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

Shigeki Yagyu,1 Takahiro Gotoh,1 Tomoko Iehara,1 Mitsuji Miyachi,1 Yoshiki Katsumi,1 Satoko Tsubai-Shimizu,1 Ken Kikuchi,1 Shinichi Tamura,1 Kunihiko Tsuchiya,1 Toshikiko Imamura,1 Akiko Misawa-Furihata,1 Tohru Sugimoto,1,2 Tadashi Sawada,1 and Hajime Hosoi1

Abstract

Background: MYCN amplification (MNA) in neuroblastoma is a strong indicator of poor prognosis. However, some MYCN nonamplified (non-MNA) cases show poor outcomes, and examining the status of the gene requires an operation, which may have surgical complications. Therefore, a new marker is needed to identify cases of non-MNA neuroblastomas with poor prognosis using less risky procedures. Aberrant hypermethylation of the DCR2 promoter has recently been associated with rapidly progressing neuroblastoma. We aimed to develop a noninvasive DCR2 methylation assay for patients with neuroblastoma using serum DNA, which predominantly originates from tumor-released DNA.

Methods: Using DNA-based real-time PCR, we simultaneously quantified a methylated-DCR2 specific sequence (M) and a reference sequence (R) located in the promoter region in serum DNA, and evaluated DCR2 methylation status as M/R ratios in 86 patients with neuroblastoma.

Results: Serum DCR2 M/R ratios were strongly correlated with those in the tumor (r = 0.67; P = 0.002). DCR2 methylation was associated with stage both in the whole neuroblastoma group and in the non-MNA group (P < 0.001), and DCR2-methylated patients showed significantly poorer 5-year event-free survival in the whole neuroblastoma group (43% versus 84%; P < 0.001), especially in the non-MNA group (12% versus 96%; P < 0.001). Among five DCR2-methylated patients whose clinical courses were followed, serum M/R ratios were close to 0 in the patients in remission, whereas the ratios increased in patients who relapsed.

Conclusions: Detection of methylated-DCR2 in serum DNA has promise as a noninvasive assay for predicting prognosis and therapeutic efficacy in neuroblastoma, especially in non-MNA cases. Furthermore, it might be a sensitive marker of tumor recurrence in DCR2-methylated cases.

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 IV, 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.

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 homozgyous 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.

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

<table>
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<tr>
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<th>No.</th>
<th>%</th>
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<tbody>
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<tr>
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<tr>
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<tr>
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<td>&lt;18 mo</td>
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<tr>
<td>&gt;18 mo</td>
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<td>+</td>
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<td>57</td>
</tr>
<tr>
<td>-</td>
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<td>Stage (INSS), in MNA(-) group (n = 68)</td>
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<tr>
<td>1</td>
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<tr>
<td>2A, 2B</td>
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<td>3</td>
<td>9</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>4S</td>
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<td>21</td>
</tr>
<tr>
<td>Nonamplification</td>
<td>68</td>
<td>79</td>
</tr>
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</table>

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 RNAeasy 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 \( \text{DCR2} \). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified for use as a loading control, as described previously (33). Primers for amplifying the \( \text{DCR2} \) 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 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 \( \text{DCR2} \) 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 \( \text{DCR2} \) 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 \( \text{DCR2} \) methylation analysis. Methylated-\( \text{DCR2} \) sequence which contained a “CGCG” sequence could be digested by BstU I, whereas the unmethylated-\( \text{DCR2} \) sequence could not be affected because all unmethylated cytidines were changed into uracils after bisulfite treatment. D, sequential analysis of \( \text{DCR2} \) M/R ratio in the diluted sample containing \( \text{DCR2} \)-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 \( \text{DCR2} \) M/R ratio. The primer set sequences used are shown in Supplementary Table S1. Full-length gels are presented in Supplementary Fig. S2.

Fig. 1. Detection of \( \text{DCR2} \) promoter aberrant methylation.
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 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 diluted with normal healthy donor DNA from 1:1 to 1:106, and each sample was done with bisulfite 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 promoter region, combined bisulfite restriction analysis with BstU1 restriction enzyme was performed (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
Table 2. Results of $\chi^2$ test for effect of DCR2 aberrant methylation on stage and MNA in patients with neuroblastoma

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>DCR2 Methylated</th>
<th>DCR2 Unmethylated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High</td>
<td>18</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Stage in MNA(-)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low</td>
<td>4</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>11</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>MYCN amplification</td>
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</tr>
<tr>
<td>Amplification</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Nonamplification</td>
<td>15</td>
<td>53</td>
<td></td>
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</table>

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.
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). 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 is 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 assay 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**

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