Chapter 2

A Novel Diagnostic Tool for Therapy Stratification of Neuroblastoma: Preoperative Analysis of Tumor Biology Using Circulating Tumor-Released DNA in Serum

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1. Introduction

Neuroblastoma (NB) patients fall into two clinically distinct subgroups; a low-risk subgroup and a high-risk subgroup. These subgroups are correlated with the age of onset [1], the extent of the disease (International Neuroblastoma Staging System) [2], pathological findings (International Neuroblastoma Pathological Criteria) [3], and genomic changes in NB tumors as represented by *MYCN* gene amplification (MNA) [4, 5]. Above all, the patterns of genetic changes can predict the subgroups of NB [6]. The low-risk, favorable NBs have mitotic disorders, and are characterized by near triploid karyotypes with whole chromosomal gains. On the other hand, the high-risk, unfavorable NBs exhibit genetic instability, and are characterized by chromosomal structural changes, including deletion of 1p or 11q, unbalanced gain of 17q, and/or MNA. MNA is the most powerful prognostic factor identified so far, and is useful regardless of the tumor stage. In the recent studies, the 5-year event free survival of patients who had NB tumors with MNA was 53% for localized NB [7] and 29% for all NBs [8], even with intensive, multimodal treatment. On the other hand, NB without *MYCN* amplification (non-MNA) falls into another two clinically distinct subgroups: a low-risk subgroup with overall survival rates of more than 95% without any intensive therapy, and a high-risk subgroup with overall survival rates of less than 40% despite the use of dose-intensive, multimodal therapy. In the high-risk non-MNA group, the development of NB depends on factors other than MNA, such as the expression profiles of other genes [9], aberrant hypermethylation of tumor suppressor genes [10], and chromosomal loss of heterozygosity (LOH) [11]. To determine the stratification of NB patients more precisely with regard to the treatment approach, a screen for these genetic aberrations should be performed before the initial treatment. Indeed, in the INRG staging system, routine assessments of MNA and 11q loss were

required for the therapeutic stratification of NB [8]. Currently, genetic alterations in NB tumors are clinically evaluated by interphase dual-color fluorescence in situ hybridization (I-FISH), array comprehensive genomic hybridization (aCGH), PCR and multiple ligation probe amplification (MLPA) [12]. However, the evaluation of tumor-related genetic aberrations requires a fresh tumor sample, which is often difficult to obtain due to the frequent occurrence of life-threatening conditions in NB patients.

Recent improvements in molecular techniques such as PCR have made it possible to detect small amounts of cell-free DNA in the serum and plasma of patients with various diseases, including cancers. In particular, tumor-derived cell-free DNA has attracted attention as a novel genetic marker for cancer. Other improvements in molecular techniques have enabled the evaluation of tumor-related genetic aberrations using small amounts of cell-free DNA in the serum. NB has been detected with several tumor-related genetic alterations, such as RAS mutations [13], or TP53 mutations [14], microsatellite instability [15], gene amplification [16, 17], aberrant promoter hypermethylation [18, 19], and allelic gain and loss of oncogenes [15, 20, 21].

We have been attempting to establish a preoperative risk stratification system for NB based on a serum-based assay system for *MYCN* gene amplification, aberrant promoter hypermethylation, and chromosomal loss of 11q. These less invasive techniques are clinically useful for the preoperative risk stratification of NB patients, because genomic changes in the tumor correlate with the tumor behavior, survival outcome, and response to therapy of NB patients. In this paper, we provide an update on the novel serum-based system for evaluating genetic aberrations for the risk stratification of patients with NB, including our recent studies.

2. Methods and results

Serum samples were collected from the patients with NB before initial treatment. Cellular components were immediately removed from the collected sera immediately by centrifugation or filtration. DNA fragments were purified from 200μl of serum according to manufacturer's protocol and were used for further genetic analysis as mentioned below.

