Translational Relevance

Preoperative assessment of serum *DCR2* methylation status can be a potent prognostic factor in patients with neuroblastoma, even in those without a *MYCN* gene amplification (MNA). Notably, this serum *DCR2* methylation assay allows us to distinguish neuroblastoma cases with poor outcome before the initial therapy. Furthermore, serial monitoring of the serum *DCR2* methylation status can be a sensitive indicator of therapeutic efficacy in *DCR2*-methylated cases. The method is also noninvasive, rapid, and sensitive, requiring only 200 μL of serum, regardless of tumor stage. In addition, our established serum *DCR2* methylation assay may allow the new risk stratification of patients with non-MNA neuroblastoma in future trials, i.e., it can help to determine the appropriate intensity of chemotherapy or to evaluate a novel therapy, especially in patients with non-MNA neuroblastoma with poor outcome.

homozygous deletions or hemizygous deletions and mutations (11–13). Furthermore, some types of aberrant hypermethylation were shown to be useful as predictors of poor prognosis (13, 14). In neuroblastomas, several tumor suppressor genes have been shown to be silenced by aberrant hypermethylation of their promoters. Examples of such genes are *CASP8* (15–17), *RASSF1A* (18–20), *HOXA9* (14), *NR1I2* (21), *CCND2* (14), *14.3.3σ* (18), and *DCR2* (15, 16, 18, 22). A positive correlation has been found between hypermethylation of the promoters of these genes and poor prognosis, suggesting that hypermethylation influences the phenotype of neuroblastoma (15, 18).

DCR2 (decoy receptor 2) is a tumor necrosis factor-α receptor superfamily gene that is located on 8p21 (18, 22). *DCR2* is negatively associated with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis because it lacks an intracellular death domain. *DCR2* is ubiquitously expressed in normal tissue, where it prevents apoptosis (22). However, *DCR2* expression was found to be silenced because of aberrant methylation of its promoter regions in some cancers (23). In neuroblastoma, the methylation profile of *DCR2* has been found to be drastically different and independent of *MYCN* status (16, 18, 22). Moreover, *DCR2* methylation was found to be associated with rapidly progressing tumors and reduced overall survival (18).

Quantification of serum DNA has been proposed as a screening tool for the early detection of lung cancer (24), and several groups have reported the clinical utility of circulating DNA in serum for genetic assessment of malignant tumors because serum DNA predominantly originates from tumor-released DNA in patients with cancer (24, 25). Our group previously reported a highly practical assay for the evaluation of *MYCN* status using serum DNA, which enables neuroblastoma with MNA to be distinguished from non-MNA neuroblastoma prior to tumor resection (26). Subsequently, we need to find an additional serum DNA–based marker to identify patients with poor prognosis even in non-MNA cases.

Recently, the detection of tumor-derived methylated genes in serum DNA has attracted attention as a novel marker because of their prognostic value and rapid accessibility as compared with tumor DNA (27–31). We hypothesized that the detection of aberrant methylation of *DCR2* in serum DNA could be a useful biomarker for predicting prognosis and therapeutic efficacy for patients with neuroblastoma, even in non-MNA cases because the methylation of *DCR2* in neuroblastoma was found to be independent of *MYCN* status. Therefore, we aimed to establish a serum DNA–based assay for evaluating the methylation status of *DCR2*, and to assess its clinical utility.

Patients and Methods

Subjects. Eighty-six children diagnosed with neuroblastoma at the Hospital of Kyoto Prefectural University of Medicine were enrolled onto this study with the informed consent of their parents. Eighteen of the patients had MNA neuroblastoma, and 68 patients had non-MNA neuroblastoma, as determined by Southern blotting or fluorescence *in situ* hybridization. According to the International Neuroblastoma Staging System (2), the 86 patients consisted of 37 in stage 1, 11 in stage 2A or 2B, 2 in stage 4S, 9 in stage 3, and 27 in stage 4, whereas the 68 patients in the non-MNA group included 34 in stage 1, 11 in stage 2A and 2B, 2 in stage 4S, 7 in stage 3, and 14 in stage 4. The patients in stages 1, 2A, 2B, and 3 at <18 mo of age were categorized as the low-risk group, whereas the other patients who were in stage 3 at ≥18 mo of age, and patients in stage 4, were categorized as the high-risk group (Table 1). The serum and tumor samples were linked to clinical and biological information and the laboratory investigators were blinded to these data. Twenty of the control sera samples were also obtained from healthy volunteers who did not have any known diagnosis of malignant disease.

Cell lines, tumor samples, and serum preparation. Five human neuroblastoma cell lines (IMR32, GOTO, KP-N-RTBM1, SK-N-AS, and KP-N-SIFA) were used in this study. Each had been established from a surgically resected tumor or metastatic bone marrow sample, and maintained as described previously (32). These cells were cultured with or without 1 μmol/L of 5-aza 2′-deoxycytidine (Sigma) for 5 days, and then harvested and used for DNA and RNA isolation (21). Primary tumor samples were obtained at surgeries which were done at the

Table 1. Clinical and biological characteristics of 86 patients with neuroblastoma

Characteristics	No.	%
Gender		
Male	50	58
Female	36	42
Age		
<18 mo	62	72
>18 mo	34	28
Mass screening		
+	49	57
-	37	43
Stage (INSS), in MNA(-) group (n = 68)		
1	37 (34)	43 (50)
2A, 2B	11 (11)	12.7 (16)
3	9 (7)	10 (10)
4	27 (14)	31 (21)
4S	2 (2)	2.3 (3)
MYCN		
Amplification	18	21
Nonamplification	68	79

NOTE: Values in parentheses show the number of the patients targeted in the non-MNA group.
Abbreviation: INSS; International Neuroblastoma Staging System.

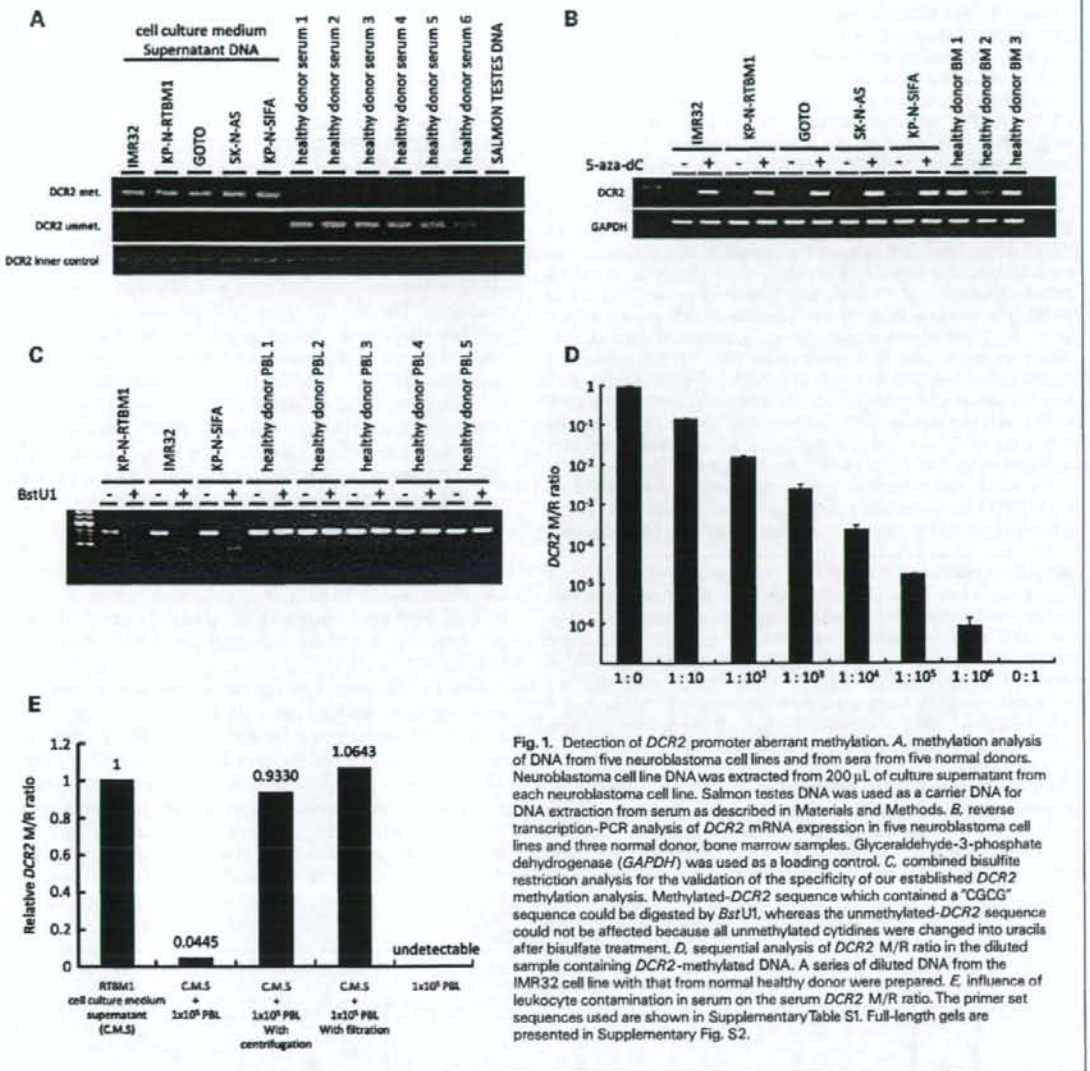

Fig. 1. Detection of *DCR2* promoter aberrant methylation. *A*, methylation analysis of DNA from five neuroblastoma cell lines and from sera from five normal donors. Neuroblastoma cell line DNA was extracted from 200 μL of culture supernatant from each neuroblastoma cell line. Salmon testes DNA was used as a carrier DNA for DNA extraction from serum as described in Materials and Methods. *B*, reverse transcription-PCR analysis of *DCR2* mRNA expression in five neuroblastoma cell lines and three normal donor, bone marrow samples. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *C*, combined bisulfite restriction analysis for the validation of the specificity of our established *DCR2* methylation analysis. Methylated-*DCR2* sequence which contained a "CGCG" sequence could be digested by *Bst*U1, whereas the unmethylated-*DCR2* sequence could not be affected because all unmethylated cytidines were changed into uracils after bisulfate treatment. *D*, sequential analysis of *DCR2* M/R ratio in the diluted sample containing *DCR2*-methylated DNA. A series of diluted DNA from the IMR32 cell line with that from normal healthy donor were prepared. *E*, influence of leukocyte contamination in serum on the serum *DCR2* M/R ratio. The primer set sequences used are shown in Supplementary Table S1. Full-length gels are presented in Supplementary Fig. S2.

Hospital of Kyoto Prefectural University of Medicine from 1980 to 2007, and stored at -80°C. Patient's serum samples were stored at -20°C. For DNA isolation, serum was centrifuged at 15,000 rpm for 10 min or filtered with a 0.45-μm filter (Kurabo Industries, Ltd.) to remove leukocytes.

