors that received preoperative chemotherapy and those that did not. The findings indicate that *CTNNB1* mutation or *RASSF1A* methylation did not occur during the period of preoperative chemotherapy, or seem to reject that the normal *CTNNB1* or unmethylated *RASSF1A* status was merely a result of effective chemotherapy for the tumors.

Overall survival of patients classified by clinical and biological characteristics

We evaluated the association of clinical and biological characteristics with overall survival in 97 patients with heptoblastoma (Fig. 3). Patients less than 2 years of age showed better overall survival than those 2 years old or over ($p < 0.001$), and patients with fetal-type tumor showed better overall survival than those with embryonal-type tumor ($p = 0.044$). Likewise, patients with a PRETEXT 1, 2 or 3 tumor or a stage 1, 2 or 3A tumor showed better overall survival than those with a PRETEXT 4 ($p = 0.003$), or a stage 3B or 4 tumor ($p < 0.001$), respectively. Patients who achieved CR or PR with cisplatin-based chemotherapy had better overall survival than those who did not respond to therapy (NC) ($p = 0.011$). Finally, patients with a tumor with unmethylated *RASSF1A* or wild-type *CTNNB1* showed better overall survival than those with a tumor with methylated *RASSF1A* or mutated *CTNNB1* ($p < 0.001$ or $p = 0.030$), respectively.

To clarify the prognostic implication of the *RASSF1A* status in unfavorable groups, we only included 33 patients with a stage 3B or 4 tumor in the next analysis and found that *RASSF1A* methylation predicted a poor outcome in this group of tumors (Fig. 4*a*).

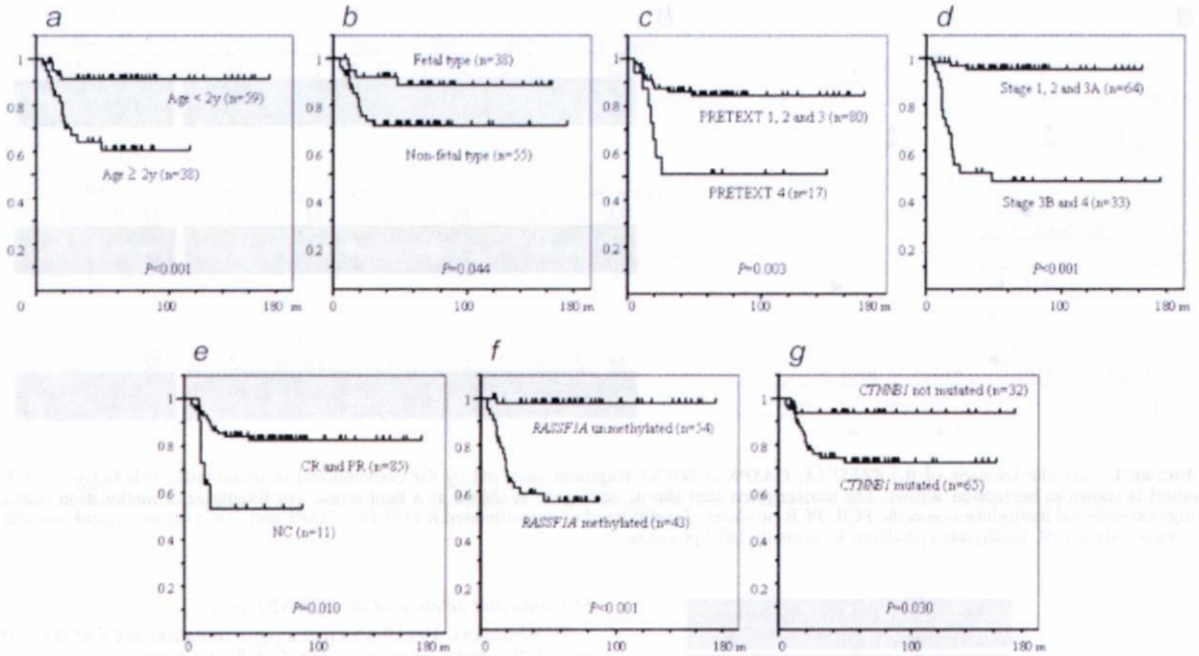

FIGURE 3 – Overall survival curves for hepatoblastoma patients based on different variables: (*a*) age, (*b*) histological type of tumor, (*c*) PRETEXT disease stage, (*d*) disease stage, (*e*) response to cisplatin-based chemotherapy, (*f*) methylation status of the *RASSF1A* gene, (*g*) mutation status of the *CTNNB1* gene.

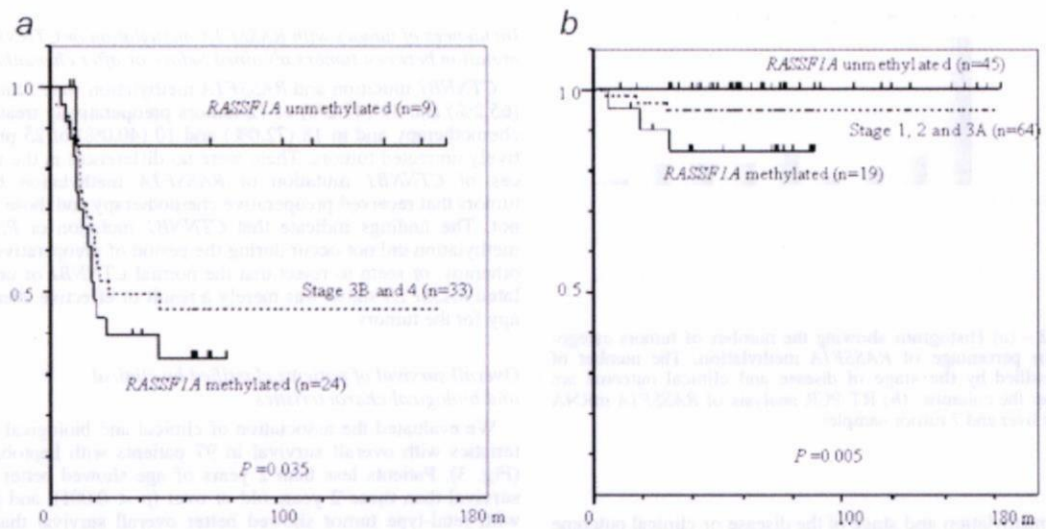

FIGURE 4 – (*a*) Overall survival curves for hepatoblastoma patients in stages 3B and 4 classified by the methylation status of *RASSF1A*. Dotted line indicates the overall survival curve of all 33 patients. (*b*) Overall survival curves for hepatoblastoma patients in stages 1, 2 and 3A classified by the methylation status of *RASSF1A*. Dotted line indicates the overall survival curve of all 64 patients.

Only 1 patient with a tumor with unmethylated *RASSF1A* died of recurrent brain metastases. When we only included 64 patients in stages 1, 2 and 3A in the next analysis, we also found that *RASSF1A* methylation predicted a poor outcome in this group of tumors (Fig. 4*b*). Three (16%) of 19 patients with a *RASSF1A*-methylated tumor died within 3 years after surgery, while all 45 patients with unmethylated *RASSF1A* were alive. These findings suggest that the *RASSF1A* methylation status is useful to identify patients who are likely to suffer recurrence or death from disease, irrespective of a favorable or unfavorable stage of the disease.

Association of RASSF1A, CASP8 or SOCS1 methylation or CTNNB1 mutation with clinical characteristics in hepatoblastoma

RASSF1A methylation was significantly associated with various factors predicting poor outcome except for the histological type

TABLE II – ASSOCIATION BETWEEN CLINICOPATHOLOGICAL FACTORS AND THE *RASSF1A* OR *CTNNB1* STATUS IN 97 PATIENTS WITH HEPATOBLASTOMA

Factors	Number of tumor	*RASSF1A*		p^1	*CTNNB1*		p^1
		Methylated	Unmethylated		Mutated	Not mutated	
Sex				0.761			0.335
Male	57	26	31		36	21	
Female	40	17	23		29	11	
Age at diagnosis				<0.001			0.262
<2 year	59	11	48		37	22	
≥2 year	38	32	6		28	10	
Histological type[2]				0.360			0.216
Fetal	38	15	23		23	15	
Non-fetal	55	27	28		40	15	
PRETEXT				0.063			0.361
1, 2, 3	80	32	48		28	52	
4	17	11	6		4	13	
Stage				<0.001			0.686
1, 2, 3A	64	19	45		42	22	
3B, 4	33	24	9		23	10	
Response to cisplatin-based chemotherapy[3]				0.010			0.218
CR, PR	85	34	51		55	30	
NC	11	9	2		9	2	
Recurrence[4]				<0.001			0.008
No	68	19	49		39	29	
Yes	24	19	5		21	3	
Outcome				<0.001			0.020
Alive	78	25	53		48	30	
Dead	19	18	1		17	2	
CTNNB1				0.007			
Not mutated	32	8	24				
Mutated	65	35	30				

CR, complete response; PR, partial response; NC, no change.
[1]Chi-square test or Fisher's exact test.–[2]Four patients, whose histological type could not be determined, were excluded.–[3]One patient who was treated only surgically was excluded.–[4]Five patients, whose tumors did not disappear and who died of the disease, were excluded.

and PRETEXT classification (Table II). *CASP8* methylation was associated with recurrent disease ($p = 0.034$), whereas *SOCS1* methylation was associated with the fetal histological type ($p = 0.020$). Nevertheless, the methylation status of both genes was unrelated to other clinical and biological characteristics, including outcome. There was no difference in the overall survival or stage distribution between patients with a tumor with only *RASSF1A* methylation and those with a tumor with *RASSF1A* and *SOCS1* or *CASP8* methylation, or with joint methylation of the 3 genes. *CTNNB1* mutation was significantly associated with recurrent disease, poor outcome and *RASSF1A* methylation. *CTNNB1* mutation includes both a point mutation and deletion of various sizes. There was no difference in the clinical characteristics, including outcome, between tumors with the point mutation and those with the deletion.

Multivariate Cox proportional hazard regression analysis was performed to clarify whether various factors independently affect overall survival in 92 patients, in whom all variables were available. Disease stage and the *RASSF1A* methylation status were shown to be independent factors predicting poor outcome, but *CTNNB1* mutation was not (Table III). The incidence of *CTNNB1* mutation (67.4%, 62/92) was higher than that of *RASSF1A* methylation (45.7%, 42/92), and tumors with the mutation included the great majority (81.0%, 34/42) of tumors with methylation. Furthermore, while patients with a tumor with mutated *CTNNB1* and unmethylated *RASSF1A* enjoyed excellent prognosis, those with a tumor with mutated *CTNNB1* and methylated *RASSF1A* suffered an unfavorable outcome ($p < 0.001$). These findings led to different results of the prognostic implication of *CTNNB1* mutation by univariate and multivariate analyses.

