were undetectable in all primary neuroblastomas and cell lines, whereas hypermethylation was readily detected in human lung adenocarcinoma-derived H1299 cell line used as a positive control (Fig. 3*b*). Our present findings ruled out the possibility that the hypermethylation of *TSLC1* promoter region contributes to the downregulation of *TSLC1* gene in unfavorable neuroblastomas. Of note, the treatment of neuroblastoma-derived SH-SY5Y and CHP-134 cells with TSA (trichostatin A) resulted in a remarkable upregulation of *TSLC1* (Fig. 4). Since TSA is a histone deacetylase inhibitor, it is possible that the acetylation status of histone plays an important role in the regulation of *TSLC1* expression.

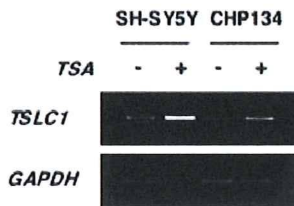

FIGURE 4 – Upregulation of *TSLC1* in cells exposed to TSA. SH-SY5Y and CHP-134 cells were treated with TSA (at a final concentration of 100 ng/ml) or left untreated. Twelve hours after treatment, total RNA was prepared and analyzed for the expression levels of *TSLC1* by semiquantitative RT-PCR. *GAPDH* was used as an internal control.

TSLC1 has an ability to suppress cell growth of neuroblastoma cells

To examine whether TSLC1 could have an ability to suppress neuroblastoma cell proliferation, we performed colony formation assays. Neuroblastoma-derived SH-SY5Y cells were transfected with or without the increasing amounts of the TSLC1 expression plasmid and maintained in fresh medium containing hygromycin for 14 days. As shown in Figure 5*a*, number of drug-resistant colonies was significantly reduced in a dose-dependent manner as compared with that in cells transfected with the empty plasmid alone. Similar results were also obtained in neuroblastoma-derived SK-N-AS cells (Supplementary Fig. 2). Next, we sought to examine a possible effect of the endogenous TSLC1 on neuroblastoma cell growth. To this end, SH-SY5Y cells were transiently transfected with control siRNA or siRNA against TSLC1. As shown in Figure 5*b*, siRNA-mediated silencing of the endogenous *TSLC1* was successful under our experimental conditions. Consistent with the present results obtained from colony formation assays, siRNA-mediated knockdown of TSLC1 resulted in an accelerated cell proliferation relative to the control cells ($p < 0.05$). Thus, it is likely that TSLC1 has an ability to suppress neuroblastoma cell proliferation.

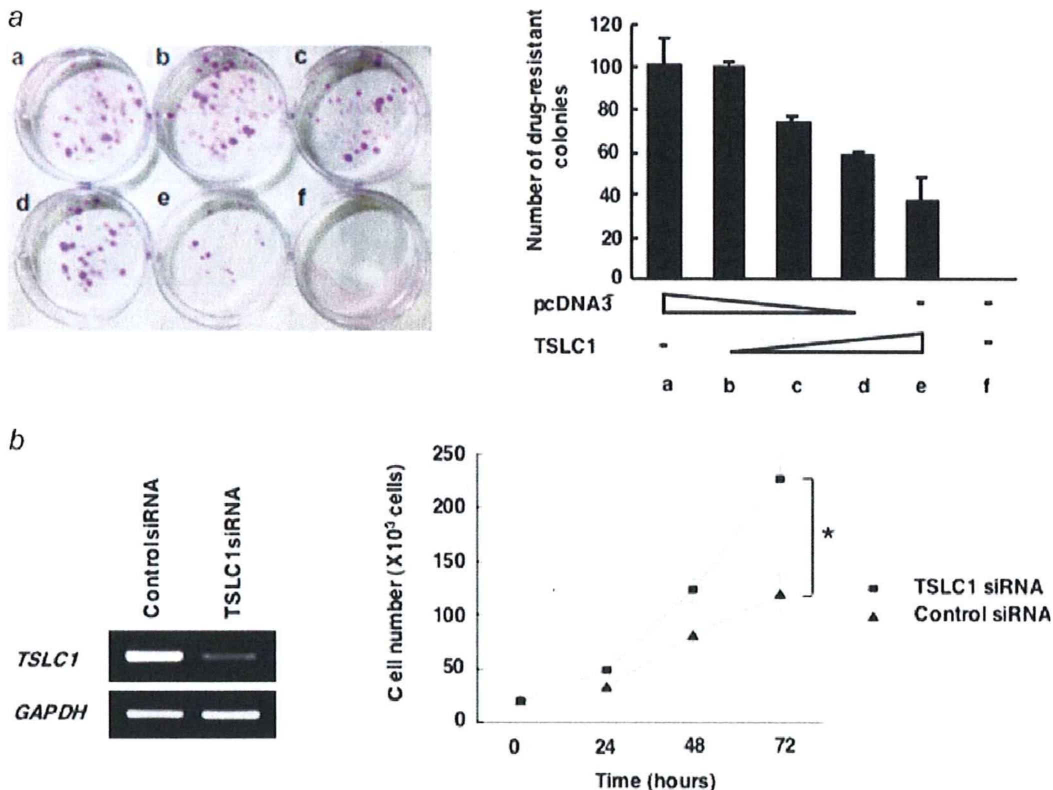

FIGURE 5 – Growth-suppressive potential of TSLC1 in neuroblastoma cells. (*a*) Colony formation assay. SH-SY5Y cells were transfected with the increasing amounts of the expression plasmid for TSLC1 (0, 250, 750 or 1,000 ng). Total amounts of plasmid DNA per transfection were kept constant (1 μg) with pcDNA3. Forty-eight hours after transfection, cells were transferred into the fresh medium containing hygromycin (at a final concentration of 200 μg/ml) and incubated for 2 weeks. Drug-resistant colonies were stained with Giemsa's solution (left panel) and number of drug-resistant colonies was scored (right panel). (*b*) siRNA-mediated knockdown of TSLC1. SH-SY5Y cells were transiently transfected with control siRNA or with siRNA against TSLC1. Forty-eight hours after transfection, total RNA was prepared and subjected to semiquantitative RT-PCR (left panel). At the indicated time periods after transfection, number of viable cells was measured in triplicate (right panel). The differences between the growth rate of control cells and TSLC1-knocked down cells were statistically significant ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Discussion

In the present study, we have demonstrated that the expression levels of a candidate tumor suppressor gene termed *TSLC1* are significantly associated with unfavorable outcome of patients with neuroblastomas. Our array-CGH studies revealed that *TSLC1* gene locates within the SRO of deletion in primary neuroblastoma at 11q. Indeed, its expression levels in primary neuroblastomas correlated with several prognostic indicators for neuroblastoma such as stage, Shimada's pathological classification, *MYCN* amplification status, *TrkA* expression levels and DNA index. Furthermore, TSLC1 had an ability to suppress neuroblastoma cell proliferation. Thus, it is likely that TSLC1 acts as a putative tumor suppressor for neuroblastoma.

As described previously, loss of *TSLC1* expression in primary esophageal squamous cell carcinoma (ESCC) preferentially correlated with invasion and metastasis,[12] and a remarkable reduction of *TSLC1* expression levels was observed in primary lung adenocarcinomas with advanced stage.[13] In addition, *TSLC1* expression was undetectable in 48% of benign (Grade I), 69% of atypical (Grade II) and 85% of anaplastic (Grade III) meningiomas.[14] Consistent with these observations, a significant downregulation of *TSLC1* was seen in unfavorable neuroblastomas bearing *MYCN* amplification as compared with favorable ones carrying single copy of *MYCN*, indicating that the decreased expression levels of *TSLC1* is one of the general properties of various human tumors including neuroblastoma. Intriguingly, there might exist an inverse relationship between the expression levels of *TSLC1* and *MYCN* amplification status in primary neuroblastoma. Indeed, our immunohistochemical analysis demonstrated that TSLC1 is detectable even in unfavorable neuroblastoma without *MYCN* amplification (Case 14). In a sharp contrast to primary neuroblastomas, the expression levels of *TSLC1* might be regulated in a *MYCN*-independent manner in neuroblastoma-derived cell lines. Although the precise molecular mechanisms behind the dysregulated expression of *TSLC1* in neuroblastoma cell lines, it might be due to certain genetic alterations occurred during the establishment of these cell lines.