Reverse transcription-PCR. RNA was extracted from cell lines and used for reverse transcription-PCR as reported previously (32). Briefly, total RNA was extracted from cell lines using the QIAamp RNeasy Protect Mini kit (Qiagen, GmbH), and reverse-transcribed to synthesize cDNA using the Superscript First-strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions. Bone marrow samples were obtained from a healthy donor for use as a positive control for the expression of *DCR2*. Glyceraldehyde-3-phosphate dehydrogenase cDNA was also amplified for use as a loading control, as described previously (33). Primers for amplifying the *DCR2* cDNA and *GAPDH* cDNA are shown in Supplementary Table S1.

DNA preparation and methylation analysis. DNA was extracted with a QIAamp DNA Mini kit (Qiagen) as per the manufacturer's protocol. For serum DNA extraction, we used 200 μL of stored serum, which contained 1 μg of salmon testes DNA (Sigma) as a carrier DNA. To investigate the methylation of DNA, genomic DNA were treated with sodium bisulfite by using an EZDNA methylation kit (Zymo Research) following the manufacturer's protocol, and subjected to methylation-specific PCR using the appropriate primer sets. Based on the sequences after bisulfate treatment, we designed methylation- and unmethylation-specific PCR primers, which recognize sequences unique to the methylated and unmethylated alleles, respectively, as shown in

Supplementary Table S1. Primers were also designed for a reference sequence in the *DCR2* promoter which is not affected by DNA methylation. To increase the sensitivity and specificity for the detection of methylated DNA, we firstly conducted nested PCR with serum DNA samples and primer sets that could amplify both methylated and unmethylated alleles, and then real-time PCR was carried out with an ABI Prism 5700 Sequence Detection System (Applied Biosystems), using nested PCR products and methylation-specific and reference sequence primer sets. The *DCR2* methylation status was calculated as a methylated-*DCR2* allele copy number/reference sequence allele copy number (M/R ratio). Standard curves were constructed in each PCR run with 4-fold serial dilutions containing nest PCR products of KP-N-RTBM1 DNA. Copy numbers were expressed as the average of two measurements. The nested PCR mixture contained Premix Ex Taq HS version (Takara Bio, Inc.), 400 nmol/L of each primer, and 2% of DMSO. The real-time PCR mixture contained SYBR Premix Ex Taq (Takara Bio), 200 nmol/L of each primer, and 2% of DMSO. All used primers are summarized in Supplementary Table S1. The nested PCR reactions were done with one cycle of 95°C for 30 s, followed by PCR amplification with 20 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 7 min. The real-time PCR reactions were done with one cycle of 95°C for 30 s, followed by PCR amplification with 40 cycles of 95°C for 5 s and 63°C for 31 s.

To assess the specificity of our established methylation status analysis for the *DCR2* promoter region, combined bisulfite restriction analysis with *Bst*U1 restriction enzyme (New England Biolabs, Inc.) was done as described previously (34). To determine the sensitivity of the methylation analysis, IMR32 cell line DNA was diluted with normal healthy donor DNA from 1:1 to $1:10^6$, and each sample was done with bisulfate modification and nested PCR as described before, and then M/R ratios were calculated. To elucidate the influence of leukocyte contamination on the serum *DCR2* M/R ratio, we added 1×10^5 of peripheral blood leukocytes to 200 μL of the KP-N-RTBM1 cell line culture medium supernatant with or without centrifugation at 15,000 rpm for 10 min, or filtered it through a 0.45-μm filter, and then M/R ratios were evaluated using bisulfate-modified DNAs obtained from each sample.

Statistical methods. Differences between the two groups were assessed using the χ^2 test. Kaplan-Meier curves were used to estimate event-free survival rates and overall survival rates and were compared with the use of the log rank statistic (35). The relation of methylation status between tumor and serum DNA was assessed by simple regression analysis. Descriptive statistical analyses were done with SPSS software. $P < 0.05$ was judged significant.

Results

Established quantitative real-time PCR–based methylation-specific PCR for the detection of* DCR2 *promoter methylation. Methylated-*DCR2* was detected in all five neuroblastoma cell line DNAs we analyzed, whereas no methylated-*DCR2* signals were detected in six healthy donor serum DNAs (Fig. 1A; Supplementary Fig. S1). In these neuroblastoma cell lines, no *DCR2* mRNA expression was observed; however, induction of *DCR2* expression occurred after a methyltransferase inhibitor treatment (Fig. 1B). In the *DCR2*-methylated neuroblastoma cell line, fragmented bands appeared after *Bst*U1 treatment, whereas normal healthy donor DNA was not digested by *Bst*U1 treatment (Fig. 1C). The *DCR2* M/R ratio gradually decreased following the serial dilution of IMR32 cell line DNA with normal human DNA. Furthermore, a *DCR2* methylation signal was detected even in an IMR32 DNA sample diluted to $1:10^6$ (Fig. 1D). Moreover, an additional centrifugation or filtration step eliminated cellular contamination, and restored the *DCR2* M/R ratio to the same level of the noncontaminated sample (Fig. 1E). These results suggest that *DCR2* expression was silenced by aberrant hypermethylation of the promoter region, and show that our real-time PCR–based methylation-specific PCR method could detect *DCR2* promoter aberrant methylation with high specificity and sensitivity in both the tumor and serum DNA.

Serum* DCR2 *methylation status as a predictor of tumor* DCR2 *aberrant hypermethylation. Of the 80 patients in which methylation status could be evaluated, both in tumor and in serum obtained before the initial therapy, *DCR2* methylation status showed a significant correlation between tumor and serum DNA. Especially in patients having *DCR2* methylation in tumor, the M/R ratios of tumor DNA and serum DNA were strongly correlated ($r = 0.67$; $P = 0.002$; Fig. 2), regardless of the patients having a localized or metastatic tumor. Not

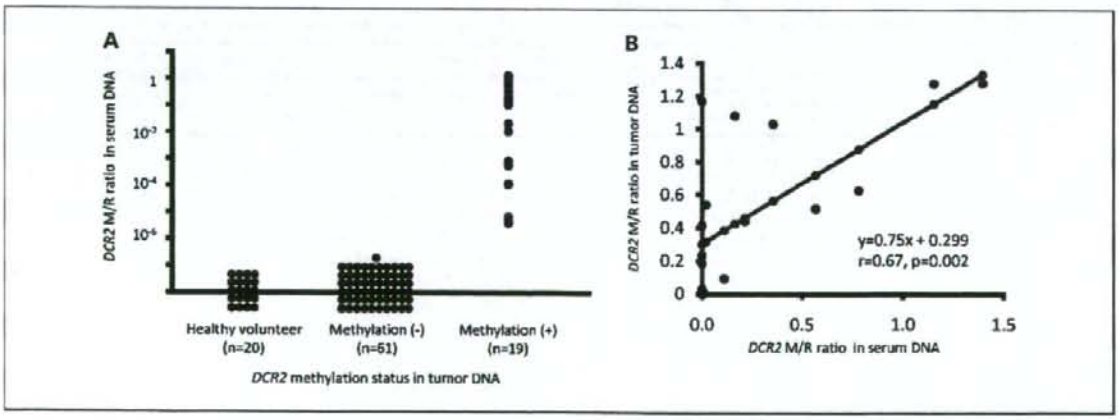

Fig. 2. *A*, distribution of serum *DCR2* M/R ratio among tumor *DCR2* methylation-positive ($n = 19$) and -negative ($n = 61$) patients who could be evaluated for both tumor and serum DNA obtained before the initial therapy, and in healthy volunteers ($n = 20$). Serum *DCR2* methylation statuses were significantly correlated with *DCR2* methylation statuses in tumor. *B*, simple regression analysis for the correlation between *DCR2* M/R ratio in tumor and in serum.

Table 2. Results of χ^2 test for effect of *DCR2* aberrant methylation on stage and MNA in patients with neuroblastoma

Characteristics	*DCR2*		*P*
	Methylated	Unmethylated	
Stage			<0.001
Low	6	53	
High	18	9	
Stage in MNA(-)			<0.001
Low	4	49	
High	11	4	
MYCN amplification			0.04
Amplification	9	9	
Nonamplification	15	53	

surprisingly, *DCR2* aberrant methylation signals were not detected in the sera of 20 healthy volunteers (Fig. 2A). This suggests that the *DCR2* methylation status in tumor could be predicted from the serum *DCR2* M/R ratio.

DCR2 ***methylation status and clinical outcome.*** Table 1 shows the clinical characteristics of our cohort. *DCR2* aberrant methylation was detected in 24 of 86 neuroblastoma tumors (28%). The frequency of *DCR2* aberrant methylation in the high-risk neuroblastoma patient group (n = 27) was significantly higher than that in the low-risk group (n = 59; 68% versus 10%; $P < 0.001$; Table 2). Especially among the patients who didn't have MNA (n = 68), the frequency of *DCR2* aberrant methylation was also strongly different between the high-risk group (n = 15) and the low-risk group (n = 53; 73% versus 8%; $P < 0.001$). However, in the frequency of *DCR2* methylation between patients with MNA and non-MNA (50% versus 22%; P = 0.04), the difference was not so significant. Figure 3 shows event-free survival and overall survival rates in *DCR2*-methylated and unmethylated patients. *DCR2*-methylated patients showed significantly poorer 5-year event-free survival than *DCR2*-unmethylated patients in the neuroblastoma group (43% versus 84%; $P < 0.001$), especially in the non-MNA group (12% versus 96%; $P < 0.001$). Furthermore, *DCR2*-methylated patients showed significantly poorer 5-year overall survival than *DCR2*-unmethylated patients in the neuroblastoma group (55% versus 85%; P = 0.008), especially in the non-MNA group (56% versus 96%; $P < 0.001$). These results indicate that *DCR2* aberrant hypermethylation is a useful biomarker for the prediction of poor prognosis, especially in patients with non-MNA neuroblastoma.