TABLE III – MULTIVARIATE ANALYSIS ON 6 CLINICOPATHOLOGICAL AND GENETIC FACTORS IN 92 PATIENTS WITH HEPATOBLASTOMA

Prognostic factors	Relative risk (95%CI)	*p* value
Age		
<2 year versus ≥2 year	1.34 (0.45–3.95)	0.600
Histological type		
Fetal versus Nonfetal	2.15 (0.69–6.74)	0.189
Stage		
1, 2, 3A versus 3B, 4	7.67 (2.13–27.61)	0.002
Response to chemotherapy		
CR, PR versus NC	1.95 (0.65–5.87)	0.234
CTNNB1		
Not mutated versus mutated	2.19 (0.47–10.23)	0.321
RASSF1A		
Unmethylated versus methylated	9.39 (1.08–82.06)	0.043

95%CI, 95% confidence interval; CR, complete response; PR, partial response; NC, no change.

Discussion

Hepatoblastoma occupies 90% of childhood liver tumors, although its incidence is relatively low.[1] Currently, 20–30% of patients who do not respond to preoperative chemotherapy, or who present with or develop metastatic disease, continue to face a poor outcome.[2,3] To improve the mortality rate, treatment strategies for hepatoblastoma refractory to the standard cisplatin and THP adriamycin regimen or with metastasis should be innovated.[33] To achieve a higher complete resection rate, more effective preoperative chemotherapy for refractory hepatoblastoma is mandatory, and such therapy offers a realistic hope for cure. In addition, novel molecular-genetic markers that predict the treatment outcome of patients are needed for better therapy planning.

Because oncogenes or tumor suppressor genes other than *CTNNB1* are rarely mutated in hepatoblastoma,[10–12] and there are no reports on the prognostic implication of *CTNNB1* mutation, we suspected that methylation of tumor suppressor genes may occur, acting as a biomarker to predict treatment outcome. Thus, we analyzed the methylation status of 13 candidate tumor suppressor genes, *RASSF1A*, *RASSF2A*, *SOCS1*, *CASP8*, *NORE1A*, *RUNX3*,

TABLE IV – TUMOR SUPPRESSOR GENES AND THE INCIDENCE OF METHYLATED HEPATOBLASTOMA TUMORS EXAMINED BY METHYLATION-SPECIFIC PCR

Pathway	Gene	Gene location	Function	Incidence of methylated tumor	References
Signal transduction	*RASSF1A*	3p21	RAS effector	43/97 (44%)	Present study
				15/39 (39%)	9
				5/27 (19%)	34
	RASSF2A	20p13	RAS effector	0/20 (0%)	Present study
	NORE1A	1q32	RAS effector	0/20 (0%)	Present study
	SOCS1	16p13	Inhibitor of JAK/STAT pathway	32/97 (33%)	Present study
				7/15 (47%)	35
	RUNX3	1p36	TGF-beta pathway	0/20 (0%)	Present study
	RARB	3p24	Retinoic acid receptor	0/27 (0%)	34
	APC	5q21	Wnt signaling pathway	0/27 (0%)	34
	SFRP1	8p12	Secreted frizzled-related protein	0/39 (0%)	9
	SFRP2	4q31	Secreted frizzled-related protein	0/39 (0%)	9
	SFRP4	7p14	Secreted frizzled-related protein	0/39 (0%)	9
	SFRP5	10q24	Secreted frizzled-related protein	0/39 (0%)	9
Cell-cycle regulation	*p16INK4A*	9p21	Cell cycle regulation	0/20 (0%)	Present study
				0/27 (0%)	34
	p14ARF	9p21	MDM2 inhibitor	0/20 (0%)	Present study
Apoptosis	*CASP8*	2q33	Activation of effector caspases	15/97 (16%)	Present study
	DCR2	8p22	Antiapoptotic decoy receptor	0/20 (0%)	Present study
	DAPK	9q34	Death-associated protein kinase	0/27 (0%)	34
Chromatin regulation and transcription	*RIZ1*	1p36	Methyltransferase superfamily	0/20 (0%)	Present study
DNA repair	*MGMT*	10q24	DNA methyltransferase	0/27 (0%)	34
Detoxification	*GSTP1*	11q13	Glutathione S-transferase	0/27 (0%)	34
Cell adhesion	*CDH1*	16q22	E-cadherin	0/27 (0%)	34
	CDH13	16q24	H-cadherin	0/27 (0%)	34
Unknown	*BLU*	3p21	Suppressor of cell cycle entry (?)	0/20 (0%)	Present study
	HOXA9	7p15-p14	Homeobox protein	0/20 (0%)	Present study
	HOXB5	17q21	Homeobox protein	0/20 (0%)	Present study

RIZ1, *BLU*, *HOXA9*, *HOXB5*, *p16INK4A*, *p14ARF* and *DCR2*, by conventional MSP.[13–22] These genes have previously been shown to be aberrantly methylated in various adult and childhood cancers and also represent important elements for several signaling pathways and cell cycle regulation (Table IV). We found that 3 genes, *RASSF1A*, *SOCS1* and *CASP8*, were methylated in a substantial number of hepatoblastoma tumors. Interestingly, univariate analysis showed that only *RASSF1A* methylation was correlated with a poor outcome, but not *SOCS1* or *CASP8* methylation. When we examined the contribution of various prognostic factors to overall survival by multivariate analysis, only the disease stage was identified as an independent factor, but not *RASSF1A* methylation.

Then we analyzed the methylation status of *RASSF1A* by quantitative MSP because this method gives more reproducible and accurate results than conventional MSP. The accuracy and reliability of quantitative MSP were proved by the inverse relationship found between the percentage of *RASSF1A* methylation and the expression (Fig. 2*b*). The incidence of tumors with hypermethylated *RASSF1A* increased from 30.9 to 44.3%, probably because quantitative MSP is more sensitive than conventional MSP. The low cutoff value of 4.8% and the slight difference in the primer locations may have also contributed to the different incidences of the methylated tumors examined by the 2 MSP methods. In our previous study of *RASSF1A* methylation in 39 hepatoblastoma tumors, multivariate analysis using the prognostic factors similar to the present ones showed an equivocal *p*-value of 0.079 with relative risk of 12.84 (95% CI, 0.74–223.13). The present multivariate analysis using the results examined by quantitative MSP and the substantial number of tumors clearly demonstrated that the methylation is an independent factor predicting treatment outcome, and its contribution ranked next to the disease stage (Table III).

RASSF1A is a gene located in the 3p21 chromosomal region where deletions and loss of heterozygosity are frequently reported in small cell lung cancer.[31] Previous studies, including ours, have repeatedly shown that promoter hypermethylation of *RASSF1A* correlated with loss of expression in various cancers, and treatment with a demethylating agent reactivated *RASSF1A* gene expression in various cancer cell lines, including a hepatoblastoma cell line HepG2.[9,34,36,37] RASSF1A inhibits tumor formation by apoptosis, and regulates microtubule dynamics and mitotic arrest *via* multiple effectors. By dysregulation of the Ras signaling pathway, *RASSF1A* methylation is correlated with poor differentiation and vascular invasion of cancer cells, and an unfavorable outcome.[36]

Among the 13 genes examined that were frequently methylated in various cancers, only 3 genes were methylated in hepatoblastoma. The present and previous studies evaluated the methylation status of at least 20 genes in hepatoblastoma and found that only 3 genes were methylated (Table IV).[9,34,35] The limited number of methylated genes suggest that this profile may be specific for hepatoblastoma,[38] and the survival and stage distribution analyses disclosed that combined *RASSF1A* and *SOCS1* or *CASP8* methylation, or joint methylation of the 3 genes are not correlated with the advanced stage of disease or a poor outcome, contrary to the findings that methylation of multiple genes were correlated with a poor outcome, reported in neuroblastoma.[39]

The present multivariate analysis identified unresectable tumor stages of disease (3B and 4) as the most significant factor predicting overall survival, followed by *RASSF1A* methylation. Downstaging of stage 3B tumors and control of metastatic lesions of stage 4 tumors by preoperative chemotherapy proceeds to subsequent complete resection, and this procedure may be critical to cure patients in such stages. Presently, JPLT or other protocols treat hepatoblastoma patients by a preoperative regimen consisting of cisplatin and adriamycin or its derivatives.[2,23] The present study showed that patients with a *RASSF1A*-methylated tumor in stage 3B or 4 were less likely to respond to preoperative therapy than those with a *RASSF1A*-unmethylated tumor in the same stage (Table II and Fig. 4*a*). In addition, in an analysis of 70 male germ cell tumors, Koul *et al.* found that the incidence of *RASSF1A* methylation is higher in cisplatin-resistant tumors than in cisplatin-sensitive tumors.[40] Therefore, we propose that patients with a *RASSF1A*-methylated hepatoblastoma tumor should be treated with a more intensive regimen with anticancer drugs other than cisplatin and adriamycin or its derivatives.

Abnormalities of the Wnt pathway are the genetic hallmark of hepatoblastoma, and *CTNNB1* mutation is the most frequent

genetic changes found in the pathway[10,41]; however, there has been only one study on the prognostic implication of *CTNNB1* mutation in hepatoblastoma, which failed to show a correlation between the mutation and outcome.[42] The present univariate analysis showed that patients with *CTNNB1* mutation had a lower overall survival rate than those without *CTNNB1* mutation (Fig. 3); however, multivariate analysis rejected the mutation as an independent factor (Table III). The great majority of tumors with *RASSF1A* methylation were included in tumors with *CTNNB1* mutation, and patients with tumors with the mutation but not with the methylation showed favorable prognosis. These findings suggest that *CTNNB1* mutation may be an early genetic event in hepatoblastoma tumorigenesis, whereas *RASSF1A* methylation may be a later event associated with tumor progression.

In the present study on various candidate tumor suppressor genes, *RASSF1A* was the most frequently methylated gene in hepatoblastoma and its methylation clearly predicted the poor outcome of patients. We believe that the *RASSF1A* status is a promising molecular-genetic marker, and we expect that this biomarker may be used to stratify patients treated in clinical trials.

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Loss of imprinting of *IGF2* correlates with hypermethylation of the *H19* differentially methylated region in hepatoblastoma

S Honda[1,4], Y Arai[2], M Haruta[1], F Sasaki[3], M Ohira[3], H Yamaoka[3], H Horie[3], A Nakagawara[3], E Hiyama[3], S Todo[4] and Y Kaneko[*,1,3]

[1]*Department of Cancer Diagnosis, Saitama Cancer Center, Research Institute for Clinical Oncology, 818 Komuro, Ina, Saitama 362-0806, Japan;* [2]*Cancer Genomics Project, National Cancer Center Research Institute, Chuo-Ku, Tokyo 104-0045, Japan;* [3]*Japanese Study Group for Pediatric Liver Tumor (JPLT), Hiroshima 734-8551, Japan;* [4]*Department of General Surgery, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan*

IGF2, a maternally imprinted foetal growth factor gene, is implicated in many childhood tumours including hepatoblastoma (HB); however, the genetic and epigenetic alterations have not comprehensively been studied. We analysed the methylation status of the *H19* differentially methylated region (DMR), loss of heterozygosity (LOH) and allelic expression of *IGF2* in 54 *HB* tumours, and found that 12 tumours (22%) with LOH, 9 (17%) with loss of imprinting (LOI) and 33 (61%) with retention of imprinting (ROI). Biallelic and monoallelic *IGF2* expressions correlated with hypermethylation and normal methylation of *H19* DMR, respectively, in two tumours with LOI and seven tumours with ROI. Quantitative RT–PCR analysis showed minimal expression of *H19* mRNA and substantial expression of *IGF2* mRNA in tumours with LOH or LOI, and substantial expression of both *H19* and *IGF2* mRNAs in tumours with ROI. Increased *IGF2* expression with predominant embryonic P3 transcript was found in the majority of HBs with ROI and foetal livers. In contrast to the earlier reports, our findings suggest that the disruption of the enhancer competition model reported in Wilms' tumour may also occur in HB. Both frequencies of LOH and LOI seem to be lower in HB than in Wilms' tumour, reflecting the different tissue origins.