Based on our present results, the presence of LOH at 11q was associated with unfavorable outcome of patients with neuroblastomas, however, there were no significant correlation between 11q LOH and the decreased expression levels of *TSLC1*. In accordance with these observations, the expression levels of *TSLC1* in neuroblastoma-derived cell lines were independent on their LOH status. These results suggest that the reduced expression levels of *TSLC1* in primary neuroblastomas are not attributed to haploinsufficiency. Alternatively, accumulating evidence strongly suggests that downregulation of *TSLC1* in various cancers including lung cancer, hepatocellular carcinoma, gastric cancer, pancreatic adenocarcinoma, prostate cancer, breast cancer, nasopharyngeal carcinoma and cervical cancer, might be due to the hypermethylation of its promoter region.[9,24–29] In a sharp contrast to these cancers, we did not detect the hypermethylation of the promoter region of *TSLC1* gene in primary neuroblastomas as well as neuroblastoma-derived cell lines under our experimental conditions. During the preparation of our article, Nowacki *et al.* found that there is no *TSLC1*-specific hypermethylation in neuroblastoma.[30] Similarly, the hypermethylation of *TSLC1* promoter region was not detectable in medulloblastoma.[31] According to the previous results, *RASSF1A* and *CASP8* gene promoters were frequently hypermethylated in primary neuroblastoma and neuroblastoma cell lines.[32] Thus, it is conceivable that, unlike the other cancers, hypermethylation of the promoter region of *TSLC1* does not contribute to its downregulation in neuroblastoma, and there might exist as yet unknown tissue-specific regulatory mechanisms of *TSLC1* transcription. Of note, the treatment of neuroblastoma-derived SH-SY5Y and CHP-134 cells with TSA (trichostatin A) resulted in a remarkable upregulation of *TSLC1*. Since TSA is a histone deacetylase inhibitor, it is likely that the acetylation status of histone plays an important role in the regulation of *TSLC1* expression. Further studies should be required to address this issue.

Several lines of evidence indicate that TSLC1 has an ability to delay the cell cycle progression.[12,16,33] Alternatively, enforced expression of TSLC1 resulted in an activation of proapoptotic caspase-3 and induction of proteolytic cleavage of its substrate PARP.[34] These findings strongly suggest that TSLC1 has an anti-proliferative and/or proapoptotic activity. In a good agreement with this notion, our present results demonstrated that enforced expression of TSLC1 in SH-SY5Y cells as well as SK-N-AS cells decreases the number of drug-resistant colonies, and enforced depletion of the endogenous TSLC1 in SH-SY5Y cells leads to an accelerated cell proliferation, which was consistent with the recent observations.[30] Collectively, our present findings suggest that TSLC1 acts as a tumor suppressor for neuroblastoma, and also might contribute to the spontaneous regression of neuroblastoma arising from neuronal apoptosis and/or differentiation.

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The methylation status of *RASSF1A* promoter predicts responsiveness to chemotherapy and eventual cure in hepatoblastoma patients

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Despite the progress of therapy, outcomes of advanced hepatoblastoma patients who are refractory to standard preoperative chemotherapy remain unsatisfactory. To improve the mortality rate, novel prognostic markers are needed for better therapy planning. We examined the methylation status of 13 candidate tumor suppressor genes in 20 hepatoblastoma tumors by conventional methylation-specific PCR (MSP) and found hypermethylation in 3 of the 13 genes. We analyzed the methylation status of these 3 genes (*RASSF1A, SOCS1* and *CASP8*) in 97 tumors and found hypermethylation in 30.9, 33.0 and 15.5%, respectively. Univariate analysis showed that only the methylation status of *RASSF1A* but not the other 2 genes predicted the outcome, and multivariate analysis showed a weak contribution of *RASSF1A* methylation to overall survival. Using quantitative MSP, we found *RASSF1A* methylation in 44.3% of the 97 tumors. *CTNNB1* mutation was detected in 67.0% of the 97 tumors. While univariate analysis demonstrated *RASSF1A* methylation, *CTNNB1* mutation and other clinicopathological variables as prognostic factors, multivariate analysis identified *RASSF1A* methylation (p = 0.043; relative risk 9.39) and the disease stage (p = 0.002; relative risk 7.67) but not *CTNNB1* mutation as independent prognostic factors. In survival analysis of 33 patients in stage 3B or 4, patients with unmethylated tumor had better overall survival than those with methylated tumor (p = 0.035). *RASSF1A* methylation may be a promising molecular-genetic marker to predict the treatment outcome and may be used to stratify patients when clinical trials are carried out.
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Key words: RASSF1A; CTNNB1; quantitative MSP; hepatoblastoma; prognostic factor

Hepatoblastoma is a rare malignant neoplasm of the liver, with an incidence of 0.5–1.5 per million children.[1] Remarkable progress in clinical outcome has been achieved in the past 20 years due to 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.[2,3] To improve the mortality of these patients, innovative treatment and potent prognostic markers for better therapy planning are needed. The present clinical factors predicting outcome include the level of alpha-feto protein, histology, disease stage and growth pattern of the tumor.[2–4] Chromosomal gains of 2q, 8q and 20 and high expression of telomerase or *PLK1* were shown to be molecular-genetic markers predicting poor outcome[5–8]; however, none have been proven to be independent prognostic factors by multivariate analysis.

We previously reported that *RASSF1A* (*RAS association domain family protein 1*) methylation, found in 39% of 39 hepatoblastoma tumors, was correlated with poor outcome by univariate analysis.[9] Nevertheless, the article had some limitations that the number of tumors was not enough, the method used to detect the hypermethylation was suboptimal, and the prognostic significance of *RASSF1A* methylation was ambiguous by multivariate analysis.

CTNNB1 (*catenin, beta-1*) mutation was reported in the majority of hepatoblastoma tumors, but reports on alterations of other oncogenes or tumor suppressor genes are rare.[10–12] Thus, we thought that epigenetic silencing of tumor suppressor genes might be involved in the tumorigenesis of hepatoblastoma and examined the methylation status of 13 candidate tumor suppressor genes, whose aberrant methylation has previously been shown in various cancers.[13–22] Conventional methylation-specific PCR (MSP) analysis showed hypermethylation in only 3 of the 13 genes, *RASSF1A*, *SOCS1* (*suppressor of cytokine signaling 1*) and *CASP8* (*caspase-8*) genes, but not in the remaining 10 genes. We examined the correlation of the methylation status of the 3 genes with various clinical characteristics in a substantial number of hepatoblastoma tumors. Furthermore, we analyzed the methylation status of *RASSF1A* by more sensitive quantitative MSP and verified the prognostic implication of methylation by multivariate analysis. We suggest that *RASSF1A* may be a promising molecular-genetic marker predicting treatment outcome that may be used to stratify hepatoblastoma patients when clinical trials are carried out.

Material and methods

Patients and samples

Tumor tissues were obtained from 97 Japanese children with hepatoblastoma and adjacent normal liver tissues were available from 3 patients. Nonmatched normal liver tissues were also obtained from 5 other hepatoblastoma patients who were not included in the present clinicopathological study. Thirty-five of 39 specimens in the previous report were included; 4 were excluded because of the lack of DNA and 62 were supplied by the Tissue Bank of the Japanese Study Group for Pediatric Liver Tumor (JPLT).[23] The median age of the 97 patients at diagnosis was 16 months (range, 2–177 months).

The clinical stage of the disease was determined at the time of initial biopsy or surgery according to the classification of the Japanese Society of Pediatric Surgeons.[24] While most tumors in stages 1 and 2, and those in 3A, occupying 3 segments of the liver, are completely resectable, tumors in stage 3B, occupying 4 segments of the liver, and those in stage 4 are not. The extent of disease was distributed in stage 1 in 6 tumors, in 2 in 33, in 3A in 25, in 3B in 11 and in 4 in 22. Patients were treated at various hospitals or institutions, mostly under the framework of JPLT-1 (1991–1999) or JPLT-2 (2000–2006) protocols.[23,25] The protocols include pre- and postoperative chemotherapy with cisplatin and THP-adriamy-

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Abbreviations: *CASP8*, *caspase-8*; CI, confidence interval; CR, complete response; *CTNNB1*, *catenin, beta-1*; JPLT, the Japanese Study Group for Pediatric Liver Tumor; MSP, methylation-specific PCR; NC, no change; PR, partial response; *RASSF1A*, *RAS association domain family protein 1*; RR, relative risk; *SOCS1*, *suppressor of cytokine signaling 1*.