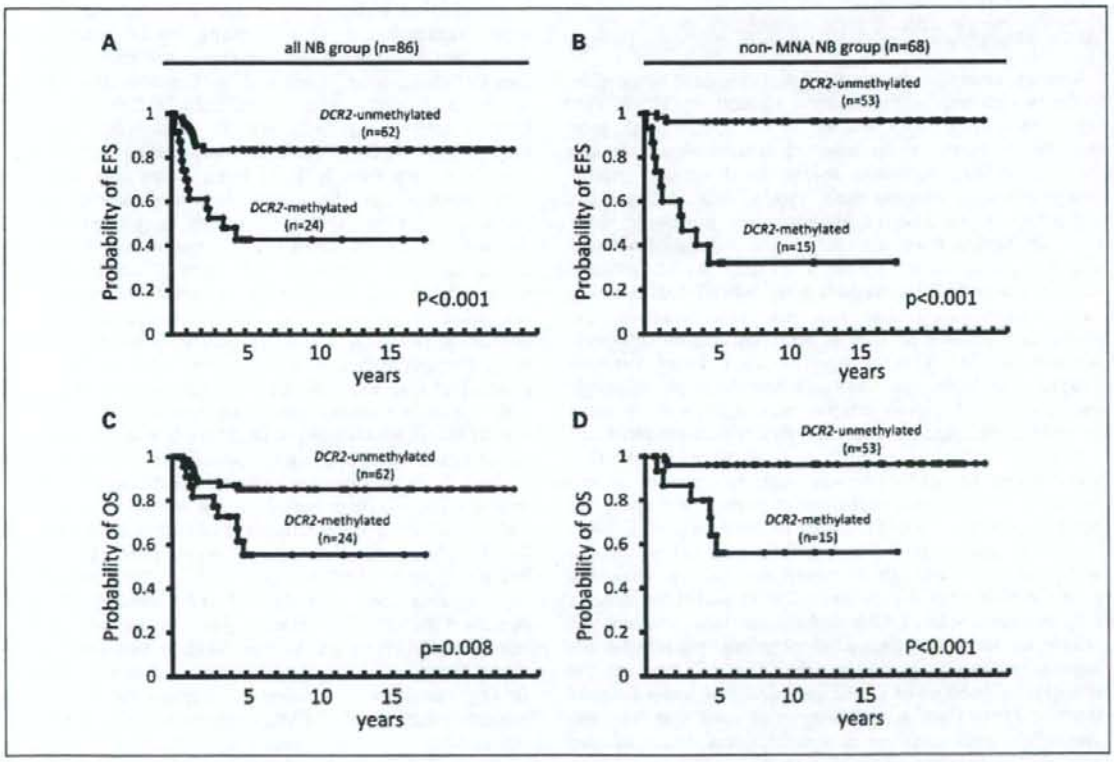

Fig. 3. Kaplan-Meier survival curves for patients with neuroblastoma according to the *DCR2* methylation status. *A*, event-free survival in all patients with neuroblastoma: methylated (n = 24) and unmethylated (n = 62); $P < 0.001$. *B*, event-free survival in patients with non-MNA neuroblastoma: methylated (n = 15) and unmethylated (n = 53); $P < 0.001$. *C*, overall survival in all patients with neuroblastoma: methylated (n = 24) and unmethylated (n = 62); P = 0.008. *D*, overall survival in patients with non-MNA neuroblastoma: methylated (n = 15) and unmethylated (n = 53); $P < 0.001$.

DCR2 *methylation status in serum as an indicator of therapeutic efficacy and minimum residual disease.* To evaluate whether an increase in serum *DCR2* M/R ratio can be used as an indicator of relapse, we measured the serum *DCR2* M/R ratio at several points in the clinical courses of five *DCR2*-methylated patients (Fig. 4). In two patients who were in complete remission (Fig. 4A and B), the serum *DCR2* M/R ratio decreased to an undetectable level. In contrast, in three patients who experienced recurrence after remission (Fig. 4C, D, and E), the serum *DCR2* M/R ratio first decreased to an undetectable level, and increased again by the time of diagnosis. Figure 4E shows the clinical course of a 3-year-old male neuroblastoma patient who was categorized, at the onset, as stage 4 with bone and bone marrow metastasis. His *DCR2* M/R ratio at the onset was 1.87×10^{-4} in the tumor and 9.71×10^{-4} in the serum, and then serum *DCR2* M/R ratio gradually decreased to an undetectable level during therapy. The patient was in remission in August 2005, at which time a bone marrow examination revealed no tumor clump. However, a *DCR2*-methylated signal was detected in the bone marrow sample (Fig. 4E, *red box*), and after 1 month, a relapse in bone marrow was disclosed. These results indicate that serial detection of *DCR2* methylation in serum or bone marrow samples is a highly sensitive indicator of therapeutic efficacy and relapse in patients with *DCR2*-methylated neuroblastoma.

Discussion

Recently, several studies revealed that epigenetic changes are likely to influence neuroblastoma phenotype (12, 14–16, 36), although little is known about the role of gene methylation in the progression of neuroblastoma. Among the tumor-related epigenetic aberrations detected in neuroblastoma tumors, aberrant methylation of *DCR2* is of greatest interest to us because of its prominent prognostic value (16, 18). Several years ago, Banelli and colleagues reported that the CpG methylation profiles of *CASP8*, *14.3.3σ*, *δNp73*, *RASSF1A*, and *DCR2* promoters were associated with malignant phenotypes of neuroblastoma (18). Especially, the methylation patterns of *14.3.3σ*, *RASSF1A*, and the intragenic segment of *CASP8* were significantly different between patients with MNA and non-MNA neuroblastoma, although the difference of overall survival rates between the patients presenting methylation or unmethylation of these genes did not reach statistical significance. Furthermore, aberrant methylation of *RASSF1A* was recently detected in all neuroblastoma tumors, regardless of their phenotype (37). Aberrant methylation of the *DCR2* promoter was also shown to be a prognostic marker of neuroblastoma, independent of MNA status. In fact, *DCR2*-methylated patients showed a poorer overall survival rate than *DCR2*-unmethylated patients (18). However, when *DCR2* methylation status was analyzed only in the non-MNA group, the methylated patients did not have a statistically poorer survival rate even though the methylation profiles of *DCR2* and *RASSF1A* were analyzed together (18). Yang and colleagues showed that high-risk disease and poor outcome of neuroblastoma were associated with the methylation of each of *DCR2*, *CASP8*, and *HIN-1* (16). However, they didn't examine whether *DCR2* methylation could be used to identify poor prognostic patients in the non-MNA group.

Detection of aberrant methylated DNA in serum can be clinically useful for disease screening, diagnosis, prognosis, and assessing occult disease progression (27–31). If *DCR2* methylation status is associated with the unique biological variables of non-MNA neuroblastoma, and if it can be detected in circulating serum DNA, it would therefore be of obvious clinical value. Furthermore, it would be especially useful for pediatric cancer patients who cannot easily undergo invasive examinations. By using real-time–based methylation-specific PCR with a reference allele located within the same promoter, we have established a rapid, noninvasive, and quantitative method for evaluating the methylation status of the *DCR2* gene promoter that requires only 200 μL of serum. In addition, aberrant methylation of *DCR2* in serum DNA was strongly correlated with methylation status in the tumor (Fig. 2). The highest sensitivity and specificity between the methylation status in the serum and in the tumor were obtained with a nested PCR for amplifying methylated and reference alleles in serum DNA. Furthermore, we found aberrant hypermethylation of *DCR2* in some patients categorized as having low-risk neuroblastoma, even though the tumor was localized in these patients. This suggests that neuroblastomas could release a methylated DNA into the systemic circulation even at an early stage of neuroblastoma. Furthermore, our serum-based *DCR2* methylation assay can reliably predict *DCR2* methylation status in tumors with high sensitivity and specificity, regardless of tumor localization. Moreover, among our 86 patients with neuroblastoma, the *DCR2*-methylated patients tended to progress to a higher stage, and to have poorer event-free survival and overall survival rates than *DCR2*-unmethylated patients. Interestingly, *DCR2* aberrant methylation was more strongly associated with poor prognosis among the non-MNA patients ($n = 68$, Table 2; Fig. 3). In fact, some cases presenting *DCR2* aberrant methylation proceeded to stage 4 during the course of the disease, although they were categorized into low-risk neuroblastoma without MNA at the onset. This suggests that preoperative assessment of serum *DCR2* methylation status can be a potent prognostic factor in patients with neuroblastoma, even in those without MNA. Additionally, our established assay rapidly provides prognostic information that can distinguish patients with poor outcome from the non-MNA group, and may allow the new risk stratification of patients with non-MNA neuroblastoma in future trials.

How epigenetic silencing of *DCR2* could result in aggressive tumor proliferation and poor prognosis of neuroblastoma is unclear. There is interest in using the death ligand TRAIL to treat malignant tumors because it can induce apoptosis in a variety of different tumor cells but not in normal cells (38). The TRAIL-induced apoptosis signal is mediated by specific interaction with TRAIL receptors DR4 or DR5, respectively, and involves adapter molecules such as Fas associated via death domain (39, 40). Downstream initiator caspases, such as caspases 8 and 10, are activated by TRAIL, and induce apoptosis either directly or through mitochondrial pathways (38–40). *DCR2*, as its name "decoy receptor" suggests, has a dominant-negative effect against TRAIL receptors because it lacks an intracellular death domain, and produces a competitive blockade of TRAIL-mediated apoptosis (39). However, TRAIL does not induce apoptosis in some cancers, and can even mediate tumor cell survival and proliferation (41, 42). Neuroblastoma cells often do not express caspase 8 and are

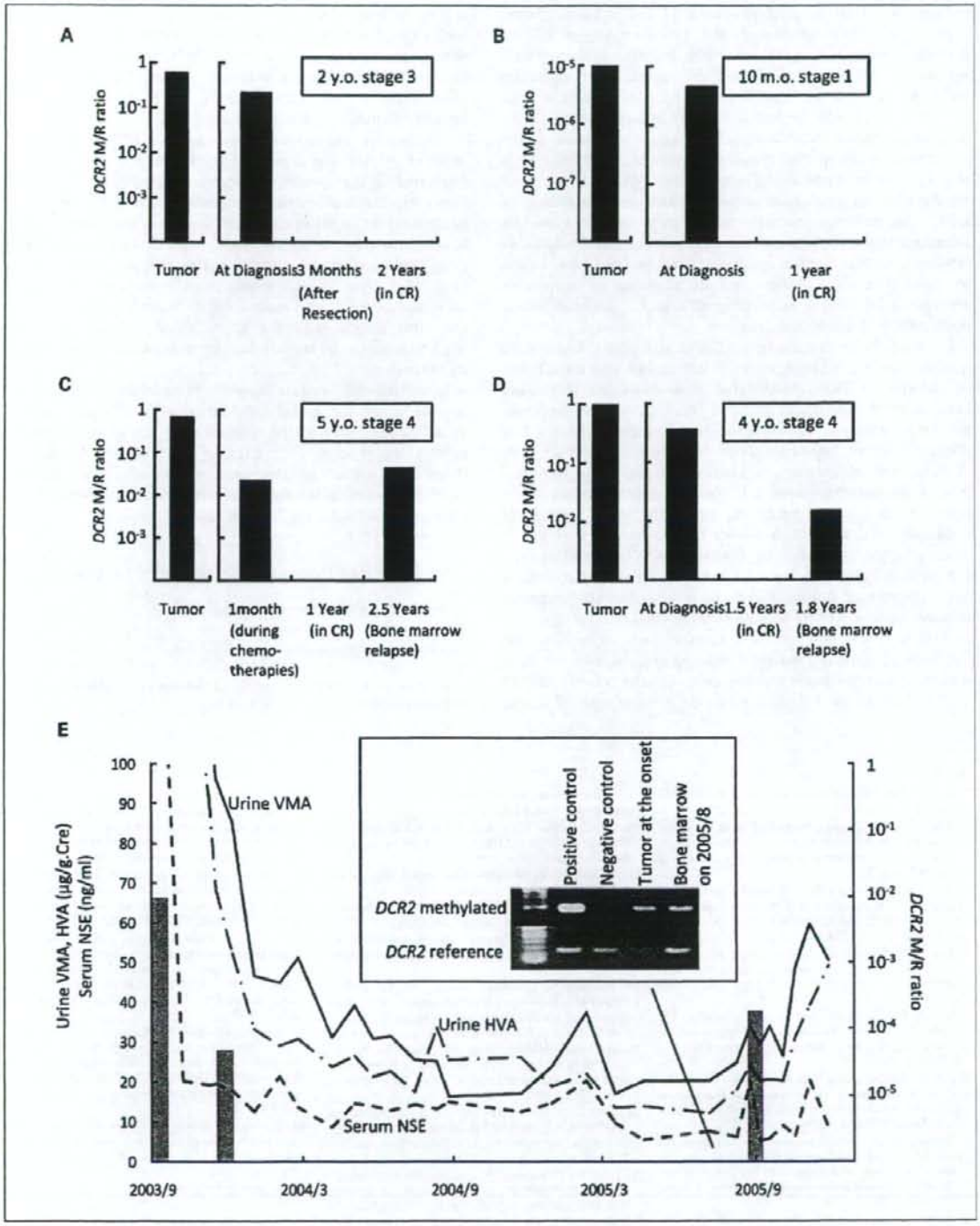

Fig. 4. Changes in serum *DCR2* M/R ratios of five patients with *DCR2* aberrant methylation during follow-up. CR, complete remission; VMA, vanillylmandelic acid; HVA, homovanillic acid; NSE, neuron-specific enolase.