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Hepatoblastoma (HB) is a rare malignant neoplasm of the liver, with an incidence of 0.5–1.5 per million children (Perilongo and Shafford, 1999). Remarkable progress in clinical outcome has been achieved in the past 20 years because of advances in chemotherapy and surgical procedures; however, the mortality rate remains 20–30% and treatment results in patients in advanced stages who are refractory to standard preoperative chemotherapy regimens are unsatisfactory (Perilongo *et al*, 2000; Fuchs *et al*, 2002). To improve the mortality of these patients, innovative treatment based on a specific molecular target is needed. The molecular mechanism involved in the development and progression of HB includes overexpression of insulin-like growth factor-II (*IGF2*) (Li *et al*, 1998b; Gray *et al*, 2000; Hartmann *et al*, 2000), downregulation of *RASSF1A* by promoter hypermethylation (Sugawara *et al*, 2007; Honda *et al*, 2008) and alterations of genes in the Wnt signalling pathway; most notably, the high incidence of *CTNNB1* (catenin, β1) mutation (Koch *et al*, 1999; Taniguchi *et al*, 2002).

IGF2 is a maternally imprinted gene and encodes a foetal peptide hormone that regulates cellular proliferation and differentiation (Foulstone *et al*, 2005). *IGF2* has four promoter regions and P3 is the most active promoter in the foetal liver, followed by P2 and P4 promoters (Li *et al*, 1998a). *PLAG1* encodes a developmentally regulated transcription factor, which positively regulates *IGF2* through binding the P3 promoter region. Although *IGF2* is downregulated in normal tissues after birth, except for liver tissues, it is overexpressed in a wide variety of childhood and adult cancers and serves as a tumour enhancer through autocrine and paracrine mechanisms (Toretsky and Helman, 1996). *IGF2* has been studied extensively over the past decade as a key molecule involving HB and Wilms' tumour (WT) pathogenesis.

The allelic expression of *IGF2* is regulated by the methylation status of the sixth CTCF (CCCTC-binding factor) site in the *H19* differentially methylated region (DMR) that represents the parental origin of the *IGF2* allele; whereas the paternal CTCF6 allele is methylated, the maternal allele is unmethylated in normal tissues (Bell and Felsenfeld, 2000; Hark *et al*, 2000; Takai *et al*, 2001). Using the enhancer competition model, *IGF2* and *H19* promoters compete on the same chromosome for a shared enhancer, and access of the maternal *IGF2* allele to this enhancer is blocked by *H19* DMR when unmethylated because of the insulator activity of CTCF binding to unmethylated *H19* DMR (Bell and Felsenfeld, 2000; Hark *et al*, 2000). It has been proved in many WTs that aberrant methylation of the maternal CTCF6 prevents the insulator binding and leads to loss of imprinting (LOI), resulting in the overexpression of *IGF2* (Steenman *et al*, 1994; Ravenel *et al*, 2001). Although LOI of *IGF2* was reported in HB, the mechanism of LOI, the concurrent overexpression of *IGF2* mRNA and loss of

*Correspondence: Dr Y Kaneko; E-mail: kaneko@cancer-c.pref.saitama.jp
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H19 mRNA expression are uncertain because of the limited number of HB tumours examined and the low frequency of the heterozygous *IGF2* polymorphic site in general populations (Davies, 1993; Montagna *et al*, 1994; Rainier *et al*, 1995; Li *et al*, 1995, 1998b; Fukuzawa *et al*, 1999; Gray *et al*, 2000; Hartmann *et al*, 2000; Ross *et al*, 2000; Albrecht *et al*, 2004; Suzuki *et al*, 2008), and some investigators stated earlier that the mechanisms of *IGF2* upregulation by LOI found in WT do not apply to HB (Li *et al*, 1995; Hartmann *et al*, 2000).

Loss of imprinting was reported in 32–38% of WTs (Ravenel *et al*, 2001; Fukuzawa *et al*, 2004; Yuan *et al*, 2005), and loss of heterozygosity (LOH), leading to uniparental disomy (UPD) of the paternal *IGF2*, was reported in 36–50% of WTs (Grundy *et al*, 1996; Fukuzawa *et al*, 2004; Yuan *et al*, 2005). In HB, although LOH of *IGF2* was reported in 20–30%, the incidence of LOI of *IGF2* was uncertain because each series included only a small number of HB tumours. In addition, it is also uncertain whether the same mechanism of LOI is involved in both WT and HB tumorigeneses because the methylation status of *H19* DMR in HB has rarely been examined (Li *et al*, 1995, 1998b; Fukuzawa *et al*, 1999).

To determine whether the alterations of *IGF2* and *H19* loci identified in WT are also found in HB, we examined the LOI and LOH status of *IGF2* using combined bisulphite restriction assay (COBRA) of the CTCF6 region that can determine the methylation status of *H19* DMR more efficiently than the method using methylation-specific restriction enzymes and Southern blot in 54 HB tumours. In addition, we evaluated promoter-specific *IGF2* transcripts, the methylation status of *IGF2* promoters and *PLAG1* mRNA expression. Our results showed that the genetic and epigenetic alterations in the *IGF2-H19* region with elevated expression of *IGF2* mRNA identified in WTs were also found in the great majority of HB tumours, although the incidences of LOH and LOI may be lower in HBs than in WTs.

MATERIALS AND METHODS

Patients and samples

Tumour tissues were obtained from 54 Japanese children with HB, and adjacent normal liver tissues were available from 5 patients. Eighteen tumour and five matched normal liver specimens were supplied by the Tissue Bank of the Japanese Study Group for Pediatric Liver Tumour (JPLT) (Matsunaga *et al*, 2004), and 36 were supplied by institutions affiliated with Saitama Cancer Center. DNA and RNA were extracted from tumour and normal tissue samples that were immediately frozen after the resection or on arrival at the centre. The median age of the 54 patients at diagnosis was 18 months (range, 1–156 months). None of patients had the Beckwith–Wiedemann syndrome or a family history of familial polyposis coli. A total of 14 and 37 tumours were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in the other 3 tumours. Pathologists in each institution and/or the JPLT pathology panel made the diagnosis of HB and verified that each sample contained 70% or more tumour cells. Informed consent was obtained from the parents, and the study design was approved by the ethics committee of Saitama Cancer Center.

COBRA of the CTCF6 site at *H19* DMR

We performed COBRA to determine the methylation status of the CTCF6 binding site at *H19* DMR, as described earlier (Watanabe *et al*, 2007). COBRA of CTCF6 showed that the mean methylation percentage ±2 s.d. of five normal livers was 52.8 ± 15.0%, and we defined more than the mean percentage +2 s.d. as the hypermethylated state.

LOH analysis of *IGF2*

High-resolution single nucleotide polymorphism (SNP) array, Affymetrix Mapping 50K-Xba array (Affymetrix, Santa Clara, CA, USA), was used to analyse chromosomal aberrations of 11p15.5 where *IGF2* resides. Genomic DNA in 43 of 54 tumours and 2 cell lines was assayed according to the manufacturer's protocol, and the genomic status of *IGF2* was determined as described earlier (Haruta *et al*, 2008).

Allelic expression analysis of *IGF2* and quantitative real-time reverse transcription-PCR analysis of *IGF2* and *H19* mRNA

The *ApaI*/*Ava*II polymorphic site in exon 9 of *IGF2* was used to evaluate the allelic expression of *IGF2* mRNA in 21 tumours whose RNA was available for this study, as described earlier (Watanabe *et al*, 2006). Quantitative real-time reverse transcription-PCR was performed to evaluate the total *IGF2* and *H19* mRNA levels in 20 tumour tissues, 2 HB cell lines (HuH6 and HepG2), foetal liver total RNA pooled from 34 foetuses (Clontech, Ohtsu, Japan) and 3 normal liver tissues adjacent to HB; the age of the patients was 16, 24 or 26 months. Of the 20 tumours, 3 and 16 were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in 1. The primers and TaqMan probes used for *IGF2* and *H19* mRNA were described earlier (Watanabe *et al*, 2007; Haruta *et al*, 2008). The expression of *IGF2* and *H19* mRNAs was normalised with *GAPDH*.

Methylation-specific PCR and bisulphite sequencing analysis of *IGF2* promoter regions

Genomic DNA from tumour and normal liver samples was treated with sodium bisulphite (Herman *et al*, 1996), and the methylation status of the P2–P4 promoter regions of *IGF2* was analysed by methylation-specific PCR (MSP), as described earlier (Beeghly *et al*, 2007). Polymerase chain reaction products were run on 2% agarose gels and visualised after staining with ethidium bromide. We confirmed the results of MSP analysis of P3 promoter by bisulphite sequencing of eight or more subcloned plasmids.

Semiquantitative RT–PCR analysis of promoter-specific transcripts of *IGF2* and *PLAG1*

P1 and P3 promoter specific expressions of *IGF2* mRNA were analysed using the primer sets described elsewhere (Lu *et al*, 2006). The primer sequences for P2-specific transcript were derived from exons 4 and 5: forward, 5′-CCCTCAGGACGTGGACAG-3′; reverse, 5′-GTGCGTTGGACTTGCATAGA-3′; and the primer sequences for P4-specific transcript were derived from exons 7, 8 and 9: forward, 5′-CGAGCCTTCTGCTGAGCTAC-3′; reverse, 5′-CGGAAACAGCA CTCCTCAAC-3′. *PLAG1* mRNA expression was analysed using the following primer sets: forward, 5′-AACGTAAGCGTGGTGAAACC-3′; reverse, 5′-TGCCACATTCTTCGCACTTA-3′ (Zatkova *et al*, 2004). Polymerase chain reaction products were run on polyacrylamide gels and visualised after ethidium bromide staining. The intensity of each band was examined using a fluorescence image analyser, FLA-3000G (Fujifilm, Tokyo, Japan). Dividing the intensity of the target transcript by that of *GAPDH* calculated the level of each transcript.

Mutation analysis of the *CTNNB1* gene

To detect point mutations and deletions of the *CTNNB1* gene, genomic DNA from each tumour sample was amplified using two sets of primers, F1, 5′-TGGCTATCATTCTGCTTTTCTTG-3′ and R1, 5′-CTCTTTTCTTCACCACAACATTTT-3′, and BCAT-3, 5′-AA

AATCCAGCGTGGACAATGG-3′ and BCAT-4, 5′-TGTGGCAAGTT CTGCATCATC-3′, respectively (Koch *et al*, 1999; Satoh *et al*, 2003). The PCR products were either directly sequenced or inserted into a vector (pGEM (R)-T Easy Vector System (Promega, Madison, WI, USA)), and six or more clones were sequenced.