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cin. Complete response (CR) was defined as the complete disappearance of tumor, and partial response (PR) as at least a 50% reduction of tumor. No change (NC) was defined as a decrease of less than 50% or an increase of tumor.[25] Seventy-two patients underwent preoperative chemotherapy, and one underwent salvage liver transplantation. The median follow-up of survivors was 66 months (range, 9–175 months). The PRETEXT system is based on hepatic surgical anatomy, described elsewhere.[26] The pathological classifications of hepatoblastoma by Haas *et al.* and the Japanese Society of Pathology divide hepatoblastoma into 2 major subtypes, namely the well-differentiated (fetal) type and the poorly differentiated (embryonal) type.[4,24]

Bisulfite treatment and conventional methylation-specific PCR (MSP) analysis

Genomic DNA from tumor samples was treated with sodium bisulfite, and the methylation status of the promoter region in various genes was analyzed by MSP, as previously described.[9,27] The genes examined were *RASSF1A*, *RASSF2A*, *NORE1A*, *SOCS1*, *CASP8*, *RUNX3*, *RIZ1*, *BLU*, *HOXA9*, *HOXB5*, *p16INK4A*, *p14ARF* and *DCR2*.[13–22] The primer sequences and their location in the original genomic sequences are listed in Table I, and the location of the analyzed fragments for *RASSF1A*, *SOCS1* and *CASP8* are shown in Figure 1*a*. While the primer sequences of *RASSF1A* are located in the promoter region, those of *CASP8* and *SOCS1* are derived from the exon 4-intron 4 region and the exon 1, respectively, because the methylation status of these regions is correlated with the expression.[15,20,30] CpGgenome™ Universal Methylated DNA (Chemicon International, Temecula, CA) and normal lymphocyte DNA were used as controls for methylated or unmethylated templates, respectively. PCR products were run on 2% agarose gels and visualized after staining with ethidium bromide.

Quantitative MSP and reverse-transcription (RT)-PCR analyses of RASSF1A

The methylation status of the *RASSF1A* promoter was also examined in all 97 tumor samples by fluorescence-based, real-time quantitative PCR using a LightCycler (Roche Diagnostics). Primers and probes designed to specifically amplify the promoter of *RASSF1A* or a reference gene, *ACTB*, were described elsewhere.[28,29] The primer sequences used for quantitative MSP and those used for conventional MSP share the 17 nucleotides with 1 nucleotide deviation in the forward primer, and the 18 nucleotides with 3 nucleotides deviation in the reverse primer, although they amplified the same *RASSF1A* CpG islands (CGIs) (Fig. 1*a* and Table I).[31] Each amplification reaction included tumor DNA samples, positive and negative controls and water blank. *ACTB* was used as a reference gene to determine the relative level of methylated DNA for *RASSF1A* in each sample. Dividing the methylated *RASSF1A/ACTB* ratio of template amounts in a sample by the methylated *RASSF1A/ACTB* ratio of template amounts in a fully methylated control and multiplying this value by 100 calculated the percentage of methylation.

To determine whether the percentage of *RASSF1A* methylation is correlated with the expression level, we performed RT-PCR analysis of the *RASSF1A* gene in 7 tumor samples with methylated or unmethylated *RASSF1A* and 1 normal liver sample available by the method described previously.[9]

Mutation analysis of the CTNNB1 gene

To detect point mutations and deletions of the *CTNNB1* gene, genomic DNA from each tumor sample was amplified using 2 sets of primers, F1, 5′-TGGCTATCATTCTGCTTTTCTTG-3′ and R1, 5′-CTCTTTTCTTCACCACAACATTTT-3′, and BCAT-3, 5′-AAAATCCAGCGTGGACAATGG-3′, and BCAT-4, 5′-TGTGGCAAGTTCTGCATCATC-3′, respectively (Suppl Fig. 1*a*).[10,32] The PCR products were either directly sequenced or inserted into a vector [pGEM (R)-T Easy Vector System (Promega, Madison, WI)], and 6 or more clones were sequenced.

Statistical analysis

Patients were grouped according to various biological and clinical aspects of disease. Significance of differences in the characteristics between patient's groups was examined using the chi-square or Fisher's exact test. Overall survival for each group of patients was estimated using the Kaplan-Meier method, and compared using the log-rank test. Time to failure was defined as the interval between surgery or preoperative chemotherapy and death from any cause. The influence of various biological and clinical factors on overall survival was estimated using the Cox proportional-hazards model calculated with Stat Flex software for Windows, version 5.0 (Artec Co., Osaka, Japan).

Results

Conventional MSP analysis of various genes in hepatoblastomas

We first examined the methylation status of 13 genes in 20 tumors, including 2 tumors in stage 1, 6 in stage 2, 6 in stage 3 and 6 in stage 4, by conventional MSP and found no methylation in 10 (*RASSF2A*, *NORE1A*, *RUNX3*, *RIZ1*, *BLU*, *HOXA9*, *HOXB5*, *p16INK4A*, *p14ARF* and *DCR2*); no further analysis was performed on these 10 genes. The remaining 3 genes, including *RASSF1A*, *SOCS1* and *CASP8*, were methylated in a substantial number of tumors. Therefore, we extended the analysis to all 97 tumors and found hypermethylation of *RASSF1A*, *SOCS1* and *CASP8* in 30 (30.9%), 32 (33.0%) and 15 (15.5%) tumors, respectively (Fig. 1*b*). All 3 genes were methylated in 3 tumors. Two of 3 genes, *RASSF1A* and *SOCS1*, *RASSF1A* and *CASP8* and *SOCS1* and *CASP8*, were methylated in 7, 3 and 5 tumors, respectively. Only 1 gene, *RASSF1A*, *SOCS1* or *RASSF1A*, was methylated in 15, 19 or 4 tumors. Conventional MSP detected unmethylated *RASSF1A* in all 8 adjacent normal liver tissues.

Correlation of the methylation status of the 3 genes analyzed by conventional MSP with overall survival

When we analyzed the correlation between the methylation status of any 1 of the 3 genes and overall survival, *RASSF1A* methylation was associated with a poor outcome ($p < 0.001$), but *SOCS1* or *CASP8* methylation was not; however, multivariate analysis using the various factors shown in Table III indicated the significant contribution of disease stage [$p < 0.001$; relative risk (RR) 9.44; 95% confidence interval (CI), 2.51–35.46], but no contribution of *RASSF1A* methylation to overall survival ($p = 0.149$; RR 2.38; 95% CI, 0.73–7.72).

Quantitative MSP analysis of RASSF1A methylation and the correlation between the percentage of the RASSF1A methylation and the expression or clinical outcome

To clarify whether *RASSF1A* methylation is an independent factor predicting outcome, we performed quantitative MSP analysis of *RASSF1A* in 97 tumors. Tumors were classified by the percentage of *RASSF1A* methylation, and about one half of tumors (46) had 0–2.5% of the methylation, and others distributed in various percentages of the methylation (Fig. 2*a*). RT-PCR detected *RASSF1A* expression in 1 normal liver sample and 2 tumor samples with less than 1% of the methylation, but did not detect the expression in tumors with more than 11% of the methylation; 2 tumors with the intermediate incidence of the methylation (4.2 or 4.8%) showed the ambiguous expression (Fig. 2*b*). Thus, there is an inverse relationship between the percentage of the *RASSF1A* methylation and the expression.

Next, we examined the dose-response relationships between the percentage of *RASSF1A* methylation and overall survival analyzed by the Kaplan-Meier method and adopted a cutoff value of 4.8%, which gave the smallest *p*-value ($p < 0.00001$). We also examined the dose-response relationships between the percentages of