resistant to TRAIL-induced apoptosis (17, 43). Furthermore, TRAIL was found to induce the proliferation of KERRY neuroblastoma cells (41), possibly because nonapoptotic signals by TRAIL are mediated by intracellular signaling molecules, such as nuclear factor-κB via TRAIL receptor DR4 or DR5 (41, 44, 45). Activation of nuclear factor-κB by TRAIL inhibits apoptosis induction, and inhibition of nuclear factor κB activation attenuates apoptosis resistance in tumor cells (41, 42). We infer that *DCR2* antagonizes TRAIL-mediated cell proliferation in neuroblastoma cells, and gene silencing of *DCR2* due to epigenetic aberrations may provide a growth advantage to neuroblastoma cells that do not express caspase 8. However, further studies are needed to explain how *DCR2* promoter hypermethylation and its silencing of expression are associated with a poor prognosis and aggressive tumor proliferation in neuroblastoma.

It is noteworthy that the serum *DCR2* M/R ratio could predict not only the methylation status in tumor, but also the efficacy of therapy in *DCR2*-methylated neuroblastoma. Although tumor-related methylated DNAs in serum have been proposed for use as prognostic markers in several cancers (25, 27–31), they have not yet been considered for clinical use as indicators of therapeutic efficacy or predictors of relapse. In neuroblastomas, a quantitative method for assessing MNA status using serum DNA (26) is becoming not only an indispensable diagnostic tool, but also a marker for monitoring therapeutic efficacy after therapies in patients with neuroblastoma. However, it cannot be used to assess samples that contain a large amount of normal tissue, such as a metastatic sample of bone marrow. This is because every normal cell has one copy of *MYCN* DNA, and the copy number ratio of *MYCN* and the reference gene is apparently reduced as a result of contamination of normal bone marrow cells. On the other hand, as *DCR2* aberrant methylation is not detected in normal tissue, and as the serum *DCR2* methylation status is closely associated with clinical course, it can be a useful biomarker to predict the therapeutic efficacy and/or relapse in bone marrow, as well as being a predictor of poor prognosis. It should also be useful for close follow-ups of cancer patients, as well as for diagnosis of pediatric cancer risk classification.

The main concern with the serum *DCR2* methylation method is that the degree of methylation could be underestimated if the serum was contaminated with leukocytes. However, the leukocytes were easily removed in the present study by centrifugation or filtration. In fact, the effect of added leukocytes on the *DCR2* M/R ratio in serum could be completely removed by additional centrifugation or filtration (Fig. 1E). Thus, it is necessary to standardize the serum collection procedure to ensure that different laboratories obtain the same results with the given blood samples. Also, our method needs to be tested on a larger set of patients to confirm its reliability.

In conclusion, we established a noninvasive, sensitive, and specific assay for quantifying aberrant methylation of the *DCR2* gene promoter that requires only 200 μL of serum. The method has promise for predicting prognosis and determining therapeutic efficacy in neuroblastoma, especially in non-MNA cases. Furthermore, it might also be useful as a marker of tumor recurrence in *DCR2*-methylated cases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Low-dose Protracted Irinotecan as a Palliative Chemotherapy for Advanced Neuroblastoma

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Summary: Management of cases of refractory neuroblastoma remains a challenge. As intensive chemotherapy sometimes results in severe regimen-related toxicity and poor quality of life, palliative chemotherapy with modest toxicity may be considered for these cases. We report 2 cases of stage 4 neuroblastoma with poor performance status that received low-dose protracted schedules of irinotecan. This regimen achieved not only disease stabilization but also dramatic improvements of quality of life for significant periods. A low-dose protracted schedule of irinotecan was tolerable even if the patient's performance status was poor, and thus might be useful as a palliative chemotherapy for advanced neuroblastoma.

Key Words: neuroblastoma, irinotecan, quality of life, performance status, palliative chemotherapy

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Intensive combination chemotherapy including high-dose chemotherapy has improved the prognosis of patients with advanced neuroblastoma.[1] However, in refractory cases, long-term intensive chemotherapy sometimes results in severe therapy-related toxicity and decreases in performance status (PS) and quality of life (QOL). These patients cannot tolerate myeloablative chemotherapy with hematopoietic stem cell transplantation because the therapy-related toxicity might be fatal. As they have little chance of being cured, palliative therapy for maintaining good QOL as long as possible is considered. To this end, regimens that are less intensive and less toxic, and that protect against tumor progression are warranted. Although several drugs, for instance retinoids or low-dose etoposide, have been tried for less intensive therapy for neuroblastoma,[1] palliative chemotherapy for refractory neuroblastoma has not been established yet.

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Irinotecan, a camptothecin analog, is a prodrug that is converted to the active metabolite SN-38 in vivo, and inhibits topoisomerase I.[2] Its antitumor activity is thought to be due to interference with S phase-specific DNA replication.[2] Although several irinotecan dosing schedules have been evaluated,[2–5] a low-dose protracted schedule is known to be less myelosuppressive and to have promising antitumor activity for neuroblastoma.[3] This schedule consists of 20 mg/m^2/d infused intravenously over 1 hour for 5 days per week for 2 consecutive weeks [(every day × 5) × 2] every 21 days. Kushner et al[4] used 5-day courses of irinotecan at 50 mg/m^2/d as a palliative therapy for refractory neuroblastoma. However, the optimal usage of irinotecan for a palliative setting has not been determined. Here, we report 2 cases of advanced neuroblastoma with a low-dose protracted schedule of irinotecan 20 mg/m^2/d [(every day × 5) × 2]. Although these cases were heavily pretreated and showed poor PS due to severe regimen-related toxicity, low-dose irinotecan not only stabilized the disease, but also markedly improved the QOL for a significant time.

CASE REPORT

Case 1

A 16-month-old girl was referred to our hospital due to exophthalmus and a left supraclavicular mass. Blood examination showed anemia (hemoglobin 8.1 g/dL) and elevation of neuron-specific enolase to 178.8 ng/mL. The levels of urinary vanillylmandelic acid and homovanillic acid were elevated to 615 and 639 μg/g creatinine, respectively. Tumor cells were present in bone marrow. Abdominal computed tomography revealed a large para-aortic mass with calcification. Magnetic resonance imaging of the head showed multiple dural and skull tumors. A ^{123}I-metaiodobenzylguanidine (MIBG) scintigram showed multiple abnormal uptake lesions on the head, neck, and abdomen. An open biopsy of the supraclavicular mass confirmed the diagnosis of stage 4 neuroblastoma without MYCN amplification. Although she received 4 courses of intensive induction therapy consisting of cyclophosphamide, cisplatin, pirarubicin, and vincristine,[6] she showed no response according to the International Neuroblastoma Response Criteria (INRC). A combination of ifosfamide and etoposide also failed because of ifosfamide-induced Fanconi syndrome. Neither a high dose of cyclophosphamide combined with doxorubicin and vincristine nor whole cranial irradiation (30 Gy) prevented tumor progression. Because she developed pneumatosis intestinalis and paralytic ileus due to chemotherapy and/or tumor involvement, she had not been able to eat for 6 months and became

emaciated. Her Lansky PS score[7] before starting irinotecan was 60. Because she could not continue intensive therapy and had little chance of being cured, we decided to try irinotecan with a low-dose protracted schedule. The parents gave written consent for the treatment after being informed of the rationale for the treatment, the known side effects of irinotecan, and the possibility of unforeseen life-threatening toxicities including the worsening of paralytic ileus. Irinotecan was initiated when the patient was 27 months old. Normally, the administration of irinotecan [(every day × 5) × 2] was repeated every 3 weeks. However, when therapy-related toxicities [eg, intestinal paralysis, infection, myelosuppression (white blood cell count < 1000/μL), and liver dysfunction (elevation of alanine aminotransferase or aspartate aminotransferase)] affected her, we suspended or delayed the irinotecan schedule until recovery from the toxicities. As a result, 14 of 25 courses of irinotecan were suspended or delayed. We did not reduce the dose of irinotecan. During 25 courses of irinotecan over a period of 22 months, the disease remained stable (no response by INRC) (Fig. 1). Grade 2 transient diarrhea occurred but was tolerable with the suspension of irinotecan or the use of loperamide. No abdominal cramps occurred. As her intestinal paralysis gradually improved after starting irinotecan, she could eat again 6 months after starting irinotecan. Her emaciated state gradually disappeared, and finally her PS score improved to 100. Myelosuppression was mild and transfusion or granulocyte-colony stimulating factor was not required. She could stay home during the intervals between irinotecan courses. Starting at the 18th interval, we prescribed oral 13-cis-retinoic acid (160 mg/m^2/d for 7 d). However, the parents stopped giving retinoic acid after the 19th interval because of its high cost. Irinotecan treatment was stopped 22 months after its start because the patient developed secondary myelodysplastic syndrome (MDS), refractory anemia with excess of blasts (RAEB), and because we worried that irinotecan contributed to her MDS. A cytogenetic study of the bone marrow revealed trisomy 11 and another karyotype abnormality [46, XX, der(9)t(9;16)(q13;?), del(16)(q?)]. Despite giving alternative therapies, such as low-dose cytarabine, she finally died of the progression of RAEB 36 months after starting irinotecan.