Statistical analysis

Student's *t*-test or Welch's *t*-test compared mRNA levels of *IGF2* and *H19* between tumours with or without *IGF2* alterations or other characteristics and the levels of *IGF2* promoter-specific transcripts between tumours with or without *PLAG1* mRNA expression. We also assessed the association between total *IGF2* mRNA levels and P2-, P3- or P4-specific *IGF2* mRNA levels by determining the Spearman rank correlation coefficient and associated *P*-value. Differences in the incidence of tumours with unmethylated P3 promoter were examined between tumours with hypermethylated *H19* DMR and tumours with normally methylated *H19* DMR by the χ^2 test. Differences in the incidences of tumours with *CTNNB1* mutation were examined between any two of three groups of tumours classified on the basis of the *IGF2* status by the χ^2 test.

RESULTS

Methylation status of the CTCF6 binding site at *H19* DMR, LOH analysis using SNP array and allelic expression analysis of *IGF2*

Combined bisulphite restriction assay showed that 21 and 33 tumours had hypermethylation and normal methylation at CTCF6, indicating LOH or LOI and retention of *IGF2* imprinting (ROI), respectively (Table 1 and Figure 1). Single nucleotide polymorphism array analysis was performed in 43 of 54 tumours; all 21 tumours with hypermethylated CTCF6 and 22 of 33 tumours with normally methylated CTCF6. Combined results of both analyses indicated that 12 tumours had LOH (10 hypermethylated CTCF6 and UPD 2 hypermethylated CTCF6 and hemizygous 11p15 deletion), 9 had LOI (hypermethylated CTCF6 and retention of heterozygosity (ROH)) and 22 had ROI (normally methylated CTCF6 and ROH). Of 21 tumours whose RNA was available, 9 and 12 tumours had heterozygous and homozygous *ApaI/AvaII* sites in exon 9 of *IGF2*, respectively. Of the nine heterozygous tumours, seven showed monoallelic expression of *IGF2*, indicating ROI, and two showed biallelic expression of *IGF2*, indicating LOI, and the results were consistent with those examined by COBRA and SNP array analyses (Table 1). From these findings, 11 tumours with normally methylated CTCF6, in which SNP array analysis was not performed, were classified as those with ROI. Thus, combined results of COBRA, SNP array and allelic expression analyses showed 12 tumours with LOH, 9 tumours with LOI and 33 tumours with ROI. In addition, one cell line (HuH6) had LOI, and the other (HepG2) had LOH (UPD) of *IGF2*.

The mean age was compared between any two of three groups of patients (i.e., LOH, LOI or ROI) by Student's *t*-test. There was no difference in the mean age between any two of the three groups of patients.

Correlation between *IGF2* and *H19* mRNA levels and the *IGF2* status (LOH, LOI or ROI)

Quantitative real-time reverse transcription-PCR analysis showed that although 15 of 20 tumours had a higher level of *IGF2* mRNA than normal liver tissues, 15 of 20 tumours had a lower level of *H19* mRNA than normal liver tissues (Table 1 and Figure 2). All 3 tumours with UPD, 1 of 1 with 11p15 loss, 1 of 3 with LOI and 10 of 13 with ROI, expressed higher levels of *IGF2* mRNA than normal liver tissues. There was no significant difference in *IGF2* mRNA levels between 3 tumours with UPD or 7 tumours with *IGF2* alterations; that is UPD, 11p15 loss or LOI, and 13 tumours with ROI. In contrast, 7 tumours with *IGF2* alterations expressed very low levels of *H19* mRNA, whereas 11 of 13 tumours with ROI expressed a substantial amount of *H19* mRNA; 2 tumours (nos. 25 and 27) with ROI expressed very low levels of *H19* mRNA. *H19* mRNA levels were higher in 13 HB tumours with ROI than in 7 HB tumours with *IGF2* alterations ($P<0.01$ by Welch's *t*-test). Although HepG2 with UPD had a higher level of *IGF2* mRNA than normal liver tissues, HuH6 with LOI had a very low level of *IGF2* mRNA. *H19* mRNA levels were very low in both cell lines.

Semiquantitative RT–PCR analysis of promoter-specific *IGF2* transcripts

Because the *IGF2* gene has four kinds of promoters, promoter-specific *IGF2* transcripts were analysed to determine the usage of each promoter. Representative results of the P3 transcript are shown in Figure 3A. All 20 tumours showed undetectable or lower levels of P1 transcripts than 3 normal liver tissues. The levels of P2, P3 and P4 transcripts were higher in 13, 15 and 10 of the 20 tumours, respectively, than those of normal liver tissues. Polymerase chain reaction cycle numbers to obtain visible levels of PCR products were 40 for P2 transcripts, 30 for P3 transcripts and 35 for P4 transcripts, indicating that the amounts of P3 transcripts were high, those of P2 transcripts were low and those of P4 transcripts were intermediate. The Spearman correlation coefficient analysis showed that the expression levels of the P2, P3 and P4 transcripts correlated with the levels of total *IGF2* mRNA (P2, $rS=0.730$; P3, $rS=0.773$ and P4, $rS=0.646$) (Figure 3B and C; data for the P2 and P4 transcripts are not shown).

The methylation status of *IGF2* promoters and its correlation with the levels of promoter-specific transcripts

In the MSP analysis of each promoter, the P2 promoter region was partially methylated in 19 tumours and normal liver tissues and the P4 promoter region was unmethylated in all 20 tumours and normal liver tissues. Therefore, the methylation status of P2 or P4 promoter region was not correlated with the expression level of P2- or P4-specific transcripts. The P3 promoter region was partially methylated in 11 tumours, HuH6 and normal liver tissues and unmethylated in 9 tumours and HepG2 (Table 1, Figure 4A and B). The results of MSP analysis in one tumour (no. 1) and HuH6 were confirmed by bisulphite sequencing (Figure 4C). Nine tumours with the unmethylated P3 promoter had higher levels of P3 transcripts than 11 tumours with the partially methylated P3 promoter ($P=0.005$) (Figure 4D). The P3 promoter was unmethylated in 5 of 7 tumours with *IGF2* alterations; UPD, 11p15 loss or LOI, but in 4 of 13 tumours with ROI. Thus, the incidence of tumours with unmethylated P3 promoter tended to be higher in tumours with hypermethylated *H19* DMR than in tumours with normally methylated *H19* DMR ($P=0.1$).

Semiquantitative RT–PCR analysis of *PLAG1* mRNA

PLAG1 positively regulates *IGF2*, and its expression was detected in 12 tumours, foetal liver RNA and 2 cell lines, but not in 8 tumour and 3 normal liver tissues (Table 1 and Figure 5). The 12 tumours with *PLAG1* mRNA expression showed higher levels of P4-specific *IGF2* transcripts ($P=0.01$) and tended to show higher levels of P3-specific *IGF2* transcripts ($P=0.051$) than the 8 tumours without *PLAG1* expression. There was no significant difference in P2- or P1-specific transcript levels between tumours with and without *PLAG1* mRNA expression.

Table 1 Genetic and epigenetic status of the *IGF2-H19* region in 54 hepatoblastoma tumours

Patients number	Age[a]/sex	Chemo[b]	%methyl CTCF6[c]	11p15 SNP[d]	ApaI site[e]	IGF2 RT-PCR	IGF2 status[f]	IGF2 mRNA	P1E[g]	P2M[h]	P2E	P3M	P3E	P4E	H19 mRNA	PLAG1 mRNA	CTNNB1 status[i]
1	48/F	+	82.8	UPD	Homo	ND	UPD	11.3	0.6	MU	17.7	U	7	3.1	0	+	M
2	5/M	–	93.4	UPD	Homo	ND	UPD	3.9	0	MU	0	U	2.1	0.9	0	–	M
3	24/M	+	76.7	UPD	Homo	ND	UPD	3.2	0	MU	2.4	MU	2.3	1.2	0	+	M
4–10	5–96/M6, F1	+6, –1	72–90	UPD	ND	ND	UPD	ND	ND	ND	ND	ND	ND	ND	ND	ND	M4, N3
11	27/M	+	87.8	Loss chr II	Homo	ND	Loss	6.1	0	MU	2.3	U	4.4	1.3	0	+	M
12	24/M	+	81.9	Loss chr II	ND	ND	Loss	ND	ND	ND	ND	ND	ND	ND	ND	ND	M
13	12/F	+	91.4	ROH	Homo	ND	LOI (m)	9.7	0	U	1.1	U	2.3	0.6	0	–	ND
14	16/M	–	86.1	ROH	Homo	ND	LOI (m)	1	0	MU	0.3	U	1.3	0.9	0	–	ND
15	26/F	+	83.1	ROH	Hetero	LOI	LOI (m, p)	0.8	0	MU	0.4	MU	0.6	0.9	0	–	M
16	24/M	+	70.9	ROH	Hetero	LOI	LOI (m, p)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M
17–21	12–84/M4, F1	+1, –3, UK1	71–91	ROH	ND	ND	LOI (m)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M3,N2
22	12/F	+	52.5	ND	Homo	ND	ROI (m)	9.2	0.8	MU	8.4	MU	2.8	2.2	3.5	+	N
23	109/F	+	49.1	ND	Homo	ND	ROI (m)	8.4	0	MU	4.3	U	4.6	2.8	0.5	+	M
24	12/M	–	56.3	ND	Hetero	ROI	ROI (m, p)	7.4	0	MU	13.2	U	4.6	2.4	1.9	+	M
25	15/M	+	55.9	ROH	Hetero	ROI	ROI (m, p)	5.7	0	MU	6.5	MU	5.1	2.2	0	+	ND
26	6/M	UK	51.1	ND	Hetero	ROI	ROI (m, p)	5.6	0.1	MU	4.7	U	1.2	0.5	2.7	+	ND
27	10/M	+	62.1	ROH	Homo	ND	ROI (m)	5	0	MU	0.9	U	5	1.1	0	–	ND
28	29/F	+	61.5	ND	Homo	ND	ROI (m)	3.7	0	MU	1.5	MU	2.5	1.4	2.5	+	M
29	26/M	+	55.4	ND	Homo	ND	ROI (m)	3.1	0.2	MU	9.3	MU	0.4	1.2	0.7	–	M
30	18/M	+	48.7	ND	Hetero	ROI	ROI (m, p)	2.4	0.1	MU	1.2	MU	1.1	1	0.8	–	ND
31	13/M	+	55.3	ND	Hetero	ROI	ROI (m, p)	2.2	0	MU	1.1	MU	1.5	1	0.6	+	N
32	60/F	+	55.7	ND	Hetero	ROI	ROI (m, p)	0.7	0.2	MU	0.2	MU	0.3	0.9	0.4	+	N
33	29/M	+	56.4	ND	Homo	ND	ROI (m)	0.5	1	MU	0.1	MU	0	0.5	1.2	–	M
34	9/M	+	56.7	ND	Hetero	ROI	ROI (m, p)	0.5	0	MU	0	MU	0.1	0.2	0.2	+	M
35–54	4–156/M11, F9	+12, –7, UK1	41–65	ROH	ND	ND	ROI (m)	ND	ND	ND	ND	ND	ND	ND	ND	ND	M11, N9
Normal livers			52.8	ND	ND	ND	ROI (m)	1	1	MU	1	MU	1	1	1	–	
Fetal livers			ND		ND	ND	ND	5.9	0.2	ND	6.4	ND	4.8	1.9	2.4	ND	
HuH6			87.3	ROH	Hetero	LOI	LOI (m, p)	0	0	MU	0	MU	0	0.1	0	+	M
HepG2			89.5	UPD	Homo	ND	UPD (m)	2.4	0	U	1.5	U	5	2.8	0	+	M

F = female; M = male; M = methylated; ROH = retention of heterozygosity; ROI = retention of imprinting; U = unmethylated; ND = not done; UK = unknown. All 20 tumours showed unmethylated promoter 4; UPD, uniparental disomy; loss chr 11, loss of chromosome 11or 11p15; LOI, loss of imprinting. [a]Age in months. [b]Chemo, chemotherapy before surgery; +6, –1 indicates that six and one tumours were treated and untreated, respectively, with chemotherapy before surgery. [c]%methyl CTCF6 indicates % methylated CTCF6 allele. [d]Results of SNP array. [e]Homo, homozygosity at *ApaI*/*AvaII* site; hetero, heterozygosity. [f]Results of SNP array analysis, methylation analysis of CTCF6 (m) and *ApaI*/*AvaII* polymorphism site analysis (p). [g]P1E, promoter 1-specific transcript. [h]P2M, the methylation status of promoter 2. [i]*CTNNB1* status: M, mutated; N, normal.