TABLE I – PRIMER SEQUENCE, GENOMIC POSITION, MSP CONDITION AND PRODUCT SIZE

Primer name	Primer sequence	Genomic position[1]	Annealing temp. (°C)	Product size (bp)	Ref.
Quantitative MSP					
ACTB-F	5′-TGGTGATGGAGGAGGTTTAGTAAGT	−1596	60	133	28
ACTB-R	5′-AACCAATAAAACCTACTCCTCCCTTAA				
TaqMan probe	5′-6FAM-TGTGTTTGTTATTGTGTGTTGGGTGGTGGT-TAMRA-3′				
RASSF1A-F	5′-GGTTTTGCGAGAGCGCGT	−72	62	168	29
RASSF1A-R	5′-GCTAACAAACGCGAACCGAAC				
TaqMan probe	5′-6FAM-GGAGGCGTTGAAGTCGGGGTT-TAMRA-3′				
Conventional MSP					
RASSF1A-UF	5′-GGGGTTTTGTGAGAGTGTGTTTAG	−74	63	175	30
RASSF1A-UR	5′-TAAACACTAACAAACACAAACCAAAC				
RASSF1A-MF	5′-GGGTTTTGCGAGAGCGCG	−73	63	169	
RASSF1A-MR	5′-GCTAACAAACGCGAACCG				
BLU-UF	5′-TTGTTTGGATTTAGGTGTGAGTT	−73	58	160	18
BLU-UR	5′-CAAAAACAACAAACCCCAACA				
BLU-MF	5′-CGTTCGGATTTAGGCGCGAGTT	−72	68	158	
BLU-MR	5′-GAAAACGACGAACCCCGACGA				
CASP8-UF	5′-TAGGGGATTTGGAGATTGTGA	+308[2]	55	321	20
CASP8-UR	5′-CCATATATATCTACATTCAAAACAA				
CASP8-MF	5′-TAGGGGATTCGGAGATTGCGA	+308[2]	58	320	
CASP8-MR	5′-CGTATATCTACATTCGAAACGA				
DCR2-UF	5′-TTGGGGATAAAGTGTTTTGATT	+101	58	146	21
DCR2-UR	5′-AAACCAACAACAAAACCACA				
DCR2-MF	5′-GGGATAAAGCGTTTCGATC	+104	59	139	
DCR2-MR	5′-CGACAACAAAACCGCG				
HOXA9-UF	5′-TAATAGTGTGTGGAGTGATTTAT	−124	56	94	22
HOXA9-UR	5′-TAATAAATTACCAACACCCA				
HOXA9-MF	5′-GCGTTTGGTTCGTTCGGTTC	−61[3]	64	123	
HOXA9-MR	5′-CAATAAAAACGCGAACGCCG				
HOXB5-UF	5′-TGAATTGGTTTTAATGATTTTTGGATT	−217	53	117	19
HOXB5-UR	5′-TTAAAAAATCACATACTTTTATTAACCAATCA				
HOXB5-MF	5′-AATCGGTTTTAACGATTTTCGGATC	−215	53	113	
HOXB5-MR	5′-AAAAAATCACGTACTTTTATTAACCAATCG				
NORE1A-UF	5′-ATTTATATTTGTGTAGATGTTGTTTGGTAT	−176		214	14
NORE1A-UR	5′-ACTTTAACAACAACAACTTTAACAACTACA				
NORE1A-MF	5′-CGTCGTTTGGTACGGATTTTATTTTTTTCGGTTC	−159		202	
NORE1A-MR	5′-GACAACTTTAACAACGACGACTTTAACGACTACG				
p14ARF-UF	5′-GGAATAGGGGAGTGGGGAT	−388	60	144	22
p14ARF-UR	5′-AATAACAACCCAAAAACCAAACA				
p14ARF-MF	5′-GGAATAGGGGAGCGGGGAC	−388	60	144	
p14ARF-MR	5′-GATAACGACCCAAAAACCGAACG				
p16INK4A-UF	5′-TTATTAGAGGGTGGGGTGGATTGT	+133	63	151	27
p16INK4A -UR	5′-CAACCCCAAACCACAACCATAA				
p16INK4A -MF	5′-TTATTAGAGGGTGGGGCGGATCGC	+133	63	150	
p16INK4A -MR	5′-GACCCCGAACCGCGACCGTAA				
RASSF2A-UF	5′-GAAGGTGTTTTTATTTTATTTTTGG	+684	59	156	13
RASSF2A-UR	5′-AAAACCTACCTCTAAAAAATCCACC				
RASSF2A-MF	5′-GTTCGTCGTCGTTTTTTAGGCG	+798	60	109	
RASSF2A-MR	5′-AAAAACCAACGACCCCCGCG				
RIZ1-UF	5′-TGGTGGTTATTGGGTGATGGT	−4782		177	17
RIZ1-UR	5′-ACTATTTCACCAACCCCAAGA				
RIZ1-MF	5′-GTGGTGGTTATTGGGCGACGGC	−4781		176	
RIZ1-MR	5′-GCTATTTCGCCGACCCCGACG				
RUNX3-UF	5′-ATAATAGTGGTTGTTAGGGTGTTG	+298[3]	60	115	16
RUNX3-UR	5′-ACTTCTACTTTCCCACTTCTCACA				
RUNX3-MF	5′-ATAATAGCGGTCGTTAGGGCGTCG	+298[3]	60	115	
RUNX3-MR	5′-GCTTCTACTTTCCCGCTTCTCGCG				
SOCS1-UF	5′-TTATGAGTATTTGTGTGTATTTTTAGGTTGGTT	+1072	60	175	15
SOCS1-UR	5′-CACTAACAACACAACTCCTACAACAACCA				
SOCS1-MF	5′-TTCGCGTGTATTTTTAGGTCGGTC	+1081	60	160	
SOCS1-MR	5′-CGACACAACTCCTACAACGACCG				

UF, unmethylated forward primer; UR, unmethylated reverse primer; MF, methylated forward primer; MR, unmethylated reverse primer.
[1]The 5′ position of the sense unmethylated or sense methylated primer sequences is numbered relative to the transcription start site of the gene concern.–[2]The number indicates the location relative to the transcription start site of *CASP8* transcript variant B (NM_033355.2).–[3]Designed for bottom strand.

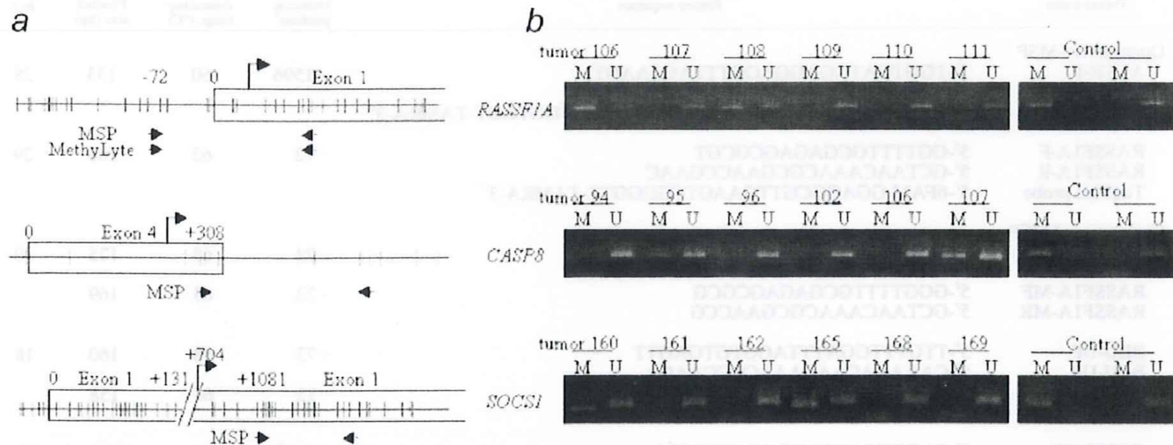

FIGURE 1 – (*a*) The location of the *RASSF1A*, *CASP8* or *SOCS1* fragment analyzed by the conventional or quantitative (MethyLyte) MSP method is shown as horizontal arrows. The transcription start site of each gene is shown as a bent arrow. (*b*) Examples of methylation status using conventional methylation-specific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

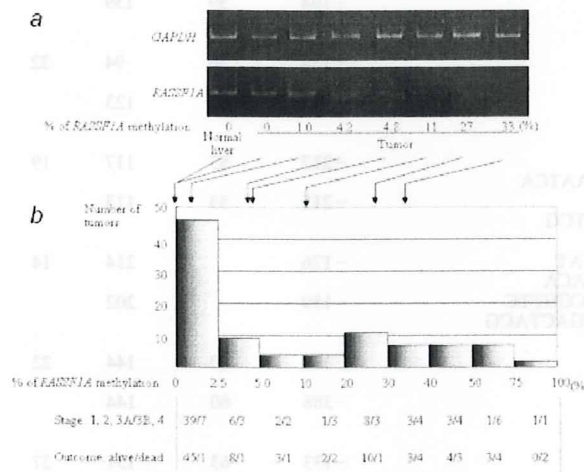

FIGURE 2 – (*a*) Histogram showing the number of tumors categorized by the percentage of *RASSF1A* methylation. The number of tumors classified by the stage of disease and clinical outcome are shown under the columns. (*b*) RT-PCR analysis of *RASSF1A* mRNA in 1 normal liver and 7 tumor samples.

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*).