Case 2

An 11-year-old girl was referred to our hospital due to a left frontal head mass and fever. Serum neuron-specific enolase was 112.3 ng/mL, and the levels of urinary vanillylmandelic acid and homovanillic acid were elevated to 107 and 168 μg/g creatinine, respectively. Abdominal computed tomography showed a large (8 cm diameter) tumor arising from the right adrenal gland. Tumor clumps were seen in the bone marrow. MIBG imaging revealed systemic multiple abnormal uptake lesions mainly derived from bone. The biopsy of the adrenal tumor confirmed the diagnosis of MYCN nonamplified stage 4 neuroblastoma with 1p loss. She received 6 courses of the same induction therapy as case 1, followed by 1 course of ifosfamide combined with etoposide and total resection of the adrenal tumor. During the intensive chemotherapy, the bone metastases progressed (progressive disease by INRC) and she became severely anorectic and emaciated (Lansky score, 30). Another course of ifosfamide/etoposide resulted in ifosfamide-induced encephalopathy. Then, 9 months after the start of induction therapy, we tried a low-dose protracted schedule of irinotecan after obtaining written informed consent as in case 1. Twenty-four courses of low-dose protracted irinotecan prevented tumor progression (no response according to INRC) for 16 months (Fig. 2). After the introduction of irinotecan, her anorexia and emaciated state gradually disappeared and her PS dramatically improved to 90. She experienced grade 4 neutropenia, grade 3 anemia and thrombocytopenia, and grade 2 diarrhea, but could tolerate them with the suspension of irinotecan or supportive

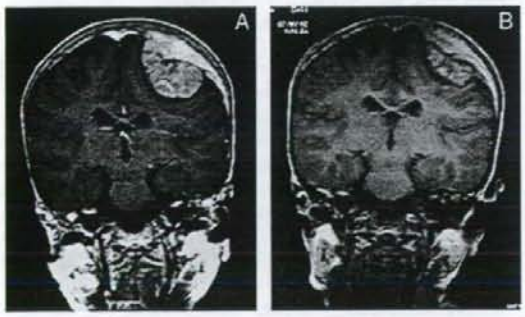

FIGURE 1. Head magnetic resonance images of case 1 before starting (A) and after 24 courses (B) of low-dose protracted irinotecan. Irinotecan reduced the left parietal metastatic tumor arising from the dura.

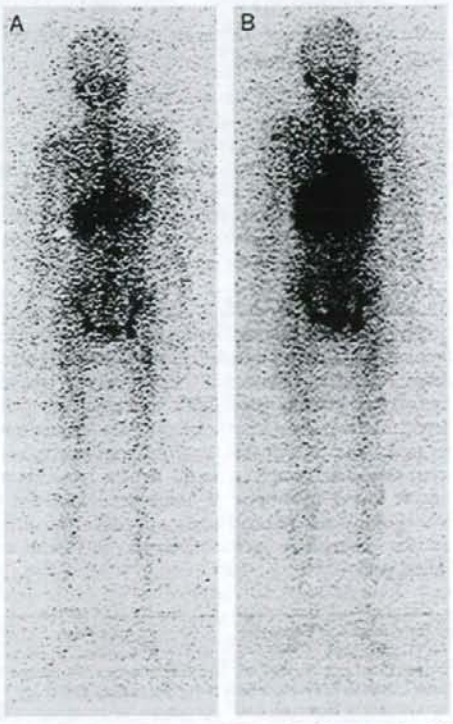

FIGURE 2. MIBG scintigram of case 2 before starting (A) and after 16 courses (B) of low-dose protracted irinotecan. During irinotecan treatment, no new lesion of abnormal uptake appeared. MIBG indicates metaiodobenzylguanidine.

therapy such as granulocyte-colony stimulating factor and transfusion; the frequency of transfusion rather decreased after starting irinotecan. Ten of 24 courses of irinotecan were suspended or delayed due to leukopenia. She could stay home during the intervals of irinotecan treatment. Two courses of a combination of irinotecan and vincristine caused severe diarrhea and hematologic toxicity. Combined use of irinotecan and 13-cis-retinoic acid (80 mg/m^2/d of 13-cis-retinoic acid for 7 days, at the intervals following the 15th and 20th courses of irinotecan) was tolerable, and may have stabilized the disease, although we were unable to determine whether irinotecan and 13-cis-retinoic acid had a synergistic antitumor effect in this case. 13-cis-retinoic acid was eventually abandoned because it was causing renal toxicity and xeroderma. Despite the use of alternative chemotherapy, she died of progressive disease 28 months after the introduction of irinotecan.

DISCUSSION

In these cases, we used low-dose protracted schedules of irinotecan as a palliative chemotherapy for advanced neuroblastoma. Our results show that irinotecan can be used in patients with poor PS, can stabilize the disease status, and can dramatically improve the QOL, which we felt was worthy of reporting.

The management of refractory neuroblastoma patients continues to be a serious challenge for physicians. For these patients, various clinical trials have been conducted to develop novel therapeutics.[3,5,8,9] On the other hand, for these patients, the purpose of therapy shifts from cure to symptom relief to improve their QOL. Such therapy, which has modest toxicity and which is well tolerated and active against neuroblastoma, includes oral etoposide, retinoids, anti-GD2 monoclonal antibodies or targeted radioimmunotherapy using ^{131}I-MIBG or ^{131}I-3F8.[1,10] Kushner et al[4] used 5-day courses of irinotecan at 50 mg/m^2/d as a palliative chemotherapy for neuroblastoma patients on an outpatient basis. This therapy was well tolerated, although the PS of the patients was not mentioned.

Our treatment using irinotecan was for an inpatient setting because of the patients' poor PS before the start of treatment, although we might be able to adapt this regimen to an outpatient clinic. Wagner et al[11] reported that home administration of intravenous low-dose protracted irinotecan (10 to 20 mg/m^2/d) combined with oral temozolomide was safe and active in patients with advanced Ewing sarcoma. A phase I study showed the feasibility of oral irinotecan (40 mg/m^2/d for 5 d for 2 consecutive weeks, repeated every 21 d) for refractory pediatric solid tumors,[9] indicating that oral administration of irinotecan can be applied more conveniently to palliative settings in pediatric oncology clinics than intravenous administration. A low-dose protracted schedule might have an advantage for patients with poor PS because any adverse effects can be detected at an early stage, allowing the physician to suspend irinotecan before it effects become severe. We suspended irinotecan immediately after worsening of diarrhea or hematologic toxicity, and resumed it after improvement of these adverse effects, as the purpose of this therapy was not to cure the disease but to prevent its progression and to palliate symptoms. This strategy improved safety and achieved long-term tolerability. Although intestinal paralysis is a contraindication of irinotecan as a rule, irinotecan reduced the intestinal paralysis in case 1. However, careful observation is essential when irinotecan is used for such cases.

What is the best dosing schedule of irinotecan for palliative therapy for advanced neuroblastoma? Irinotecan at 50 mg/m^2/d for 5 days every 21 days achieved stable disease in 9 of 44 patients with resistant neuroblastoma after receiving more than 5 courses, and in 3 of 44 patients after receiving more than 10 courses, respectively.[4] On the other hand, in a phase II trial of irinotecan, 50 mg/m^2/d for 5 days every 21 days resulted in only 1/18 partial response for refractory neuroblastoma.[5] A phase I study demonstrated that the maximum tolerated dose of irinotecan on a protracted schedule was 20 mg/m^2/d in heavily pretreated patients with childhood cancer.[3] This study also showed that 1 of 5 refractory neuroblastoma cases achieved partial response and that in the other 4 cases the disease stabilized for 1 to 6 months.[3] These results, taken together with our own experiences, indicate that a dosage of 20 mg/m^2/d is also appropriate in a palliative setting.

Although we also tried irinotecan in combination with other antitumor drugs such as retinoids or vincristine, we were unable to determine whether they had a synergistic effect. Further investigations of drug combinations are needed to identify more effective palliative therapies with modest toxicity.

Some cases of stage 4 neuroblastoma without MYCN amplification have survived for long periods when they were treated with a combination of multiple cycles of chemotherapy with modest toxicity, cis-retinoic acid, anti-GD2 immunotherapy, and/or targeted radiotherapy.[8] Neuroblastoma differentiates into ganglioneuroma either spontaneously or during treatment with some drugs, including irinotecan. In vivo treatment with irinotecan leads to differentiation of neuroblastoma xenografts.[12] Our 2 cases were also without MYCN amplification, so that low-dose protracted irinotecan might have prevented tumor progression.

We stopped irinotecan due to the complication of RAEB in case 1, in view of the possibility that irinotecan contributed to her MDS. Therapy-related myeloid malignancy is rare in neuroblastoma: the Childhood Cancer Survivor Study demonstrated that only 1 of 897 cases with neuroblastoma developed acute myeloid leukemia.[13] Because she was administered various chemotherapeutic agents including cyclophosphamide, ifosfamide, doxorubicin, pirarubicin, and etoposide (cumulative doses of 17.7 g/m^2, 14 g/m^2, 75 mg/m^2, 200 mg/m^2, and 1100 mg/m^2, respectively) before irinotecan, the causative agent(s) of the MDS could not be identified. Trisomy 11 is rare in both de novo and therapy-related MDS.[14,15] However, irinotecan has not yet been reported as a cause of therapy-related MDS. Further investigation is needed to clarify whether irinotecan can cause a second malignancy.

In summary, we used low-dose protracted schedules of irinotecan as a palliative chemotherapy for advanced neuroblastoma. This dosing schedule of irinotecan was well tolerated, and resulted in disease stability and improved PS and QOL for significant periods for 2 heavily pretreated patients with refractory neuroblastoma. Therefore, low-dose protracted irinotecan might be an attractive modality for palliative treatment for advanced neuroblastoma patients.

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RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: a prognostic marker

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The tumour suppressor gene *RASSF1A* is known to be frequently silenced by promoter hypermethylation in neuroblastoma tumours. Here we explored the possible prognostic significance of aberrant promoter hypermethylation of *RASSF1A* in serum DNA samples of patients with neuroblastoma as a surrogate marker for circulating tumour cells. We analysed the methylation status of the *RASSF1A* gene in matched tumour and pretreatment serum DNA obtained from 68 neuroblastoma patients. Hypermethylation of *RASSF1A* in tumour samples was found in 64 patients (94%). In contrast, serum methylation of *RASSF1A* was observed in 17 patients (25%). Serum methylation of *RASSF1A* was found to be statistically associated with age ⩾12 months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and *MYCN* amplification ($P<0.001$). The influence of serum *RASSF1A* methylation on prognosis was found to be comparable with that of the currently most reliable marker, *MYCN* amplification on univariate analysis (hazard ratio, 9.2; 95% confidence interval (CI), 2.8–30.1; $P<0.001$). In multivariate analysis of survival, methylation of *RASSF1A* in serum had a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this association did not reach statistical significance ($P=0.194$). These findings show that the methylation status of *RASSF1A* in the serum of patients with neuroblastoma has the potential to become a prognostic predictor of outcome.

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Keywords: *RASSF1A*; methylation; serum; DNA; neuroblastoma

Neuroblastoma is the most common extracranial solid tumour in children and is characterised by a wide range of clinical behaviours, from spontaneous regression to rapid progression with a fatal outcome (Maris *et al*, 2007). The clinical outcome is associated with disease stage, age at diagnosis, *MYCN* amplification and histological classification. Although numerous genetic abnormalities, including *MYCN* amplification, are associated with tumour progression and poor outcome, the molecular mechanisms responsible for the pathogenesis of aggressive neuroblastoma remain unclear. Identifying such molecular changes may contribute to improved clinical management and outcome prediction of newly diagnosed neuroblastomas.