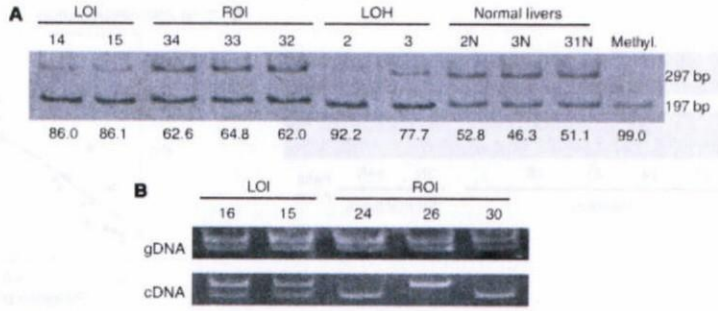

Figure 1 Analysis of *IGF2* alterations. (**A**) Examples of the methylation status of CTCF6 analysed by a combined bisulphite restriction assay (COBRA). Bisulphite-modified PCR products were digested with *MluI*. Upper and lower lanes indicate unmethylated and methylated fragments, respectively. Numbers above lanes indicate the tumour number. Numbers below lanes show the percentage of methylated DNA fragments containing CTCF6. The mean value of the DNA methylation percentages calculated from three COBRA experiments is shown in Table 1. MethyL, control methylated DNA. The *IGF2* status is shown above the tumour numbers. LOI, loss of *IGF2* imprinting; LOH, loss of heterozygosity in the *IGF2* region; ROI, retention of *IGF2* imprinting. (**B**) Electrophoretic pattern of genomic DNA PCR products or RT–PCR products after *AvaII* digestion. Reverse transcriptase–PCR analysis shows LOI in two tumours and ROI in three tumours.

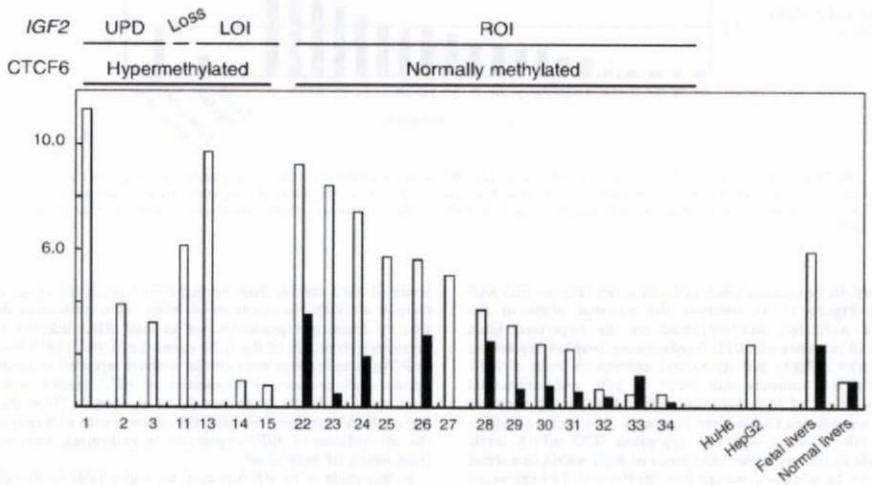

Figure 2 Results of quantitative real-time RT–PCR analysis of *IGF2* and *H19* mRNAs. Relative mRNA (*Y* axis) of total *IGF2* (open rectangles) and *H19* (closed rectangles) is plotted in 3 tumours with UPD, in 1 tumour with 11p15 loss, in 3 tumours with LOI, in 13 tumours with ROI, in 2 cell lines, in foetal liver total RNA and in adjacent normal liver tissues (a mean value of 3 samples). Tumours in each group are arranged in order by the levels of *IGF2* mRNA. Numbers below *X* axis indicate the tumour number shown in Table 1. *IGF2* status (UPD, loss of 11p15, LOI and ROI) and methylation status of CTCF6 at *H19* DMR (hypermethylated or normally methylated) are shown above the graph. Nine tumours (nos. 1–3, 11, 13–15, 25 and 27) and two cell lines expressed a minimal amount of *H19* mRNA, which was shown as zero in the graph. Similarly, HuH6 expressed a minimal amount of *IGF2* mRNA, which was shown as zero in the graph.

Incidences of tumours with *CTNNB1* mutation between any two groups of tumours classified on the basis of the *IGF2* status

DNA was available for *CTNNB1* mutation analysis in 48 of 54 HB tumours. The results are described in Table 1. There were no differences in the incidences of *CTNNB1* mutation between 7 tumours with *IGF2*-LOI and 29 tumours with *IGF2*-ROI or 12 tumours with *IGF2*-LOH, and between 29 tumours with *IGF2*-ROI and 12 tumours with *IGF2*-LOH.

DISCUSSION

In this study, biallelic and monoallelic *IGF2* expressions correlated with hypermethylation and normal methylation of CTCF6,

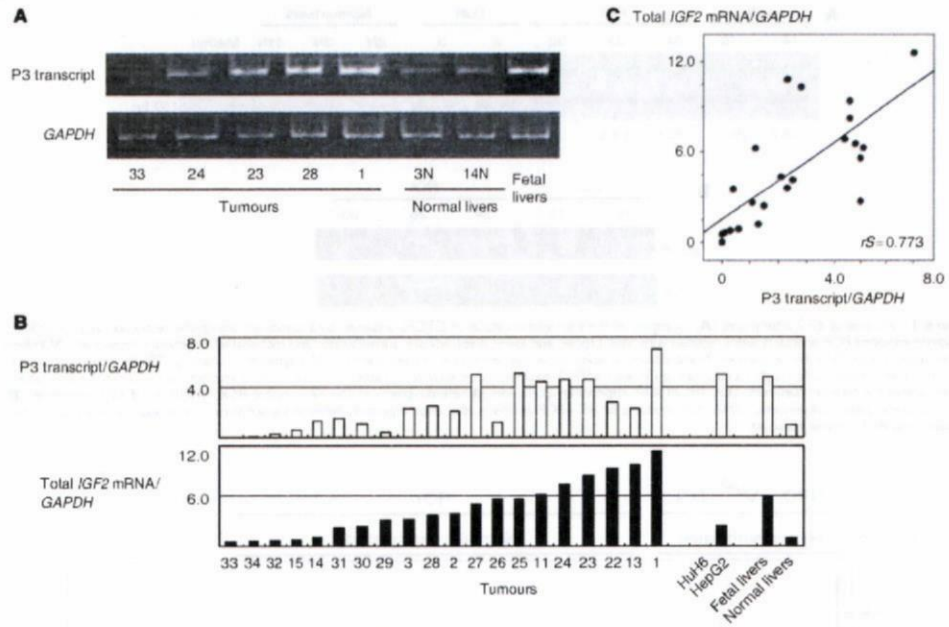

Figure 3 (**A**) Representative data of RT–PCR analysis of P3 transcripts. (**B**) Expression levels of P3 transcripts (upper lane) and total *IGF2* mRNA (lower lane) are plotted in 20 tumours, in 2 cell lines, in foetal liver tissues and normal liver tissues (a mean value of 3 samples). Tumours are arranged in order by total levels of *IGF2* mRNA. Numbers below *X* axis indicate the tumour number. (**C**) Correlation between levels of P3 transcript (*X* axis) and total *IGF2* mRNA (*Y* axis).

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respectively, in two tumours with LOI and seven tumours with ROI (Table 1, Figure 1). In addition, the paternal origin of the duplicated *IGF2* loci was confirmed by the hypermethylated CTCF6 in 10 tumours with UPD. Furthermore, very low expression levels of *H19* mRNAs and substantial expression levels of *IGF2* mRNAs in HB tumours with UPD or LOI, and substantial expression levels of both *IGF2* and *H19* mRNA in HB tumours with ROI were found (Table 1 and Figure 2). Two (nos. 14 and 15) of three HB tumours with LOI expressed *IGF2* mRNA levels comparable to but not higher than those of *IGF2* mRNA in normal liver tissues. In addition, one cell line, HuH6, with LOI expressed minimal expression of *IGF2* mRNA, although Hartmann *et al* (2000) found the moderate expression in the same cell line. These findings may be explained by the speculation that such tumours expressed increased levels of *IGF2* mRNA at the critical time of tumorigenesis, but not at the time of surgical resection or after many passages of cell culture. From these findings, the hypothesis established for WT that the hypermethylation of maternal *H19* DMR causes LOI, and that LOI or duplication of paternal *IGF2* (UPD) results in overexpression of *IGF2*, may be also applied to HB.

Although the expression levels of *IGF2* mRNA were reported to be higher in WTs with UPD than in WTs with ROI in two series of WTs (Wang *et al*, 1996; Haruta *et al*, 2008), conflicting results were reported in *IGF2* mRNA levels between WTs with LOI and WTs with ROI (Wang *et al*, 1996; Ravenel *et al*, 2001). The present and earlier studies showed that all HB tumours with UPD and the majority of HB tumours with LOI or ROI expressed the higher levels of *IGF2* mRNA than normal liver tissues (Li *et al*, 1998b; Gray *et al*, 2000; Hartmann *et al*, 2000). This study also showed that P3 transcripts predominated in total *IGF2* mRNAs in HB tumours irrespective of the *IGF2* status (i.e., UPD, 11p15 loss, LOI or ROI); these findings were similar to those reported in foetal liver tissues showing elevated expression of *IGF2* mRNA with predominance of the P3 transcript (Li *et al*, 1998a). Thus, the high *IGF2* mRNA expression of many HB tumours with ROI may mimic the upregulation of *IGF2* expression in embryonic liver tissues, from which HB may arise.