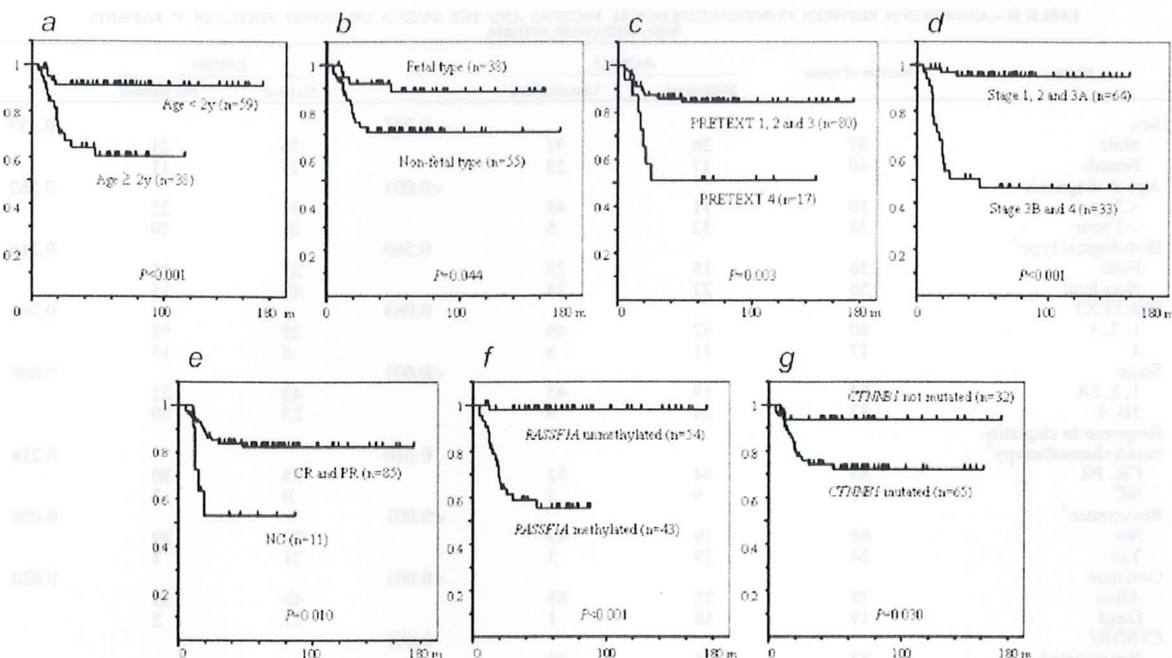

FIGURE 3 – Overall survival curves for hepatoblastoma patients based on different variables: (*a*) age, (*b*) histological type of tumor, (*c*) PRETEXT disease stage, (*d*) disease stage, (*e*) response to cisplatin-based chemotherapy, (*f*) methylation status of the *RASSF1A* gene, (*g*) mutation status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

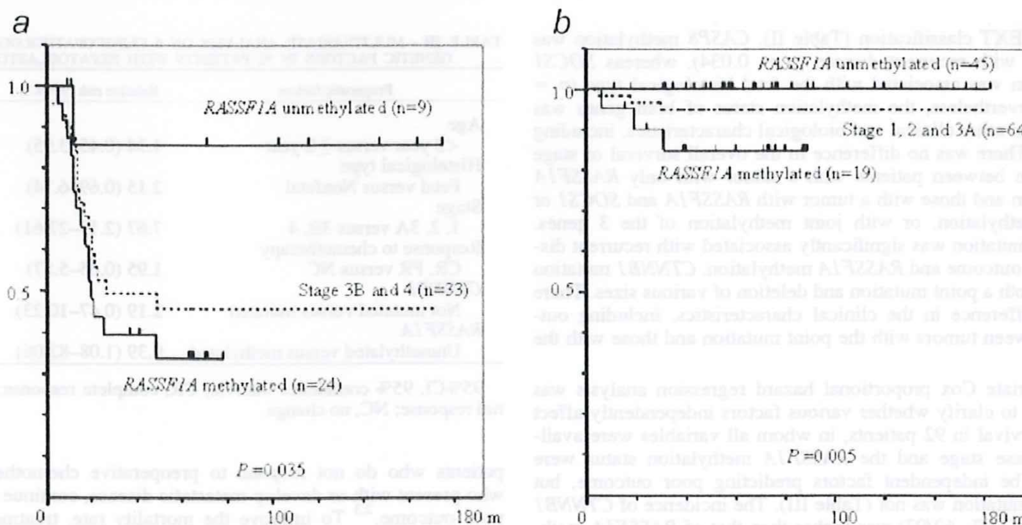

FIGURE 4 – (*a*) Overall survival curves for hepatoblastoma patients in stages 3B and 4 classified by the methylation status of *RASSF1A*. Dotted line indicates the overall survival curve of all 33 patients. (*b*) Overall survival curves for hepatoblastoma patients in stages 1, 2 and 3A classified by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

Acknowledgements

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ORIGINAL ARTICLE

Clinical significance of minimal residual disease in patients with t(8;21) acute myeloid leukemia in Japan

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Abstract To examine the prognostic significance of minimal residual disease (MRD) in t(8;21) acute myeloid leukemia (AML), 96 bone marrow samples from 26 Japanese patients in complete remission (CR) were analyzed regarding the *RUNX1/MTG8* transcript using real-time reverse transcriptase polymerase chain reaction assay. All patients were treated with intensive chemotherapy. The median copy number of the *RUNX1/MTG8* transcript, measured after each treatment course decreased over time. However, an increase in the MRD level was documented in three patients after the second consolidation, and all of them subsequently relapsed. The relapse-free survival (RFS) did not differ between the patients whose MRD levels were below or above 1,000 copies/μg after the first consolidation, with respective 2-year rates of 62 and 86% ($P = 0.21$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Although these findings need to be confirmed with a larger number of patients, our data indicate that the MRD level at a given time during the early course in CR does not predict the outcome in Japanese patients.

Keywords Acute myeloid leukemia · t(8;21) · *RUNX/MTG8* · Minimal residual disease · Prognosis

1 Introduction

t(8;21)(q22;q22) is one of the most common karyotype abnormalities in acute myeloid leukemia (AML), occurring

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in 7–8% of adult patients [1–3]. This translocation leads to the formation of the chimeric *RUNX1(AML1)/MTG8(ETO)* transcript, which enables detection by polymerase chain reaction (PCR) assay. Since the introduction of real-time reverse transcriptase (RT)-PCR [4], prognostic significance of minimal residual disease (MRD) quantified using this method has been intensively investigated. Several studies from Western countries showed that MRD levels during or after treatment are associated with a risk of relapse on the basis of results from 21–51 patients [5–9].

We previously reported that Japanese patients with t(8;21) AML could have a more favorable outcome than the Western patients [10]. Marcucci et al. [11] also showed the difference in the outcome between the white and non-white patients enrolled in successive Cancer and Leukemia Group B trials. Given that clinical characteristics of t(8;21) AML can differ according to ethnicities, prognostic significance of MRD may also differ between Japanese and Western patients.

Here we examine the relationship between MRD status during intensive chemotherapy and the outcome in Japanese patients with t(8;21) AML.

2 Patients and methods

2.1 Study patients

We retrospectively reviewed the medical records of a total of 46 adults, who were newly diagnosed to have t(8;21) AML, at nine collaborating hospitals between January 2000 and December 2005. Induction therapy was given to 45 patients, and 41 (91%) achieved complete remission (CR). Data on MRD after the first or second consolidation were available for 27 of the 41 CR patients. We excluded one patient who received low-dose cytarabine-containing therapy, leaving 26 patients eligible for this study. We did not exclude any patient who relapsed after the first consolidation therapy. All patients provided their informed consent before the initiation of any medical procedure.

2.2 Diagnosis of t(8;21) AML and MRD evaluation

The diagnosis of t(8;21) AML was established based on chromosomal analysis (G-banding) and/or detection of the *RUNX1/MTG8* fusion gene by real-time RT-PCR. The molecular quantification of the *RUNX1/MTG8* fusion gene was performed as described previously [12]. The results were reported as the number of transcript copies, which were normalized by means of *GAPDH* and then converted into copies/μg RNA. The molecular quantification of the *RUNX1/MTG8* fusion gene was conducted each time after the induction and consolidation therapies. Bone marrow samples were used for all the MRD analyses.

2.3 Statistical analysis

The relapse-free survival (RFS) was calculated as the time from diagnosis to relapse or death, using the Kaplan–Meier product limit method. A log rank test was applied to assess the difference between the groups. The estimated survival was calculated as of 7 May 2008. Differences in distribution of categorical variables were compared with the Fisher's exact test. All analyses were conducted using the STATA version 9.2 software program (StataCorp, College Station, TX).