In recent years, changes in the status of DNA methylation, known as epigenetic alterations, have turned out to be one of the most common molecular alterations in human neoplasia including neuroblastoma (Misawa *et al*, 2005; Sugino *et al*, 2007). Several potential tumour-suppressor genes have been described as frequently silenced by hypermethylation in neuroblastomas. Methylation of promoter CpG islands is known to inhibit transcriptional initiation and cause permanent silencing of downstream genes. Loss of heterozygosity of chromosome 3p21.3 is one of the most frequent alterations in solid tumours. Located within this 3p21.3 locus, the RAS-association domain family 1, isoform A gene (*RASSF1A*) encodes a RAS effector that has been identified as a tumour suppressor of many different cancer types (Dammann *et al*, 2000). *RASSF1A* falls into the category of genes frequently inactivated by methylation rather than mutational events. This gene is silenced and inactivated by promoter region hypermethylation in many adult and childhood cancers, including neuroblastoma (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). *RASSF1A* has been shown to play important roles in cell cycle regulation, apoptosis and microtubule stability as a tumour suppressor gene (Agathanggelou *et al*, 2005).

It is well known that DNA fragments are frequently and abundantly found in the serum of cancer patients, with significantly higher levels in patients with metastasis (Hesson *et al*, 2007). A number of studies have evaluated the potential of circulating tumour-related methylated DNA in serum for the molecular diagnosis and prognosis of various types of cancer (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Mori *et al*, 2005). Methylation-specific PCR assay is a sensitive and specific assay for tumour-related DNA methylation in serum. Several studies have investigated the prospect of using DNA methylation as a surrogate marker for circulating tumour cells in serum samples from breast cancer or melanoma patients (Fiegl *et al*, 2005; Koyanagi *et al*, 2006). However, no studies of neuroblastoma have assayed serum samples for aberrant DNA methylation. Therefore, this study

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investigated whether it is possible to detect *RASSF1A* epigenetic alterations in the serum of neuroblastoma patients, and aberrant *RASSF1A* methylation in patient pretherapeutic serum is of prognostic significance in neuroblastoma using a series of matched neuroblastoma tumour and serum DNA.

MATERIALS AND METHODS

Patients and sample collection

Clinical data were collected retrospectively by reviewing the medical database at the Hospital of Kyoto Prefectural University of Medicine for the period between 1985 and 2004. After approval by the Institutional Review Board, 68 neuroblastoma patients were identified on the basis of histological examination of tumour specimens that met the following criteria: the patient had an available tumour specimen; a serum specimen was available; and the patient either died or had >1 year of follow-up time. The clinical data included information regarding tumour stage, age at diagnosis, sex, *MYCN* gene status and outcome. Staging was evaluated according to the criteria of the International Neuroblastoma Staging System (Maris *et al*, 2007). Patients of any age who had stage 1 or 2 disease and those younger than 12 months with stage 3 or 4S disease were given either surgery or surgery with chemotherapy (Matsumura and Michon, 2000). Patients aged 12 months or older with stage 3 and any patients with stage 4 disease were treated according to the protocol by the Japanese Neuroblastoma Study Group (Sawaguchi *et al*, 1990; Tsuchida and Kaneko, 2000; Kaneko *et al*, 2002; Suita *et al*, 2007). The patients with stage 4 disease underwent high-dose chemotherapy with autologous stem-cell rescue after the initial chemotherapy. Instead of pre-specified sample size determination, power analysis was conducted after collecting clinical data to guarantee statistical power and to evaluate whether *RASSF1A* methylation is a prognostic marker for survival. In a realistic scenario, a study of 68 patients had power of 96% to detect a single marker with hazard ratio larger than 5.

Tumour samples at the time of diagnosis and before the administration of chemotherapy were frozen immediately and stored at −80°C until DNA extraction. In addition, match-paired serum samples were assessed. Peripheral blood was obtained before any therapy or surgery. To avoid contamination of serum DNA by the DNA from WBCs, serum was prepared exclusively from the liquid fraction of clotted blood after centrifugation at 1000 × **g** for 10 min and stored it at −20°C until DNA extraction. For the extraction of free DNA, we used 200 μl of stored serum, which supplemented with 1 μg salmon testes DNA (Sigma, St Louis, MO, USA) as a carrier DNA. DNA was extracted from tumour samples using a QIAmp DNA Mini Kit and from serum samples using a QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Analysis of DNA methylation

Treatment of tumour DNA and serum DNA with sodium bisulphite was performed with an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) following the protocol of the manufacturer. Methylation-specific PCR was performed with primers specific for either methylated or unmethylated DNA spanning the region within the *RASSF1A* gene (Figure 1). The primers used were methylation-specific RAM-1 (5′-GTG TTAACGCGTTGCGTATC-3′) and RAM-2 (5′-AACCCCGCGAACT AAAAACGA-3′) and unmethylation-specific RAU-1 (5′-TTTGGT TGGAGTGTGTTAATG-3′) and RAU-2 (5′-CAAACCCCACAAACT AAAAACAA-3′), as described earlier (Lo *et al*, 2001). PCR conditions consisted of an initial incubation for 10 min at 95°C followed by 35 cycles for tumour samples or 40 cycles for serum samples of denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 60 s, followed by a final extension step of 72°C for 10 min. Lymphocyte DNA and *in vitro* methylated (using *SssI* CpG methylase; New England Biolabs, Beverly, MA, USA) lymphocyte DNA were used as unmethylated and methylated controls, respectively. The PCR products obtained were analysed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Samples were scored as methylation positive when methylated alleles were visualised as bands in the methylated DNA lane and as methylation negative when bands were seen only in the unmethylated DNA lane. The analysis of the samples in this study was performed by an analyst blinded to the clinical and biological information.

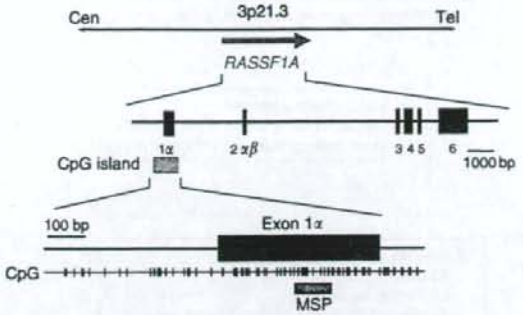

Figure 1 Genomic structure of the *RASSF1A* gene. Vertical tick marks, CpG sites; blue boxes, exons; green box, CpG island in the promoter; red box, region analysed by methylation-specific PCR.

Statistical analysis

The primary end point was overall survival defined by the period from diagnosis of the primary tumour to any cause of death. The relationship between clinicopathological variables and methylation status of the *RASSF1A* gene was shown initially using contingency tables and χ^2 test. Survival curves for *RASSF1A* methylation were derived by the Kaplan–Meier method. Univariate analysis was conducted using Cox's proportional hazard models and log-rank test. Performance of *RASSF1A* methylation as a prognostic marker was also analysed after adjustment for known prognostic factors by (i) subset analysis of stage 3 patients using contingency tables and Fisher's exact test and (ii) multivariate Cox's proportional hazard models including age, sex and tumour stage. Two-sided *P*-values <0.05 were considered as significant. SAS 9.13 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

RESULTS

A total of 124 patients with histologically confirmed neuroblastoma or ganglioneuroblastoma were treated at the Hospital of Kyoto Prefectural University of Medicine between January 1985 and May 2004. Sixty-eight patients met the criteria of this retrospective study. The detailed patient disposition is shown in Figure 2 and the baseline characteristics of patients are presented in Table 1. Of the 68 patients, 24 were classified as stage 1, 11 as stage 2, 11 as stage 3, 18 as stage 4 and 4 as stage 4S. At the time of diagnosis, 42 patients (62%) were younger than 12 months, and 26 (38%) were older. We found no significant differences between included and excluded patients for age or stage statistically. Twelve patients (18%) had tumours with *MYCN* amplification, and *MYCN* amplification was not detected in the tumours from 56 (82%) patients by southern

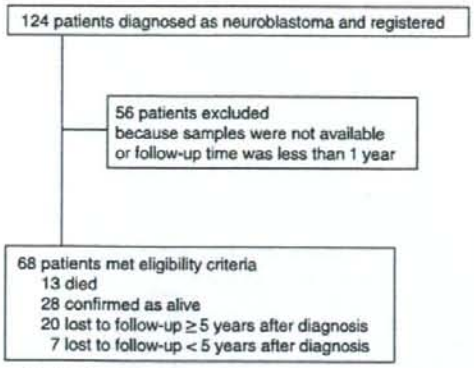

Figure 2 Patient disposition.

Table 1 Characteristics of patients

Characteristic	No. of patients (%)
Sex	
Male	27 (39.7)
Female	41 (60.3)
Age at diagnosis	
< 12 months	42 (61.8)
⩾ 12 months	26 (38.2)
Stage	
1	24 (35.3)
2	11 (16.2)
3	11 (16.2)
4	18 (26.5)
4S	4 (5.9)
MYCN	
Non-amplified	56 (82.4)
Amplified	12 (17.6)
Diagnosis	
GNB	7 (10.3)
NB	61 (89.7)
Serum RASSF1A	
Unmethylated	51 (75.0)
Methylated	17 (25.0)

blot analysis or fluorescence *in situ* hybridisation. The median follow-up time was 72 months, with a range from 9 to 248 months.

Detection of *RASSF1A* promoter methylation in tumours

This study initially investigated the hypermethylation status of the *RASSF1A* tumour suppressor genes in 68 neuroblastoma tumours. Only four (one each at stage 1, 2, 4S and 3) tumours showed no methylation of *RASSF1A* (Supplementary Table). All other neuroblastoma tumours (64 of 68; 94%) showed methylated *RASSF1A*. Hypermethylation in tumours was observed very frequently in all of the stages of neuroblastoma examined, including stage 1, 2 and 4S tumours (Supplementary Table) and no correlation between *RASSF1A* methylation and known prognostic factors including stage, age and *MYCN* amplification was detected. No relationship between *RASSF1A* methylation in tumours and outcome was also observed. *RASSF1A* methylation was not observed in any of the three benign ganglioneuromas.

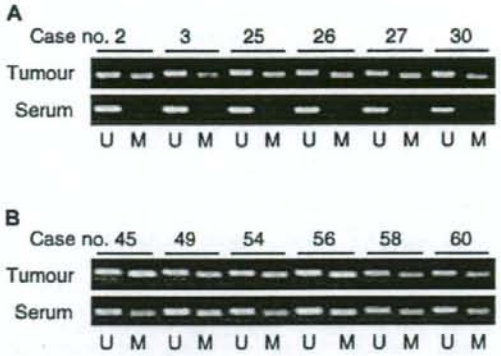

Figure 3 *RASSF1A* methylation status of tumour and serum DNA in neuroblastoma patients. M, methylated; U, unmethylated. The sizes of the PCR products for methylated and unmethylated primers are 93 and 105 bp, respectively. (**A**) Cases stage 1 and 2 with good prognosis tumour DNAs are methylated but absent in the serum DNAs. (**B**) In contrast, in stage 3 and 4 *MYCN*-amplified cases, methylated DNAs are detected in both tumour and serum samples.