In this study of 54 HB tumours, we found LOH in 12 (22.2%), LOI in 9 (16.7%) and ROI in 33 (61.1%). Hepatoblastoma tumours can be classified into those with LOH and those with ROH, and tumours with ROH can be further classified into those with LOI and those with ROI. For data comparison, the frequencies of LOH and LOI in the earlier and present series of HB tumours are shown in Tables 2 and 3, respectively (Davies, 1993; Montagna *et al*, 1994; Li *et al*, 1995; Rainier *et al*, 1995; Fukuzawa *et al*, 1999; Gray *et al*, 2000; Hartmann *et al*, 2000; Ross *et al*, 2000; Albrecht *et al*, 2004; Suzuki *et al*, 2008). Both frequencies of LOH and LOI were similar between the earlier and present series of HB tumours. When we compared the frequencies of LOH and LOI between HB and WT, the frequencies of LOH and LOI are lower in HB tumours than in WT tumours (Table 4). The present and earlier studies showed that levels of *IGF2* mRNA are higher in normal liver tissues than in normal kidney tissues, and in foetal liver tissues than in foetal kidney tissues, but showed similarly high levels in both WTs and HBs (part of the data not shown) (Hedborg *et al*, 1994; Haruta

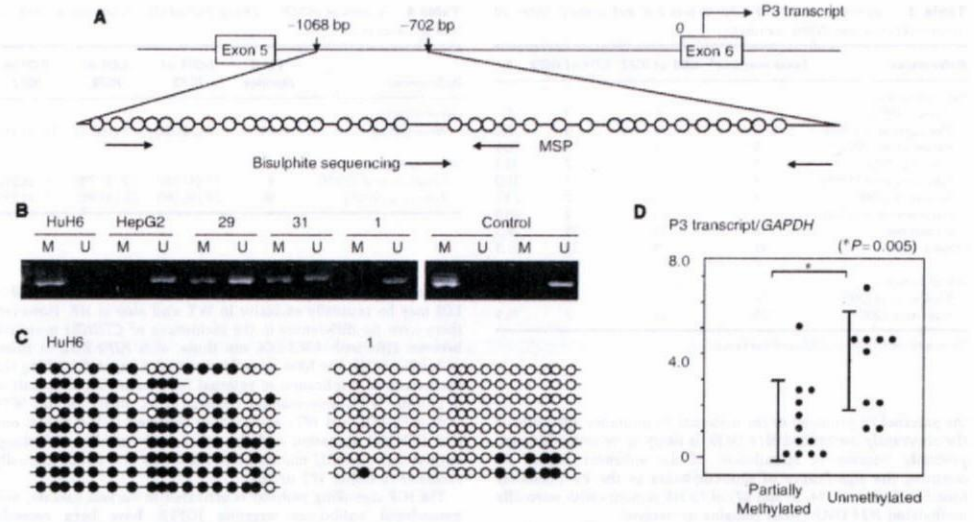

Figure 4 (**A**) Diagram of the *IGF2* P3 promoter region. Individual CpG dinucleotides located upstream of exon 6 (from −1068 to −702 bp) are represented by circles. Horizontal arrows indicate locations of PCR primers used for MSP and bisulphite sequencing. (**B**) Examples of the promoter methylation status using methylation-specific PCR. Polymerase chain reaction products of methylated or unmethylated P3 promoters from HB tumours are shown. Numbers above horizontal bars indicate the tumour number. M, methylated promoter; U, unmethylated promoter. (**C**) Bisulphite sequencing analysis of the methylation status of P3 promoter in HuH6 and one tumour (no. 1), which displayed complete methylation and complete unmethylation, respectively. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. (**D**) Levels of P3 transcripts in tumours with partially methylated P3 promoter and tumours with unmethylated P3 promoter.

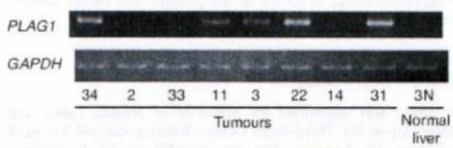

Figure 5 Representative data of RT–PCR analysis of *PLAG1* mRNA. Numbers below lanes indicate the tumour number.

Table 2 Incidences of LOH of *IGF2* in previous and present series of hepatoblastoma and Wilms' tumours

References	Total number	LOH of *IGF2*[a]	No-LOH of *IGF2*	%
Hepatoblastoma				
Montagna *et al* (1994)	13	3	10	23.1
Fukuzawa *et al* (1999)	7	2	5	28.6
Gray *et al* (2000)	10	2	8	20.0
Hartmann *et al* (2000)	24	6	18	25.0
Albrecht *et al* (2004)	56	13	43	23.2
Suzuki *et al* (2008)	17	4	13	23.5
Total number	127	30	97	23.6
Present study	54	12	42	22.2
Wilms' tumour				
Grundy *et al* (1996)	260	93	167	35.8
Yuan *et al* (2005)	62	26	36	41.9

[a]Tumours with LOH of 11p15, but no informative *IGF2* locus are included.

et al, 2008), indicating that embryonal kidney tissues might be more susceptible to IGF2 stimulation than embryonal liver tissues. These findings might be related to higher incidences of UPD or LOI in WT than in HB.

The *IGF2* gene has four promoter regions and each promoter can initiate transcription producing a distinct *IGF2* transcript with different 5′-untranslated regions with a common translated region in the 3′-side (Li *et al*, 1998a). The *IGF2* gene is transcriptionally regulated in a development-dependent and tissue-specific manner. In the foetal liver, promoters P2, P3, and P4 are active and expressed monoallelically; P3 is the most active promoter and P1 is inactive. However, in the adult liver, P1 becomes dominant and is biallelically expressed, and P2, P3 and P4 activities are decreased or lost (Li *et al*, 1998a). In foetal liver tissues, P3 promoter methylation is inversely correlated with the P3 transcript expression. The inverse correlation between P3 promoter methylation and P3 transcript expression was reported earlier in seven HB tumours (Li *et al*, 1998b). This study confirmed the upregulation of P2, P3 and P4 transcripts and downregulation of P1 transcript, and the inverse correlation between P3 promoter methylation and P3 transcript expression in the majority of 20 HB tumours. Although P2, P3 and P4 transcripts were all correlated to the total amount of *IGF2* mRNAs, the earlier and present studies showed that the P3 transcript was most abundant and seemed to play a major role in the tumorigenesis of HB (Li *et al*, 1998b). Increased *IGF2* expression with the predominant P3 transcript was reported earlier in WTs with LOI or ROI (Vu and Hoffman, 1999). This study also showed that HB tumours with hypermethylated *H19* DMR tended to have an unmethylated P3 promoter, indicating that

Table 3 Incidences of LOI of *IGF2* in previous and present series of hepatoblastoma and Wilms' tumours

References	Total number[a]	LOI of *IGF2*	ROI of *IGF2*	%
Hepatoblastoma				
Davies (1993)	3	0	3	0
Montagna *et al* (1994)	5	1	4	20.0
Ranvier *et al* (1995)	5	1	4	20.0
Li *et al* (1995)	3	1	2	33.3
Fukuzawa *et al* (1999)	4	1	3	25.0
Ross *et al* (2000)	13	3	10	23.1
Hartmann *et al* (2000)	5	3	2	60.0
Total number	38	10	28	26.3
Present study	42	9	33	21.4
Wilms' tumour				
Ravenel *et al* (2001)	36	15	21	41.7
Yuan *et al* (2005)	29	22	7	75.9

[a]Tumours with LOH of *IGF2* were excluded.

Table 4 Incidences of LOH, LOI and ROI of *IGF2* in hepatoblastoma and Wilms' tumours

References	Total number	LOH of *IGF2*	LOI of *IGF2*	ROI of *IGF2*
Hepatoblastoma				
Present study	54	12 (22.2%)	9 (16.7%)	33 (61.1%)
Wilms' tumour				
Fukuzawa *et al* (2004)	41	17 (41.5%)	13 (31.7%)	11 (26.8%)
Yuan *et al* (2005)	58	29 (50.0%)	22 (37.9%)	7 (12.1%)

the paternal P3 promoter or the maternal P3 promoter upstream of the aberrantly methylated *H19* DMR is likely to be unmethylated, probably because of stimulation of the enhancer signal. In contrast, the significance of unmethylation in the P3 promoter found in 4 (nos. 23, 24, 26 and 27) of 13 HB tumours with normally methylated *H19* DMR (ROI) remains unresolved.

PLAG1 located in 8q11 encodes a developmentally regulated transcription factor, and positively regulates *IGF2*. The P3 promoter region of *IGF2* contains *PLAG1* consensus-binding sites, and PLAG1 transactivates the transcription from embryonic *IGF2* promoter P3 in HB cell lines, HuH6 and HepG2 (Zatkova *et al*, 2004). *PLAG1* mRNA was highly expressed in most HB tumours compared with normal liver tissues. In this study, HB tumours with *PLAG1* mRNA expression showed and tended to show higher levels of P4 and P3 transcripts, respectively. Thus, the correlation of *PLAG1* mRNA expression with increased levels of P3 transcripts reported by Zatkova *et al* (2004) may be confirmed; furthermore, the correlation of *PLAG1* mRNA expression with increased levels of P4 transcripts was also suggested.

WTs can be classified at least into two groups; one has intralobar nephrogenic rest that is associated with *WT1* abnormality and the other has perilobar nephrogenic rest associated with *IGF2*-LOI (Ravenel *et al*, 2001). *CTNNB1* mutation is frequently found in WTs with *WT1* abnormality, but rare in WTs without *WT1* abnormality (Maiti *et al*, 2000). These findings suggest that WTs with no *WT1* abnormality may include a substantial number of tumours with *IGF2*-LOI, and that *CTNNB1* mutation and *IGF2*-LOI may be mutually exclusive in WT and also in HB. However, there were no differences in the incidences of *CTNNB1* mutation between HBs with *IGF2*-LOI and those with *IGF2*-ROI, or those with *IGF2*-LOH. We have recently reported a paper describing the occurrence of duplication of paternal *IGF2* or *IGF2*-LOI in half of WTs with *WT1* abnormalities (Haruta *et al*, 2008). Of two WTs with *IGF2*-LOI and *WT1* abnormality reported in that paper, one had *CTNNB1* mutation and the other had not. These findings suggest that *CTNNB1* mutation and *IGF2*-LOI may not be mutually exclusive in either WT or HB.

The IGF signalling pathway is activated in various cancers, and monoclonal antibodies targeting IGF1R have been recently developed; IGF1R is a transmembrane tyrosine kinase receptor, and both IGF1 and IGF2 are ligands for IGF1R (Foulstone *et al*, 2005). Early clinical trials using anti-IGF1R monoclonal antibodies showed promising results in refractory Ewing's sarcomas and rhabdomyosarcomas (Ryan and Goss, 2008). Because 20–30% of HB tumours do not respond to the current chemotherapy consisting of cisplatin and adriamycin (Perilongo *et al*, 2000; Fuchs *et al*, 2002), and the great majority of HB tumours overexpresses *IGF2*, as shown in the present and earlier studies, HB may be the next target tumour for antibody therapy.