3 Results

3.1 Patient characteristics

The characteristics of the 26 patients are shown in Table 1. The median age was 50 years (range, 25–64 years), with 19 males and 7 females. Details of treatments are also summarized in Table 1. For induction therapy, 24 received idarubicin and cytarabine, and 2 received daunorubicin and cytarabine. Consolidation therapy included high-dose cytarabine in 12, and standard-dose cytarabine in 14 patients. The median follow-up of the surviving patients was 39.2 months (range, 14.0–92.4 months).

3.2 Clinical outcome

Of the 26 patients, 17 had continued first CR until the time of last observation. Relapse occurred in the remaining nine patients at a median of 9.9 months (range, 7.5–81.6 months). Five patients died due to the primary disease ($n = 3$), sudden cardiac disorder ($n = 1$) and cardiac arrhythmia ($n = 1$). The probability of RFS was 73% at 2 years for the entire population. The rate was 67% for patients who received high-dose cytarabine for consolidation therapy, whereas it was 79% for patients who received standard-dose cytarabine ($P = 0.87$).

3.3 Kinetics of MRD of each patient

The MRD levels were measured in a total of 96 samples from the 26 patients. Samples were available from 18 patients after induction therapy, 20 patients after the first consolidation, 18 patients after the second consolidation, and 13 patients after the third consolidation. The kinetics of MRD of each patient is shown in Fig. 1. The median copy number of the *RUNX1/MTG8* transcript decreased over time, for example 4,750 copies/μg after induction, 480 copies/μg after the first consolidation, 240 copies/μg after the second consolidation, and <100 copies/μg after the third consolidation. All the 15 patients whose MRD data

Table 1 Characteristics of the patients with t(8;21) AML at diagnosis

Variables		Number
Age (years)	Median, range	50 (25–64)
Sex	Male/female	19/7
Karyotypic abnormality[a]		
(A) t(8;21)(q22; q22) without additional karyotypic abnormality		7
(B) t(8;21)(q22; q22) with loss of sex (Y) chromosome		6
(C) t(8;21)(q22; q22) with abnormal chromosome 9		2
(D) t(8;21)(q22; q22) with ≥3 additional abnormalities		7
(E) t(8;21)(q22; q22) with loss of X chromosome		1
(F) Other karyotypic abnormality[b]		1
White blood cell count (/μL)	Median, range	7750 (900–54970)
Lactate dehydrogenase level (IU/L)	Median, range	441 (186–3354)
Extramedullary involvement	Present/absent	5/21
Induction therapy		
Idarubicin 12 mg/m^2 d1–3 + cytarabine 100 mg/m^2 d1–7		24
Daunorubicin 50 mg/m^2 d1–5 + cytarabine 100 mg/m^2 d1–7		2
Consolidation therapy	High-dose cytarabine-based chemotherapy	12
	No. of courses (2/3/4)	1/8/3
	Standard cytarabine-based chemotherapy	14
Hematopoietic stem cell transplantation	In first complete remission (autologous/allogeneic)	2/1
	In other stage (autologous/allogeneic)	0/3[c]

[a] Two patients were diagnosed by the detection of *RUNX1/MTG8* fusion gene using reverse transcriptase-polymerase chain reaction

[b] 46,XX,t(2;19)(q37;p13),t(8;21)(q22;q22)

[c] Patients who underwent allogeneic stem cell transplantations in second complete remission

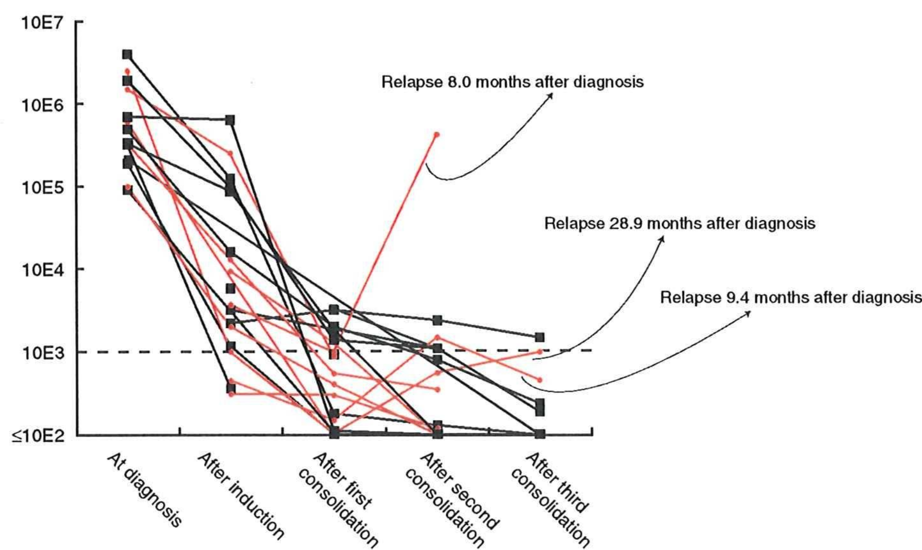

Fig. 1 Kinetics of the *RUNX1/MTG8* level in bone marrow. Kinetics of the *RUNX1/MTG8* level (copies/μg RNA) is shown for the 17 patients who remained in remission (*squares*) and for the 9 patients who had experienced a relapse (*circles*). The increases of the *RUNX1/MTG8* level were documented in three patients and all of them subsequently relapsed

were available both after induction and first consolidation showed reduction in varying degrees. On the other hand, the increments were documented in 3 of the 16 patients who had MRD levels measured both after the first and second consolidation, and all of them subsequently relapsed (Fig. 1).

3.4 Effect of MRD level on relapse-free survival

We next evaluated the prognostic relevance of the MRD level at a specific time point. Given that an increase in the MRD level was observed in none of the patients after the first consolidation, but in three patients after the second

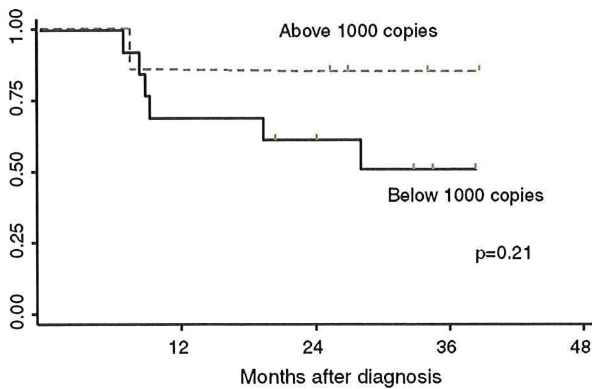

Fig. 2 Relapse-free survival according to the level of minimal residual disease after the first consolidation course. No difference was found between the patients with the *RUNX1/MTG8* level above ($n = 7$) and below 1,000 copies/µg RNA ($n = 13$)

consolidation, we examined the effect of MRD level after the first consolidation on RFS. The copy number of the *RUNX1/MTG8* transcript at this time point was less than 1,000 copies/µg in 13 patients (65%). Six patients (30%) exhibited less than 100 copies/µg. Figure 2 compares RFS according to the MRD level after the first consolidation. Here, a cutoff of 1,000 copies/µg was chosen in accordance with the findings of Tobal et al. [6]. RFS did not differ between the patients with an MRD level below or above 1,000 copies/µg ($P = 0.21$), with respective 2-year rates of 62 and 86%. The results were similar when we used different cutoffs such as 100 copies/µg ($P = 0.74$), the median value of 480 copies/µg ($P = 0.28$) or 3 log reduction from the time of diagnosis ($P = 0.41$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Rather, an inferior RFS was observed in patients whose MRD level was less than the median value ($P = 0.03$).

4 Discussion

This is the first report from Japan, which investigated the prognostic value of MRD in t(8;21) AML. The study highlights two principal results. First, an increase in the *RUNX1/MTG8* level strongly predicted a subsequent relapse, and it was observed after the second consolidation or later. Second, unlike previous studies from Western countries [5–9], the MRD data obtained during the early course in CR did not correlate with outcome. A lack of difference in RFS by MRD level in this study might be attributable to the relatively favorable outcome of the patients with a higher *RUNX1/MTG8* level. Although the cutoffs of the MRD level vary from study to study, the RFS rate of 69% at 2 years for the patients with lower MRD level was closely comparable with other studies [5–9]. In contrast, the 2-year RFS rate was 85% for our patients with higher *RUNX1/MTG8* level, which was much better than 10–40% in those reports [5–9]. Although it is not clear why the prognosis of such "poorer responders" was different between the Western reports and ours, this difference might contribute to the more favorable overall outcome observed in Japanese patients with t(8;21) AML [10]. Recent studies have shown that the kinase domain mutations of the *KIT* gene are detected in a substantial proportion of patients with t(8;21) AML and are associated with poor prognosis [13–15]. Further investigations on molecular pathogenesis may therefore provide further insights into this issue. On the other hand, it has been well documented that non-leukemia stem cells in t(8;21) AML patients during CR possess the AML1-MTG8 fusion gene.[16] Therefore, in patients with a high MRD, AML1-MTG8 transcripts might derive from non-leukemia cells. Further basic research on the leukemia genesis of t(8;21) AML are thus warranted.

