Neuroblastoma is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome (1). The current risk classification of patients with neuroblastoma is based on the age of onset, the extent of disease at the time of diagnosis as defined by the International Neuroblastoma Staging System, and evaluation of genetic aberrations, such as MYCN amplification (MNA; ref. 2). MNA is considered the strongest prognostic factor and is routinely assessed for therapy stratification (2–4), but it is a big concern that some cases without MNA also have a poor prognosis (5). Hero and colleagues recently reported that spontaneous regression is often seen in localized infantile neuroblastoma without MNA, and they suggested that a wait-and-see strategy avoiding chemotherapy and surgical procedure was justified in such patients (6). Nevertheless, some patients in their cohort showed local progression, progression to stage IVS, or progression to stage IV. Thus, it is important to have additional biomarkers with prognostic value for the management of non-MNA cases of neuroblastoma. We screened many biological markers for neuroblastoma, such as Trk A expression (7) and chromosome allelic loss (8–10), but none of them were found to be useful for risk classification among non-MNA patients.

Recent studies have revealed that epigenetic alterations, such as silencing of tumor suppressor gene by aberrant hypermethylation of its promoter, often play important roles in the pathogenesis of human cancers, and some of these alterations are thought to cause loss of function comparable with
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.

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>
<thead>
<tr>
<th>Characteristics</th>
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NOTE: Values in parentheses show the number of the patients targeted in the non-MNA group.
Abbreviation: INSS; International Neuroblastoma Staging System.
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 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 bisulfite treatment, we designed methylation- and unmethylation-specific PCR primers, which recognize sequences unique to the methylated and unmethylated alleles, respectively, as shown in

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 BstU1, whereas the unmethylated-DCR2 sequence could not be affected because all unmethylated cytidines were changed into uracils after bisulfite 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.
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 performed 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), 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 BstU1 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 digested with normal healthy donor DNA from 1:1 to 1:106, 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 M/R ratios, we added 1×105 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 χ2 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 BstU1 treatment, whereas normal healthy donor DNA was not digested by BstU1 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 1:106 (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

![Graph](image-url)
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 \( (n = 59; 68\% \text{ versus } 10\%; P < 0.001; \text{ Table 2}) \). 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 \( (n = 53; 73\% \text{ versus } 8\%; P < 0.001) \). However, in the frequency of DCR2 methylation between patients with MNA and non-MNA \( (50\% \text{ versus } 22\%; P = 0.04) \), 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 \( (43\% \text{ versus } 84\%; P < 0.001) \), especially in the non-MNA group \( (12\% \text{ versus } 96\%; P < 0.001) \). Furthermore, DCR2-methylated patients showed significantly poorer 5-year overall survival than DCR2-unmethylated patients in the neuroblastoma group \( (55\% \text{ versus } 85\%; P = 0.008) \), especially in the non-MNA group \( (56\% \text{ versus } 96\%; P < 0.001) \). These results indicate that DCR2 aberrant hypermethylation is a useful biomarker for the prediction of poor prognosis, especially in patients with non-MNA neuroblastoma.

<table>
<thead>
<tr>
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<th>( P )</th>
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<td>Unmethylated</td>
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<tr>
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<tr>
<td>High</td>
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<td>MYCN amplification</td>
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<tr>
<td>Nonamplification</td>
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</tr>
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</table>

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

\[ P < 0.001 \]

\[ P < 0.001 \]

\[ P < 0.001 \]

\[ P = 0.008 \]

\[ P < 0.001 \]
**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 $1.87 \times 10^{-4}$ in the tumor and $9.71 \times 10^{-5}$ 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, 14.3.3-σ, Np73, RASSF1A, and DCR2 promoters were associated with malignant phenotypes of neuroblastoma (18). Especially, the methylation patterns of 14.3.3-σ, 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 MSA 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.

Acknowledgments

We thank Dr. Ikuyo Ueda for technical advice about methylation-specific PCR and Ryoko Murata for her secretarial assistance.

References

Serum Assay of DCR2 Aberrant Methylation in Neuroblastoma


Circulating Methylated-DCR2 Gene in Serum as an Indicator of Prognosis and Therapeutic Efficacy in Patients with MYCN Nonamplified Neuroblastoma

Shigeki Yagyu, Takahiro Gotoh, Tomoko Iehara, et al.


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