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Plasma midkine level is a prognostic factor for human neuroblastoma

Shinya Ikematsu,[1] Akira Nakagawara,[2] Yohko Nakamura,[2] Miki Ohira,[2] Masaki Shinjo,[3] Satoshi Kishida[4] and Kenji Kadomatsu[4,5]

[1]Department of Bioresources Engineering, Okinawa National College of Technology, Okinawa 905-2192; [2]Division of Biochemistry, Chiba Cancer Research Center Research Institute, Chiba 260-8717; [3]Department of Public Health and Epidemiology, Okinawa Prefectural College of Nursing, Okinawa 902-0076; [4]Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

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Neuroblastoma is the third-most-common solid tumor of childhood. To date, no reliable blood marker for neuroblastoma has been established. The growth factor midkine is highly expressed in human carcinomas and its knockdown leads to tumor growth suppression in animal models. The present study evaluated the plasma midkine level in human neuroblastoma patients. Plasma samples were obtained from patients found through mass screening, as well as from sporadic neuroblastoma patients. The total number of cases examined was 756. Among them, prognostic information was available for 175 sporadic cases and 287 mass-screening cases. Midkine levels were significantly higher in neuroblastoma patients, including both mass-screening cases and sporadic cases, than in non-tumor controls ($P < 0.0001$). The midkine level was significantly correlated with the statuses of *MYCN* amplification, *TRKA* expression, ploidy, stage and age ($P < 0.0001$, < 0.0001, $= 0.004$, < 0.0001 and < 0.0001, respectively), which are known prognostic factors for neuroblastoma. There was a striking correlation between high plasma midkine level and poor prognosis ($P < 0.0001$). Within sporadic cases, the midkine level was also strikingly higher than in non-tumor controls ($P < 0.0001$), and correlated with the statuses of *MYCN* amplification and stage ($P = 0.0005$ and $= 0.003$, respectively). There was a significant correlation between high plasma midkine level and poor prognosis ($P = 0.04$). Taken together, the present data indicate that plasma midkine level is a prognostic factor for human neuroblastoma. (*Cancer Sci* 2008; 99: 2070–2074)

Neuroblastoma (NBL) is the third-most-common malignant tumor of childhood, accounting for 15% of cancer-related death.[1] In spite of an enormous amount of research devoted to curing this disease, its prognosis remains poor. NBL has several established prognostic factors, i.e. *MYCN* amplification, *TRKA* expression level, ploidy, stage and age.[1,2] Cases with tumors with an amplified *MYCN* gene, low *TRKA* expression or diploidy show poor prognosis. Cases at stage 3 or 4, or at ages older than 18 months also show poor prognosis. Since molecular fingerprints within tumor tissues, such as *MYCN* amplification, *TRKA* expression level and ploidy, require a tumor biopsy or its removal, a blood marker for NBL has long been awaited.[1,2] A blood marker would not only be useful for the initial diagnosis but would also be beneficial for the sequential monitoring of the tumor status.

The growth factor midkine (MK) was originally found in embryonal carcinoma cells, and has been implicated in cancer development.[3–5] MK is highly and frequently expressed in human carcinomas, including Wilms' tumor, tumors of the digestive tract, brain tumors, urinary bladder tumors and breast tumors, whereas its expression is scarcely detected in normal adult tissues.[6–10] Strong MK expression is also detected in pre-cancerous stages of human colorectal cancer and human prostate cancer.[11,12] Knockdown of MK expression leads to suppression of xenografted tumors of mouse colorectal cancer cells and human prostate cancer cells.[13,14]

We previously reported that the plasma MK level was correlated with the values of established prognostic factors through a study of 220 cases, including 82 non-mass-screening (sporadic) cases and 122 mass-screening cases.[15] However, in that study, information on the prognosis of patients was too limited to determine whether the plasma MK level could be a prognostic factor. In the present study, we measured plasma MK levels of 756 NBL cases, which consisted of 286 sporadic cases, 387 mass-screening cases and 83 unknown cases. Among them, prognostic information was available for 175 sporadic cases and 287 mass-screening cases. This enabled us to evaluate the plasma MK level as a prognostic factor.

Mass screening for NBL started in 1985 in Japan, but was discontinued in 2004 because of the lack of apparent beneficial effects on the cure rate of NBL. Mass-screening cases are grouped into the favorable prognosis group, and most of the mass-screening cases are thought to have spontaneously regressed. Therefore, nowadays, sporadic NBL patients are the major subject of therapy. However, information obtained from mass-screening cases has been useful, especially to understand tumor phenotype with favorable prognosis. This is the reason why we enrolled 387 mass-screening patients in this study. Accordingly, we evaluated plasma MK levels in two categories: first, the entire set of NBL cases including mass-screening cases and sporadic cases; and second, the set of sporadic cases.

Here we report that the plasma MK level is a prognostic factor for NBL.

Materials and Methods

Plasma samples. Clinical data of 756 neuroblastoma patients are summarized in Table 1. The same archive samples were used as those without malignant tumors ($n = 17$; eleven were <1-year old and six were >1-year old).[15]

Enzyme-linked immunoassay for human MK. An enzyme-linked immunoassay for human MK was performed as described previously.[16] Briefly, human MK was produced using *Pichia pastoris* GS115 by transfection with a human MK expression vector, which was constructed into pHIL-D4 (Invitrogen, Carlsbad, CA, USA). This yeast-produced human MK was used to immunize rabbits and chickens to raise antibodies. The rabbit antihuman MK antibody (50 μL of 5.5 μg/mL in 50 mM Tris HCl (pH 8.2), 0.15 M NaCl, 0.1% NaN_3) was coated onto the wells of microtiter plates (Polysorp plates, Nunc, Rochester, New York, USA) for 20 h at room temperature. After washing with 0.05% Tween-20 in phosphate-buffered saline (PBS), the wells were

[5]To whom correspondence should be addressed.
E-mail: kkadoma@med.nagoya-u.ac.jp

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Table 1. Plasma samples

	n
No malignant tumors	17
<1 year	11
>1 year	6
Neuroblastomas	756
Stage 1,2,4S	372
Stage 3,4	330
Unknown	54
MYCN amplification –	643
MYCN amplification +	97
Unknown	16
High *TrkA* expression	425
Low *TrkA* expression	159
Unknown	172
Mass screening	387
Sporadic	286
Stage 1,2,4S	62
Stage 3,4	209
Unknown	15
MYCN amplification –	207
MYCN amplification +	73
Unknown	6
High *TrkA* expression	109
Low *TrkA* expression	113
Unknown	64
Hyperdiploidy/pentaploidy	96
Diploidy/tetraploidy	136
Unknown	54
<18 months	101
>18 months	183
Unknown	2
Unknown	83
Hyperdiploidy/pentaploidy	379
Diploidy/tetraploidy	263
Unknown	114
<18 months	506
>18 months	242
Unknown	8

blocked with 300 μL of 0.1% casein, 0.01% Microcide I (aMReSCO) in PBS for 20 h at 37 C. Plasma samples (10 μL each) were mixed with 100 μL of 50 mM Tris HCL (pH 8.4), 0.5 M KCl, 0.1% casein, 0.5% bovine serum albumin (BSA), 0.01% Microcide I and 0.1 μg/mL peroxidase-labeled chicken antihuman MK antibody. Aliquots of 50 μL of this mixture were added to wells prepared as described above, and further subjected to chromogenic detection at optical density at 450 nm (OD_{450}) using tetramethylbenzidine as the substrate. This assay system shows linearity from 0 to 5 ng/mL of MK, and there is no cross-reaction with pleiotrophin, a close homolog of MK.[5]

Statistical analysis. The Kruskal–Wallis test was used to evaluate the statistical differences between stages. The Mann–Whitney *U*-test was used to further evaluate the difference between the two groups. The Mann–Whitney *U*-test was used for analysis of the other prognostic factors. Survival time was measured from the date of initial diagnosis to the date of death or last contact. The Kaplan–Meier method was used to compare survival between the groups defined by plasma MK levels, and survival differences were analyzed using the log-rank test. All analyses were carried out using StatView for Windows (ver. 5.0; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Plasma MK levels of NBL patients and the relationship of plasma MK to established prognostic factors for NBL. The entire set of 756 NBL cases consisted of 387 cases found through mass screening, 286 sporadic NBL cases and 83 unknown cases (Table 1). Plasma MK level of the NBL cases was 23–1 062 520 pg/mL, whereas that of non-tumor controls was 146–517 pg/mL (Fig. 1a). The values of NBL cases were significantly higher than those of controls ($P < 0.0001$). We set the cut-off value average ± 4SD of non-tumor controls at 900 pg/mL (Fig. 1a). The group of cases with levels higher than 900 pg/mL was designated high MK, whereas cases with lower than 900 pg/mL were grouped into low MK.

MYCN amplification, *TRKA* expression level, ploidy, stage and age are well-known prognostic factors for NBL.[1] The values of each factor were determined for all 756 NBL cases. As shown in Figure 1(b–f), MK levels were significantly correlated with all the prognostic factors. Thus, MK levels were significantly higher in *MYCN*-amplified cases ($P < 0.0001$, *versus* *MYCN*-nonamplified), in cases with low *TRKA* expression ($P < 0.0001$, *versus* high *TRKA* expression), in diploidy cases ($P = 0.004$), in cases at stage 3 and 4 ($P < 0.0001$, *versus* stage 1, 2, and 4S) and in cases older than 18 months ($P < 0.0001$, *versus* younger than 18 months). These groups in which MK levels were high, i.e. *MYCN*-amplified, low *TRKA* expression, diploidy, stage 3 and 4 and older than 18 months, are known to have a poor prognosis. The data indicate close correlations between MK levels and known prognosis factors and are consistent with our previous report.[13]

Figure 2(a) shows Kaplan–Meier survival curves based on plasma MK levels for all NBL cases. A high MK level was closely associated with poor prognosis of NBL patients ($P < 0.0001$), indicating that the MK level alone can be a prognostic factor for NBL patients. It was interesting that a high MK level was associated with poor prognosis within the unfavorable NBL group based on ploidy, i.e. diploidy ($P = 0.02$) (Fig. 2b). This was also the case within favorable NBL groups, that is, groups with *MYCN* non-amplification, age <18 months or high *TRKA* expression ($P = 0.02$, 0.001 or 0.02, respectively), although the survival differences between high MK and low MK were very small (data not shown).

Analysis for sporadic NBL cases. We examined 286 sporadic NBL cases, among which prognostic information was available for only 175. Plasma MK level was significantly higher in sporadic NBL cases than in non-tumor controls ($P < 0.0001$) (Fig. 3a). It was closely related to the values of two prognostic factors, i.e. *MYCN* amplification and stage ($P = 0.0005$ and 0.003, respectively) (Fig. 3b,c), but not to those of age, *TRKA* expression level and ploidy (data not shown).

Kaplan–Meier analysis revealed that a high MK level was correlated with poor prognosis in the sporadic NBL patients ($P = 0.04$) (Fig. 4a). The Kaplan–Meier data on MK was further compared with those on known prognostic factors. Survival based on ploidy exhibited a significant difference ($P = 0.025$) (Fig. 4b). *MYCN* amplification, *TRKA* expression level and stage also showed significant differences ($P = 0.003$, 0.01 and 0.008, respectively), whereas age could not be a prognostic factor for the sporadic NBL cases examined (data not shown). The Cox hazard ratio was 1.71 for MK level, 2.27 for ploidy, 2.70 for *MYCN* amplification, 2.38 for *TRKA* expression and 1.84 for stage.