It should be noted that the patients were not treated with uniform regimens due to the retrospective nature of the study. We therefore restricted the analysis to patients who were given intensive chemotherapy. Accordingly, all but two patients received the same induction therapy consisting of idarubicin and cytarabine, and the other two received daunorubicin and cytarabine, another standard induction regimen for AML. Regarding consolidation therapy, 46% of the patients received high-dose cytarabine, while others received standard-dose cytarabine. However, there was no difference in RFS between these two groups. Notwithstanding, such limitations make it necessary to confirm our results with a larger number of patients in prospective studies.

In conclusion, our data raise an important issue that the clinical significance of MRD in t(8;21) AML may differ between Japanese and Western patients. The MRD level measured at a given time during the early course in CR may not be useful in predicting the outcome of Japanese t(8;21) AML patients. Although this needs to be verified by future studies, clinicians should note the possibility of such potential differences among ethnicities.

Acknowledgments We wish to thank all the staff and resident members of the participating institutions. A complete list of participating institutions appears in the Appendix.

Appendix

This study was conducted at the following institutions: Toyohashi Municipal Hospital, Toyohashi; Yamanashi Prefectural Central Hospital, Kofu; National Hospital Organization Nagoya Medical Center, Nagoya; Komaki

City Hospital, Komaki; Nagoya University Hospital, Nagoya; Yokkaichi Municipal Hospital, Yokkaichi; Okazaki City Hospital, Okazaki; Meitetsu Hospital, Nagoya; Fujita Health University Hospital, Toyoake, Japan.

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EWS/ETS Regulates the Expression of the Dickkopf Family in Ewing Family Tumor Cells

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Abstract

Background: The Dickkopf (DKK) family comprises a set of proteins that function as regulators of Wnt/β–catenin signaling and has a crucial role in development. Recent studies have revealed the involvement of this family in tumorigenesis, however their role in tumorigenesis is still remained unclear.

Methodology/Principal Findings: We found increased expression of DKK2 but decreased expression of DKK1 in Ewing family tumor (EFT) cells. We showed that EFT-specific EWS/ETS fusion proteins enhance the DKK2 promoter activity, but not DKK1 promoter activity, via ets binding sites (EBSs) in the 5′ upstream region. EWS/ETS-mediated transactivation of the promoter was suppressed by the deletion and mutation of EBSs located upstream of the DKK2 gene. Interestingly, the inducible expression of EWS/ETS resulted in the strong induction of DKK2 expression and inhibition of DKK1 expression in human primary mesenchymal progenitor cells that are thought to be a candidate of cell origin of EFT. In addition, using an EFT cell line SK-ES1 cells, we also demonstrated that the expression of DKK1 and DKK2 is mutually exclusive, and the ectopic expression of DKK1, but not DKK2, resulted in the suppression of tumor growth in immuno-deficient mice.

Conclusions/Significance: Our results suggested that DKK2 could not functionally substitute for DKK1 tumor-suppressive effect in EFT. Given the mutually exclusive expression of DKK1 and DKK2, EWS/ETS regulates the transcription of the DKK family, and the EWS/ETS-mediated DKK2 up-regulation could affect the tumorigenicity of EFT in an indirect manner.

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Introduction

The Wnt/β–catenin signaling pathway is known to regulate development, differentiation, and a variety of biological phenomena. Recent findings support notion that the aberration of canonical Wnt/β–catenin signaling is involved in malignant transformation [1,2,3]. Mutations in components of the pathway have been observed in primary human cancers. These mutations often allow ligand-independent Wnt/β–catenin signaling in tumor cells. Among the components, the tumor suppressor Adenomatous polyposis coli (APC) and the scaffold protein Axin are frequently mutated in colon cancer [4] and hepatocellular carcinoma [5] respectively. Mutations in β–catenin itself are also found in a number of cancers. These changes induce the stabilization of β–catenin in the cytoplasm and an abnormal accumulation of free β–catenin in the nucleus, resulting in the aberrant activation of Wnt target genes through T-cell factor family members.

A number of activators and antagonists in the Wnt/β–catenin signaling pathway have been cloned and investigated. The Dickkopf (DKK) family is comprised of secreted protein modulators of Wnt/β–catenin signaling [6,7]. In human, the family consists of DKK1, DKK2, DKK3/REIC and DKK4, all of which have two cysteine-rich domains. DKK1 interacts with low-density lipoprotein receptor (LRP) 5/6, a component of the Wnt receptor complex, and inhibits canonical Wnt/β–catenin signaling (Mao et al. 2001). DKK2 is structurally very similar to DKK1 and also interacts with LRP5/6, but its effect on Wnt/β–catenin signaling is thought to be rather agonistic [8,9]. DKKs have been found to be important in multiple developmental processes such as limb development [10,11,12] and bone formation [13,14].

In addition, it has been recently reported that DKKs play a crucial role in cell transformation [15]. Hyper-methylation of the promoter and gene silencing of DKK1 were observed in tumor cells, including colorectal cancer [16] and malignant melanoma

cells [17]. Given evidence that ectopic expression of DKK1 suppresses features of transformation in tumor cells [18,19,20], DKK1 might inhibit tumorigenicity. However, the expression of DKK1 is elevated in some tumor cells including myeloma cells [21], hepatoblastoma cells and Wilm's tumor cells [22]. Therefore, the molecular function of DKK1 in cancer is controversial and still not fully elucidated. DKK3/REIC is also proposed as a tumor suppressor. The overexpression of DKK3/REIC inhibits tumor growth in prostate cancer [23], melanoma [17] and hepatocellular carcinoma [24]. The down-regulated expression of DKK3/REIC in osteosarcoma [25,26], hepatoblastma [26] and prostate cancer [27] further supports this notion. Although these studies indicate that the modulation of DKK expression contributes to tumorigenicity, the underlying molecular mechanism is not fully understood.

Ewing family tumor (EFT) is a pediatric cancer arising from bone and soft tissues. In EFT, a specific translocation results in production of the fusion protein EWS/ETS, where the C-terminal of EWS, including the RNA-binding domain, is replaced with a DNA-binding domain of the ets gene family, such as FLI1, ERG, E1AF, ETV1 and FEV [28]. The consequent fusion proteins have been proposed to act as an aberrant transcriptional regulator and believed to play an important role in the initiation and development of EFT. EWS/FLI1, transactivates the expression of cyclin D1 [29], cyclin E [30] and TERT [31] through the Sp1, E2F or ets DNA-binding sites located in each promoter, but suppresses the expression of p21 [32] and TGFBRII [33,34].

In this paper, we present evidence of enhanced DKK2 but suppressed expression of DKK1 in EFT cells. The experiments including those using inducible EWS/ETS expression systems in human primary bone marrow-derived mesenchymal progenitor cells (hMPCs) [35] demonstrated that the expression of DKKs is regulated by the EFT-specific chimeric protein, EWS/ETS. We further address the role of DKKs in the tumorigenicity of EFT.

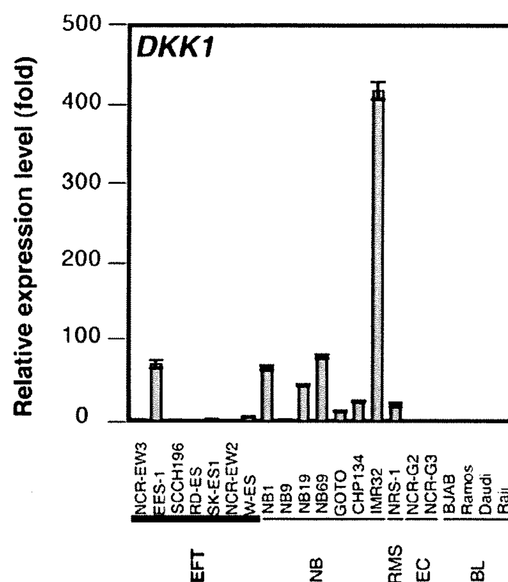
Materials and Methods

Animals

All the animals used in this study were treated in accordance with regulation on Animal Experimentation at National Research Institute for Child Health and Development.