Table 2 Associations between clinical factors and serum *RASSF1A* methylation status

Characteristic	Methylated no.	Unmethylated no.	Total no.	*P*-value
Age at diagnosis				0.002
< 12 months	5	37	42	
⩾ 12 months	12	14	26	
Stage				< 0.001
1/2/4S	3	36	39	
3	3	8	11	
4	11	7	18	
MYCN				< 0.001
Non-amplified	5	51	56	
Amplified	12	0	12	

Detection of *RASSF1A* promoter methylation in serum

The hypermethylation status of *RASSF1A* in the matched serum DNA samples was then determined and compared with the pattern of hypermethylation found in the corresponding tumour DNA samples (Figure 3). *RASSF1A* hypermethylation was detected in 17 of 68 (25%) matched serum DNA samples (Table 1). The detailed overview is shown in Supplementary Table.

Correlation of serum *RASSF1A* methylation status with clinical factors

The methylation status of *RASSF1A* in the pretherapeutic serum of the 68 patients was analysed for association with known prognostic factors (Table 2). Serum *RASSF1A* methylation showed a significant statistical association with age ⩾12 months ($P = 0.002$). *RASSF1A* methylation in serum was detected more frequently in disseminated stage 4 tumours than local-regional (stage 1, 2 and 3) and 4S tumours ($P < 0.001$). Furthermore, serum-methylated *RASSF1A* was significantly correlated with *MYCN* amplification ($P < 0.001$). Notably, all cases with *MYCN* amplification showed *RASSF1A* methylation of serum DNA (Supplementary Table).

Table 3 Univariate analysis of survival

Characteristic	Hazard ratio	95% CI	*P*-value
Age at diagnosis			
<12 months	Reference		
⩾12 months	23.6	3.1–181.9	0.002
Sex			
Male	Reference		
Female	1.0	0.3–3.0	0.983
Stage			
1/2/3/4S	Reference		
4	19.8	4.4–89.5	<0.001
MYCN			
Non-amplified	Reference		
Amplified	8.2	2.7–24.7	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	9.2	2.8–30.1	<0.001

CI = confidence interval.

Analysis of prognostic significance of *RASSF1A* methylation in serum

The association of pretherapeutic serum methylation status of *RASSF1A* with clinical outcome was analysed in 68 patients with known follow-ups. Univariate analyses revealed prognostic significance for age at diagnosis ⩾12 months, stage 4 and *MYCN* amplification ($P=0.002$, $P<0.001$ and $P<0.001$, respectively; Table 3) in this cohort, as expected. Patients with serum-methylated *RASSF1A* had significantly worse overall survival than patients with serum-unmethylated *RASSF1A* ($P<0.001$, log-rank test; Figure 4A). The 5-year survival was more than 90% in patients without serum methylation of *RASSF1A*, whereas lower than 50% in patients with serum methylation of *RASSF1A* (hazard ratio, 9.2; 95% confidence interval (95% CI), 2.8–30.1; $P<0.001$); Table 3). *RASSF1A* methylation in serum and the known prognostic factors were also correlated with relapse-free survival as well as with overall survival ($P<0.001$; Table 4; Figure 4B). Furthermore, a subset analysis revealed that stage 3 patients also had a trend towards poorer prognosis when *RASSF1A* was methylated in serum. When limited to cases in stage 3, two of the three patients with serum-methylated *RASSF1A* died, whereas all eight patients with serum-unmethylated *RASSF1A* are alive ($P=0.055$, Fisher's exact test). In a multivariate analysis including age, sex and tumour stage, serum *RASSF1A* methylation was still associated with poor outcome with a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this did not reach statistical significance ($P=0.194$; Table 5).

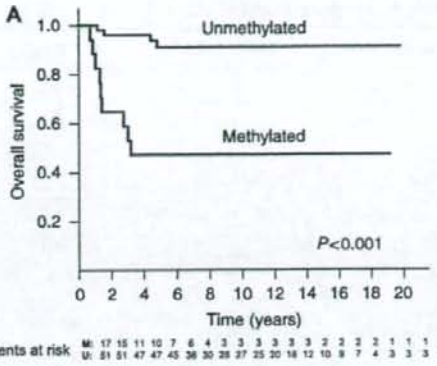

Figure 4 Kaplan–Meier survival curves of 68 neuroblastoma patients: correlation of pretherapeutic serum *RASSF1A* methylation status with overall survival (**A**) and relapse-free survival (**B**). M, methylated: patients with serum methylation of *RASSF1A*. U, unmethylated: patients without serum methylation of *RASSF1A*. The 5-year overall survival was more than 90% in patients without methylation, whereas lower than 50% in patients with methylation ($P<0.001$).

DISCUSSION

In patients with malignancies, aberrant methylation of serum DNA has been reported (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Fiegl *et al*, 2005; Mori *et al*, 2005; Koyanagi *et al*, 2006). We have detected cell-free tumour DNA in serum of neuroblastoma patients (Gotoh *et al*, 2005). Prognosis in stage 4 neuroblastoma patients with metastases is poor despite intensive chemotherapy (Maris *et al*, 2007). Therefore, this study aimed to explore the possible prognostic significance of aberrant promoter hypermethylation of *RASSF1A*, which has been found frequently in neuroblastoma tumours, using pretherapeutic serum of neuroblastoma patients as a surrogate marker for circulating tumour cells.

We first investigated the *RASSF1A* methylation status in 68 neuroblastoma tumour DNA samples in comparison with matched serum DNA samples. The methylation of *RASSF1A* was observed in this study in 94% of primary tumours. Our results show that promoter hypermethylation of *RASSF1A* occurs at a high frequency in primary neuroblastoma tumours and no correlation between *RASSF1A* methylation and known prognostic factors including stage, age and *MYCN* amplification, or outcome was seen. The high proportion of *RASSF1A* promoter methylation in tumours agrees with earlier reports in the literature, which have found *RASSF1A* to be hypermethylated in 52–94% of tumour DNA samples (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004, 2007; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). Several earlier studies with one exception (Yang *et al*, 2004) failed to find a statistical correlation between *RASSF1A* methylation in tumours and poor outcome (Astuti *et al*, 2001; Harada *et al*, 2002; Banelli *et al*, 2005; Michalowski *et al*, 2008). We also did not observe any relationship between *RASSF1A* methylation in tumours and prognosis. *RASSF1A* hypermethylation in tumours can be a relatively early event in neuroblastoma tumorigenesis as it is detectable in non-advanced early-stage tumours with high frequency. Although the prognostic significance of epigenetic changes of single genes in neuroblastoma tumour DNA remain controversial, a few studies have indicated that poor prognosis is associated with the CpG island methylator phenotype

Table 4 Univariate analysis of relapse

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
< 12 months	Reference		
⩾ 12 months	12.5	2.8–55.3	<0.001
Sex			
Male	Reference		
Female	1.0	0.4–2.9	0.972
Stage			
1/2/3/4S	Reference		
4	14.3	4.0–51.0	<0.001
MYCN			
Non-amplified	Reference		
Amplified	7.2	2.6–20.0	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	6.8	2.4–19.1	<0.001

CI = confidence interval.

Table 5 Multivariate analysis of survival

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
< 12 months	Reference		
⩾ 12 months	1.2	1.0–1.5	0.066
Sex			
Male	Reference		
Female	0.6	0.1–2.5	0.452
Stage			
1/2/3/4S	Reference		
4	8.4	1.5–46.4	0.014
Serum RASSF1A			
Unmethylated	Reference		
Methylated	2.4	0.6–9.2	0.194

CI = confidence interval.

(Abe *et al*, 2005; Banelli *et al*, 2005; Yang *et al*, 2007), suggesting that aberrant methylation of multiple genes is likely to contribute to neuroblastoma pathogenesis.

As a next step, we analysed *RASSF1A* methylation status in 68 paired serum DNA samples. In contrast to tumours, *RASSF1A* methylation was detected in neuroblastoma patient serum from only 25% (17 out of 68). To investigate the clinical significance of the serum *RASSF1A* methylation, associations with established prognostic factors and outcome were evaluated. *RASSF1A* methylation in serum was found to be statistically associated with established prognostic factors. Serum *RASSF1A* methylation was more frequently detected in neuroblastoma patients with age ⩾12 months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and *MYCN* amplification ($P<0.001$). Furthermore, the presence of methylation of *RASSF1A* in serum was associated with poorer outcome. The influence of serum *RASSF1A* methylation on prognosis was found to be comparable with that of the currently most reliable marker, *MYCN* amplification in univariate analysis. A subset analysis of stage 3 patients showed a trend associating poor survival with serum *RASSF1A* methylation ($P=0.055$), although the data were limited due to the small number of patients in the subgroup. In multivariate analysis of survival, methylation of *RASSF1A* in serum had a hazard ratio of 2.42, but this association did not reach statistical significance ($P=0.194$). Further validation studies using a larger set of patients are necessary to confirm our findings.

The presence of tumour-derived DNA within the blood stream has been identified earlier (Müller *et al*, 2003; Fiegl *et al*, 2005; Mori *et al*, 2005). Recently, one study showed that the detection of circulating tumour cells was correlated with tumour-related methylated DNA in patients with melanoma (Koyanagi *et al*, 2006), suggesting that circulating tumour cells are a potential source of circulating methylated DNA. Our study suggests that methylated *RASSF1A* DNA in serum is a surrogate marker for circulating neuroblastoma cells. Another recently published study showed that *RASSF1A* methylation was also detectable in ovarian cancer patient's serum at a high frequency from methylated tumour cases including several stage I tumours (Ibanez de Caceres *et al*, 2004). In the earlier study, there was no statistical association between tumour stage and positive detection in serum. However, some other studies have shown limited detection of *RASSF1A* methylation in the serum of patients with other neoplasms (Murray *et al*, 2004; Hesson *et al*, 2007). These differing results may suggest that free neoplastic DNA from ovarian cancer can access the blood stream more readily than that from other neoplasms including neuroblastoma.