Discussion

In the present study, we first evaluated the plasma MK level using the entire set of NBL cases including both the mass screening and sporadic cases. As predicted from our previous data,[15] we found that MK level is correlated with established prognostic factors (*MYCN*, *TRKA*, ploidy, stage and age). Since

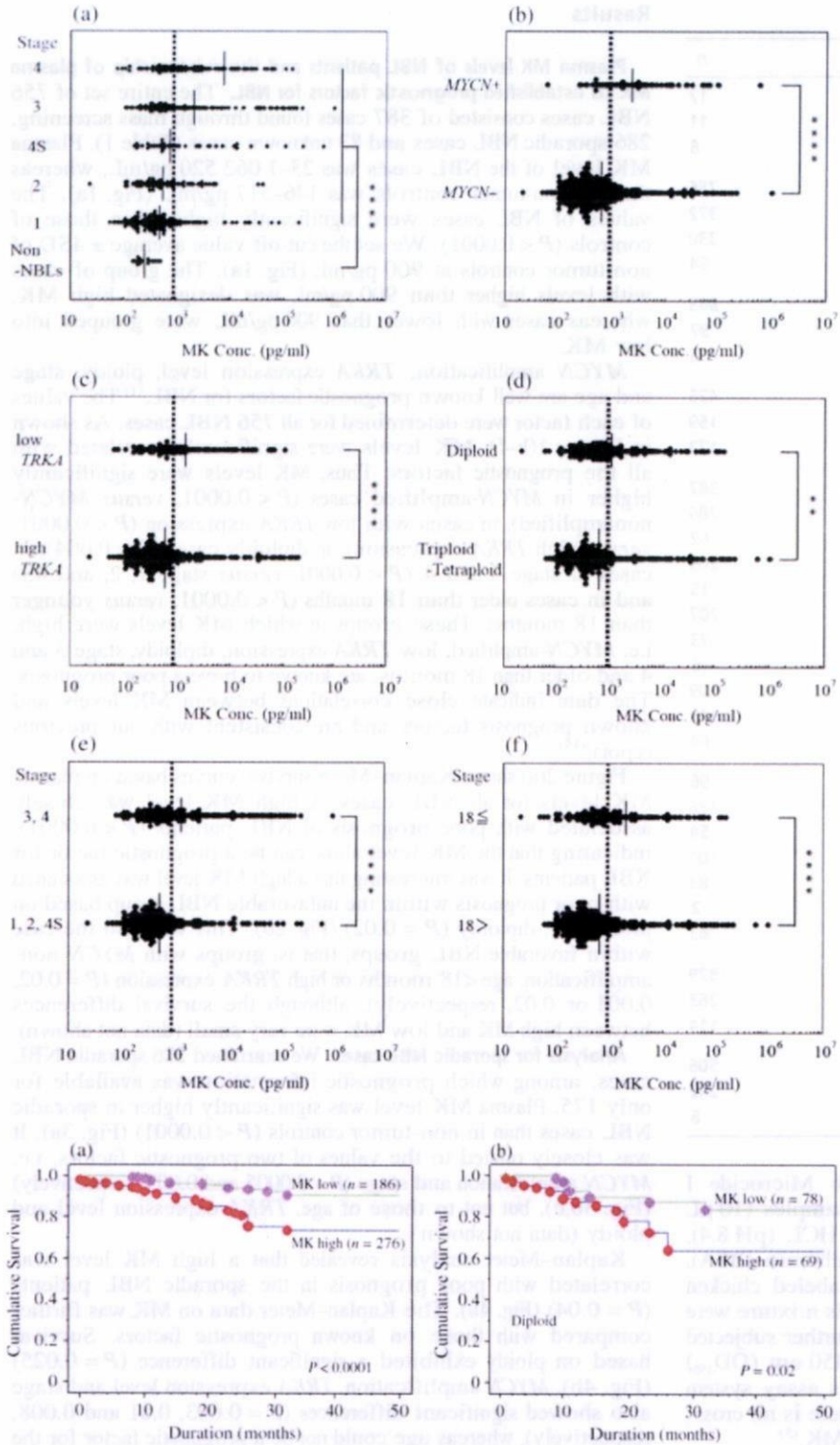

Fig. 1. Plasma midkine (MK) levels of the entire set of neuroblastoma (NBL) cases and the relationship of MK level to established prognostic factors for NBL. Blood MK levels are presented with dots. Each dot represents a NBL patient or a non-NBL control as indicated. (a) MK level distribution of the NBL patients through stages. Non-NBL, non-NBL controls. ****$P < 0.0001$. (b) NBL cases divided into *MYCN* amplification (MYCN+) and nonamplification (MYCN–). ****$P < 0.0001$. (c) NBL cases divided into low *TRKA* expression (low *TRKA*) and high *TRKA* expression (high *TRKA*). ****$P < 0.0001$. (d) NBL cases divided into diploid and triploid/pentaploid. **$P = 0.004$. (e) NBL cases divided into stage 3 or 4 (Stage 3, 4) and stage 1, 2 or 4S (Stage 1, 2, 4S). ****$P < 0.0001$. (f) NBL cases divided into age >18 months and <18 months. ****$P < 0.0001$.

Fig. 2. Kaplan–Meier curves for neuroblastoma (NBL) cases. 'MK low' was defined as a blood midkine (MK) level less than 900 pg/mL, whereas 'MK high' was more than 900 pg/mL. Cumulative survival rates of MK low and high groups were estimated for the entire set of NBL cases (a) and cases with diploidy (b).

mass screening has been discontinued, sporadic NBL are the major subject of therapy. We therefore further evaluated the MK level of only the sporadic cases. Our study revealed that, within sporadic cases, blood MK level alone could be a predictor of prognosis. MK level was also significantly correlated with *MYCN* amplification and stages.

However, blood MK level could not predict prognosis of patients in the intermediate risk group (*MYCN* non-amplification and stage 3 or 4) (data not shown). It could not predict the prognosis of patients within the high-risk group or low-risk group either (data not shown). This indicates that a single molecule may not be satisfactory for predicting the prognosis or judging the precise status of NBL for the decision of therapy, since, like other carcinomas, a complex of molecules is thought to contribute to carcinogenesis and development of NBL.[17,18] There are several blood markers predicting clinical outcome of neuroblastoma patients; i.e. serum lactate dehydrogenase, ferritin, neuron-specific enolase, disialoganglioside GD2 and NM23H1.[19–23] Therefore,

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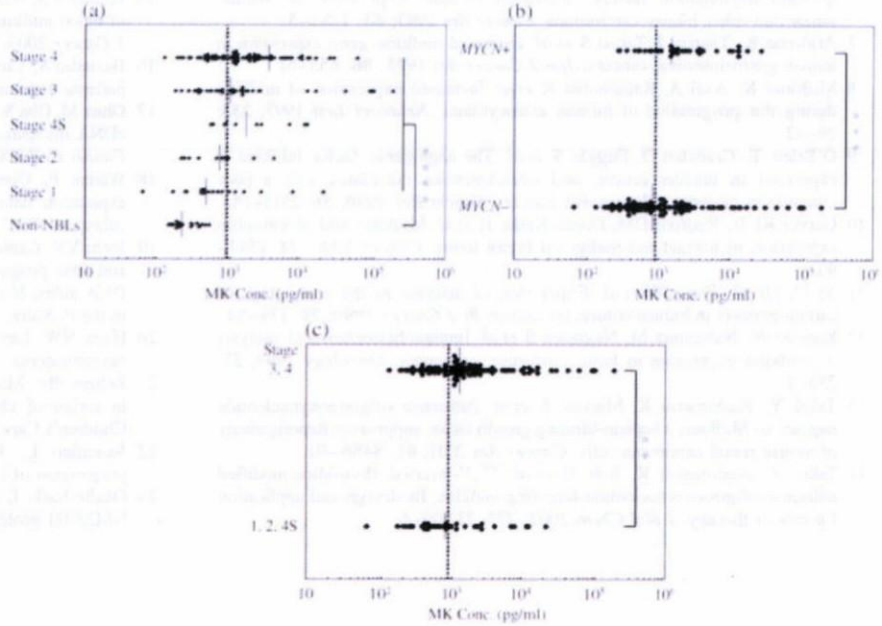

Fig. 3. Analysis for sporadic neuroblastoma (NBL) cases. Blood midkine (MK) levels of sporadic NBL cases are shown. (a) MK level distribution of the sporadic NBL patients through stages. Non-NBLs, non-NBL controls. ****$P < 0.0001$. (b) Sporadic NBL cases divided into *MYCN* amplification (MYCN+) and non-amplification (MYCN–). ***$P = 0.0005$. (c) Sporadic NBL cases divided into stage 3 or 4 (Stage 3, 4) and stage 1, 2 or 4S (Stage 1, 2, 4S). **$P = 0.003$.

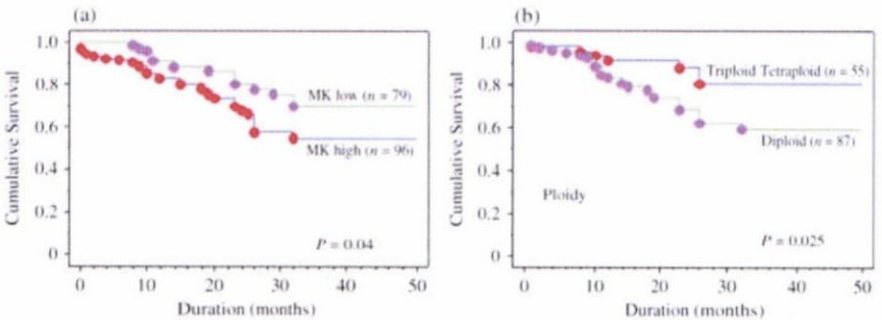

Fig. 4. Kaplan–Meier curves for sporadic neuroblastoma (NBL) cases. Cumulative survival rates of sporadic NBL cases were compared based on the following criteria. (a) Midkine (MK) low or high. (b) Diploid or triploid/pentaploid.

it is reasonable to expect that a combination of the plasma levels of MK and other blood biomarkers will facilitate accurate prognosis and accurate evaluation of tumor status. In addition, many efforts are being made to identify molecular changes associated with NBL with unfavorable prognosis.[17,18] Such studies will provide other biomarkers for NBL.

It is interesting that MK levels of stage 4s were lower in the present study than those in the previous study. Twelve cases were only available for stage 4s in the previous study. In the present study, 39 cases of stage 4s were available for the analysis of the entire set of NBL (Fig. 1a) and 15 cases for the sporadic NBL (Fig. 3a). Therefore, it is conceivable that midkine level deduced in the present study is more reliable because of the increased number of cases analyzed.

This is the first report indicating the plasma MK level as a prognosis factor for a human carcinoma. MK is frequently and highly expressed in malignant tumors regardless of the tissue type,[5] similar to mutations in the p53 gene. An elevated serum MK level is also detected in more than 80% of human adult carcinomas.[16] Although the MK level has not been evaluated as a prognosis factor for human carcinomas except for NBL, further assessment of the MK level will be useful in potentially establishing it as a new biomarker for other carcinomas.

Tumor growth is suppressed by the knockdown of MK expression.[13,14] MK is barely detectable in normal adult tissues. Furthermore, the present study has established that high blood MK level is closely related to poor prognosis, at least in NBL. Therefore, our data also support the idea that MK is a candidate molecular target for cancer therapy. Indeed, MK-deficient mice carrying a *MYCN* transgene show delayed development of NBL as compared with wild-type mice (Kishida and Kadomatsu, unpublished data). A therapy targeting MK for NBL is currently being studied in our laboratory.

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