Plasmid construction

To construct a luciferase reporter vector using the 5′upstream region of the DKK2 gene, the −1955/+49 genomic fragment of the gene was amplified by PCR from human lymphocyte genomic DNA and cloned into the *EcoRV* site of the reporter vector pGL4 (Promega) to generate pGL4-DKK2. Serial deletions of pGL4-DKK2 were generated by digestion with restriction enzymes and subsequent self-ligation. The resultant reporter vectors were designated pGL4-DKK2Δ*KpnI* (−1741/+49), pGL4-DKK2Δ*NheI* (−1241/+49) and pGL4-DKK2Δ*SacI* (−521/+49). Mutagenesis of putative ets binding sites (EBS) in the DKK2 5′upstream region was performed using KOD-plus (TOYOBO). The primers used for the mutagenesis were as follows: for the −1585/−1573 genomic fragment of the DKK2 5′upstream region (designated EBS-1): 5′-CTACCTTAAA GAAACCTTAT TCAAAAGATA3′ and 5′-AGATTTTTCA CATTTTAGTG TGTGGGGTTT-3′; for the −904/−895 genomic fragment of the DKK2 5′upstream region (designated EBS-2): 5′-GCACCTTGCC AAGGAAGACA GGATCTCAAA-3′ and 5′-CTTCTAGCCC CAGTGAATTA CAAGAGAAGC-3′. A flag-tag and a Gateway cassette were amplified from pifw [36] by PCR and the product was inserted into the *EcoRV* site of pcDNA^{TM}3 (Invitrogen) (termed pcDNA3-flagDEST). Full-length EWS/FLI1 type II, EWS/ERG and EWS/E1AF cDNAs were amplified from cDNAs prepared from NCR-EW2 [37], W-ES [38] and NCR-EW3 cells [37], respectively, by



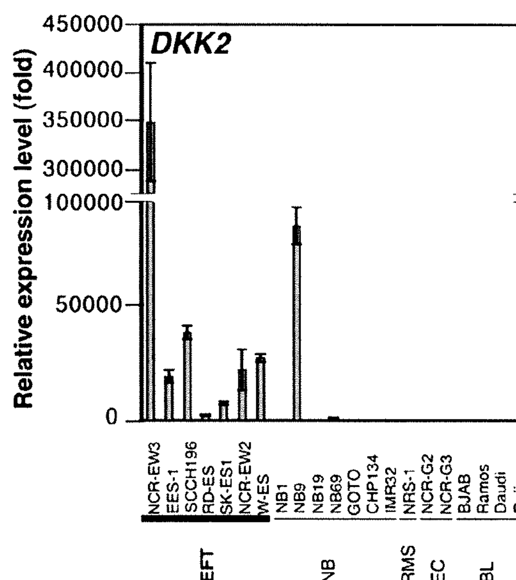


Figure 1. The expression pattern of the DKK family in Ewing's family tumor (EFT). A, B, Real-time RT-PCR analysis using pediatric tumor cell lines for DKK1 (**A**) and DKK2 (**B**) expression. EFT: Ewing's family tumor, NB: neuroblastoma, BL: Burkitt lymphoma, RMS: Rhabdomyosarcoma, EC: embryonal carcinoma. Data are normalized to the mRNA level in SCCH196 (for DKK1) and Ramos (for DKK2) which is arbitrarily set to 1. Signal intensity was normalized using that of a control housekeeping gene (human GAPDH gene). Data are relative values with the SD for triplicate wells.
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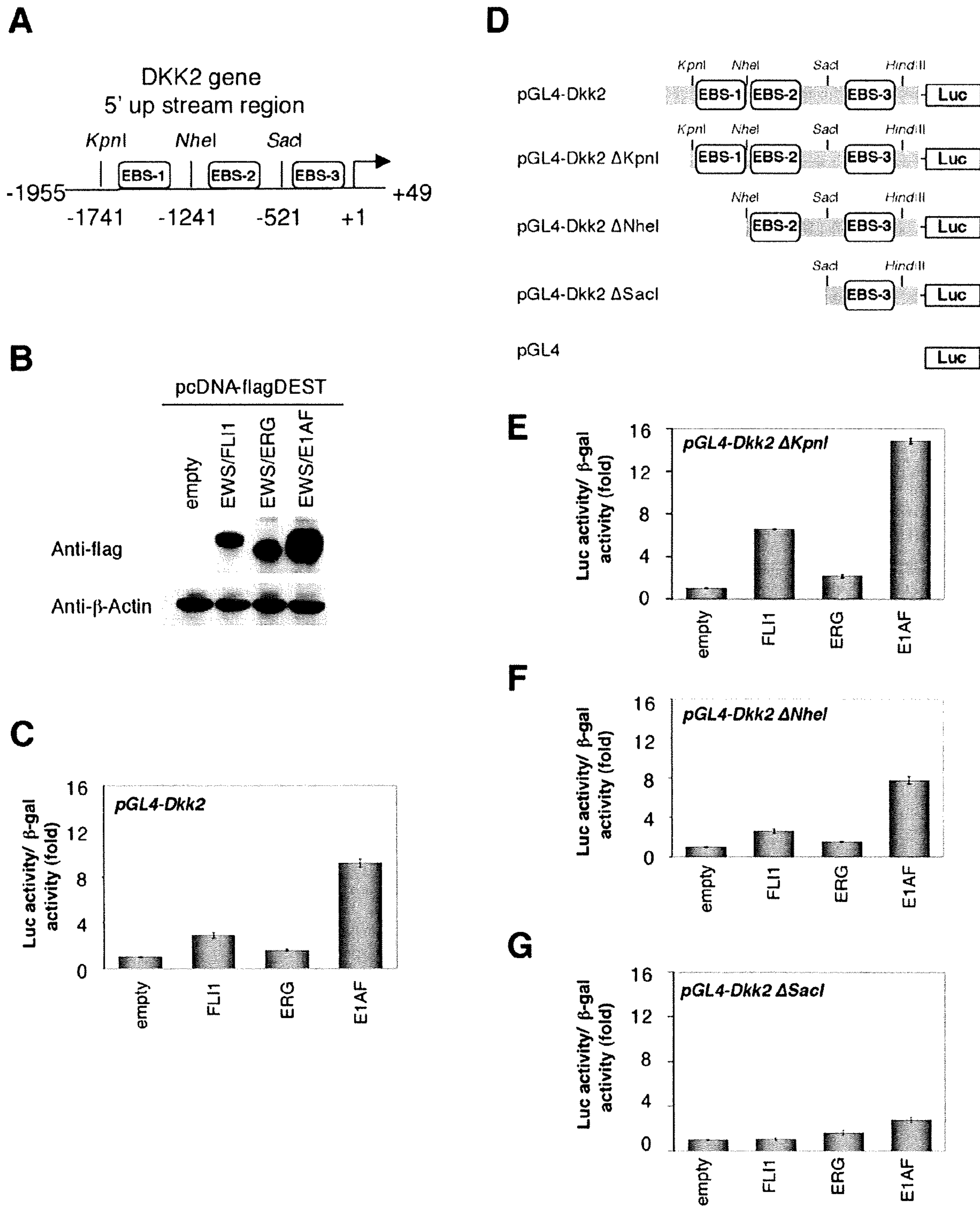

Figure 2. The effect of EWS/ETS expression on the activity of the DKK2 promoter. A, Schematic representation of the 5'upstream region of the DKK2 gene. The nucleotide numbering represents the distance from the translation start site (+1). *KpnI* (−1741), *NheI* (−1241), and *SacI* (−521) sites were used to construct a series of deletion mutants of the DKK2 promoter (see Materials and Methods). Consensus ets binding sites are boxed (EBS-1, EBS-2 and EBS-3). **B,** Western blot analysis for EWS/ETS expression. HEK293 cells were transiently transfected with pcDNA3-flagDEST or pcDNA3-flagEWS/ETS and then analyzed by anti-flag Western blotting. **C,** The effect of EWS/ETS expression on the activity of the DKK2 promoter. HEK293 cells were co-transfected with reporter plasmids and the expression vector. Luciferase activity was measured after 48 hours. Data are relative values with the SD for triplicate wells. Data are normalized to the value for the empty vector (pcDNA3-flagDEST) which is arbitrarily set to 1. The