In conclusion, this is the first study to examine epigenetic changes in a tumour suppressor gene, *RASSF1A*, the promoter of which is hypermethylated at a high frequency in neuroblastoma tumours, using serum DNA in a cohort of neuroblastoma patients. This study demonstrates the utility of detecting circulating methylated *RASSF1A*, which can be measured in serum, as a potentially predictive marker of neuroblastoma outcome. *RASSF1A* methylation in serum could have useful clinical applications in neuroblastoma management, if our results are confirmed in larger studies. However, we should not forget the limitation when attempting to translate our findings into the clinical fields as highly sensitive methylation analysis could be tricky because of incomplete bisulphite conversion by inexperienced analysts.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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SPECIAL ARTICLE

From the Department of Pediatrics, The University of Chicago, Chicago, IL; Section of Paediatrics, Institute of Cancer Research and Royal Marsden Hospital, Surrey; Children's Cancer and Leukaemia Group Data Centre, University of Leicester, Leicester, United Kingdom; Children's Oncology Group Statistics and Data Center, University of Florida, Gainesville, FL; Section for Paediatric Surgery, Division of Surgery, Rikshospitalet University Hospital, Oslo, Norway; Children's Cancer Research Institute, St Anna Kinderkrebsforschung, Vienna, Austria; Division of Oncology, The Children's Hospital of Philadelphia, Department of Pediatrics, The University of Pennsylvania, Philadelphia, PA; Institute of Medical Biostatistics, Epidemiology and Informatics, University of Mainz, Mainz; Department of Pediatric Oncology and Hematology, Children's Hospital, University of Cologne, Cologne, Germany; Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan; Service de Biostatistiques, Institut Curie, Paris, France; Department of Hematology–Oncology, Gaslini Institute, Largo Gaslini, Genoa, Italy; Unidad de Oncologia Pediatrica, Hospital Infantil La Fe, Valencia, Spain; and the Department of Pediatrics, University of California School of Medicine, San Francisco, CA.

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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The International Neuroblastoma Risk Group (INRG) Classification System: An INRG Task Force Report

Susan L. Cohn, Andrew D.J. Pearson, Wendy B. London, Tom Monclair, Peter F. Ambros, Garrett M. Brodeur, Andreas Faldum, Barbara Hero, Tomoko Iehara, David Machin, Veronique Mosseri, Thorsten Simon, Alberto Garaventa, Victoria Castel, and Katherine K. Matthay

ABSTRACT

Purpose

Because current approaches to risk classification and treatment stratification for children with neuroblastoma (NB) vary greatly throughout the world, it is difficult to directly compare risk-based clinical trials. The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification.

Patients and Methods

The statistical and clinical significance of 13 potential prognostic factors were analyzed in a cohort of 8,800 children diagnosed with NB between 1990 and 2002 from North America and Australia (Children's Oncology Group), Europe (International Society of Pediatric Oncology Europe Neuroblastoma Group and German Pediatric Oncology and Hematology Group), and Japan. Survival tree regression analyses using event-free survival (EFS) as the primary end point were performed to test the prognostic significance of the 13 factors.

Results

Stage, age, histologic category, grade of tumor differentiation, the status of the *MYCN* oncogene, chromosome 11q status, and DNA ploidy were the most highly statistically significant and clinically relevant factors. A new staging system (INRG Staging System) based on clinical criteria and tumor imaging was developed for the INRG Classification System. The optimal age cutoff was determined to be between 15 and 19 months, and 18 months was selected for the classification system. Sixteen pretreatment groups were defined on the basis of clinical criteria and statistically significantly different EFS of the cohort stratified by the INRG criteria. Patients with 5-year EFS more than 85%, more than 75% to ≤ 85%, ≥ 50% to ≤ 75%, or less than 50% were classified as very low risk, low risk, intermediate risk, or high risk, respectively.

Conclusion

By defining homogenous pretreatment patient cohorts, the INRG classification system will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world and the development of international collaborative studies.

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INTRODUCTION

Neuroblastoma (NB) is remarkable for its broad spectrum of clinical behavior, with some tumors regressing or maturing, whereas others progress despite intensive multimodality treatment.[1,2] This diversity in behavior correlates closely with a number of clinical and biologic features,[2] and combinations of prognostic variables are used for risk-group assignment and treatment stratification. However, the factors selected by various cooperative groups to define risk are not uniform. For example, the International Society of Pediatric Oncology Europe Neuroblastoma Group (SIOPEN) uses age, surgical risk factors defined by imaging, and *MYCN* status for risk-group assignment of locoregional tumors, whereas the Children's Oncology Group (COG) uses age, postsurgical staging, *MYCN* amplification, histology, and DNA ploidy.[3,4] Furthermore, the increasing number of genetic features included in more recently developed clinical trials to guide therapy decisions[5-7] further complicates comparisons.

To facilitate comparison of clinical trials performed throughout the world, the William Guy Forbeck Research Foundation sponsored an international conference more than 20 years ago. The outcome of the conference was published as the International Neuroblastoma Staging System (INSS).[8,9] During the last two decades, there have been major advances in understanding the genetics

of NB. Although the unfavorable prognostic factor *MYCN* amplification[10] is used by all cooperative groups for risk-group stratification and therapeutic decisions, other prognostically significant genetic features[5-7,11] have not been consistently incorporated into risk classification schemas. Furthermore, only some cooperative groups include tumor histology for risk-group assessment.[12,13]

To develop a consensus approach to pretreatment risk stratification, a task force of investigators with expertise in NB from the major pediatric cooperative groups around the world was established in 2004. A new International Neuroblastoma Risk Group (INRG) Staging System (INRGSS) was designed to stratify patients at the time of diagnosis before any treatment, as detailed in the companion article by Monclair et al.[14] In the INRGSS, extent of locoregional disease is determined by the absence or presence of image-defined risk factors (L1 and L2, respectively). Stage M will be used for widely disseminated disease, and MS describes metastatic NB limited to skin, liver, and bone marrow without cortical bone involvement in children age 0 to 18 months with L1 or L2 primary tumors. In addition, the Task Force's recommendations for defined standard operating procedures for molecular diagnostic testing of NB tumor tissue, criteria for the evaluation of bone marrow metastatic disease by immunocytochemistry and RT-PCR and for the assessment of metastatic disease by MIBG will be described in future reports.

PATIENTS AND METHODS

INRG Task Force Members

In 2004, investigators from the major cooperative groups, COG (North America and Australia), the German Pediatric Oncology and Hematology Group (GPOH), the Japanese Advanced Neuroblastoma Study Group (JANB), the Japanese Infantile Neuroblastoma Co-operative Study Group (JINCS), SIOPEN and China with expertise in NB were contacted by AD]P and SLC and invited to participate in an initiative to establish the INRG classification system. The major goal of the Task Force was to develop a consensus approach for pretreatment risk stratification of NB, based on statistical analyses of prognostic factors.

The leaders of the cooperative groups were asked to nominate six individuals with expertise in one or more of the following categories: clinical trials related to NB, chemotherapy, surgery, pathology, biology, radiology, nuclear medicine and statistics. In addition, young investigators were invited, and 52 investigators were identified. Four committees were formed: Surgery, Chair—Tom Monclair; Statistics, Chair—Wendy B. London; Biology, Chair—Peter F. Ambros; and Metastatic Disease, Chair—Katherine K. Matthay. The four chairs of the committees and the co-chairs of the INRG Task Force (A.D.J.P. and S.L.C.) comprised the INRG Executive Committee. Four international conferences were held: June 2004 in Genoa, Italy; September 2005 in Whistler, Vancouver, Canada sponsored by the William Guy Forbeck Research Foundation; May 2006 in Los Angeles, CA; and September 2006 in Geneva, Switzerland.

Table 1. Number of Patients in the International Neuroblastoma Risk Group Analytic Cohort by Country or Cooperative Group of Origin

Country or Cooperative Group	No.	%
COG	4,235	48.1
SIOPEN: Previous European Neuroblastoma Study Group (ENSG)	917	10.4
SIOPEN: Italy	304	3.5
SIOPEN: Spain	410	4.7
SIOPEN: LNESG1 trial	526	6.0
Germany	1,938	22.0
Japan	470	5.3
Total	8,800	100

Abbreviations: COG, Children's Oncology Group; SIOPEN, International Society of Pediatric Oncology Europe Neuroblastoma Group.

Patient Cohort

Data were collected on patients enrolled on COG, GPOH, JANB, JINCS, or SIOPEN trials. Enrollment cutoff of 2002 was chosen to allow at least 2 years of follow-up at the 2004 data freeze. Eligibility for inclusion in the INRG cohort included (1) confirmed diagnosis of NB, ganglioneuroblastoma (GNB), or ganglioneuroma (GN) maturing; (2) age no older than 21 years; (3) diagnosis between 1990 and 2002; and (4) informed consent. In addition to date of diagnosis and follow-up data, information on 35 potential risk factors were requested: age, INSS stage, Evans stage, Shimada classification, Shimada histologic category, Shimada grade, Shimada mitosis-karyorrhexis index (MKI), International Neuroblastoma Pathology Classification (INPC), INPC histologic category, INPC grade of tumor differentiation, INPC MKI, *MYCN* status, DNA ploidy (defined as DNA index ≤ 1.0 v > 1.0), 11q loss of heterozygosity (LOH), 11q aberration, unbalanced 11q LOH, 1p LOH, 1p aberration, 17q gain, serum ferritin, serum lactate dehydrogenase (LDH), six primary tumor sites, and eight metastatic sites. Analyses were performed on 8,800 unique patients.

Statistical Considerations

Objective, inferential criteria formed the initial basis for definition of the risk groups. However, because there were too few patients who had known values for all the factors and challenges of reaching international agreement, the final decision regarding the delineation of pretreatment risk groups was made by consensus on the basis of treatment strategies and overall survival (OS), in addition to event-free survival (EFS) results.

Survival Analyses

The primary analytic end point was EFS. Time to event was defined as time from diagnosis until time of first occurrence of relapse, progression, secondary malignancy, or death, or until time of last contact if none of these occurred. EFS was selected as the primary end point because the majority of patients with non–high-risk disease who have an event successfully achieve treatment salvage, and it is difficult to discriminate subsets using OS because of fewer events (deaths) in the lower-risk cohorts, resulting in lower power. Univariate analyses using a log-rank test, at a 5% significance level and without adjustment for multiple testing, were performed to identify factors statistically significantly predictive of EFS to be carried forward into the survival-tree regression. Kaplan-Meier curves were examined for each factor (data not shown).[15] Cox proportional hazards regression models were used to identify the most highly statistically significant variable to create a given split or "branch" in the survival tree.[16-19] The survival tree methodology, rather than attempting to develop a prognostic index, was used to develop the classification because the consensus of the clinical and scientific participants involved was that the survival tree approach was more intuitive, reflected the customary format for risk-group presentation in this disease, and could be used more easily internationally. The assumption of proportional hazards was tested. For practical reasons, all factors were analyzed as binary variables. All EFS and OS values are reported at the 5-year time point ± the SE.

Methods to Dichotomize Age, LDH, and Ferritin

Age was dichotomized using methods previously described by London et al (n = 3,666 COG patients from the INRG database).[20] Excluding these 3,666 patients, the analysis to identify an optimal age cutoff was repeated (data not shown). For LDH and ferritin respectively, the median value was used to dichotomize the cohort, and two binary variables were created for the survival-tree analysis.

Justification for Utilizing Underlying Components of Histologic Classification

The INPC and Shimada histology systems use age at diagnosis and histologic features of the tumor to categorize tumors as favorable versus unfavorable. This results in a duplication of the prognostic contribution