2.1. Detection of amplified *MYCN* gene in the sera of NB patients

The *MYCN* oncogene can be detected in the serum of patients with MNA-NB. We previously established a system for quantitatively evaluating system of *MYCN* gene copy number using serum DNA of NB patients [17]. We simultaneously quantified the dosages of the *MYCN* gene located on 2p24 as a target, and the *NAGK* gene located on 2p12 as a reference by real-time PCR using DNA samples obtained from paired serum and tumor samples before the initial treatment. Also, we evaluated the *MYCN* gene copy number to obtain the *MYCN*/*NAGK* ratio. The *MYCK*/*NAGK* ratios of serum DNA and tumor DNA were strongly correlated, and the serum *MYCN*/*NAGK* ratio in the MNA group was significantly higher than the ratio in the non-MNA group. Notably, the sensitivity and specificity of the serum *MYCN*/*NAGK* ratio as a diagnostic test were both 100% when the serum *MYCN*/*NAGK* ratio cutoff was set at 10.0

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(Figure 1). We also reported that the serum-based *MYCN* status was an indicator of therapeutic efficacy. Among six MNA patients whose clinical courses were followed, the serum ratios decreased to within the normal range in the patients in remission (n=3), whereas the ratios increased to high levels in the patients who relapsed (n=2) or failed to achieve remission (n=1). These data strongly suggested that the serum-based quantitative *MYCN* status was a useful tool for preoperatively determining the stratification for therapy and for the evaluation of therapeutic efficacy during the course of treatment, when tumor cells were not available for a molecular analysis. Considering that it is often impossible to obtain tumor samples from patients with advanced NB for biological studies due to their life-threatening conditions, these data may have important clinical implications.

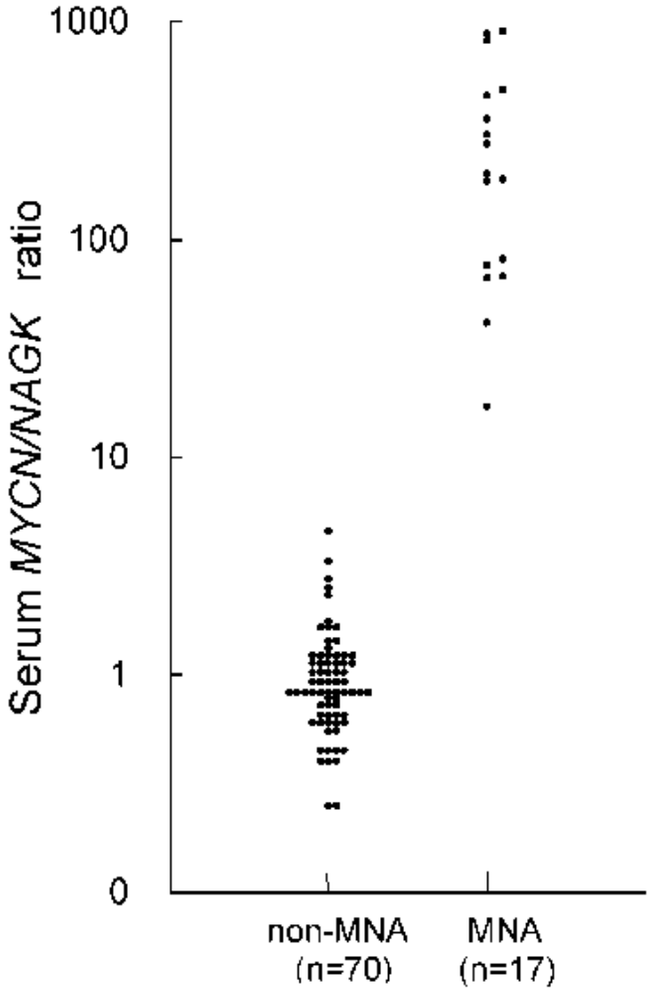

Figure 1. A scatter plot of serum *MYCN*/*NAGK* ratio according to tumor *MYCN* status. The serum *MYCN/NAGK* ratio was significantly ($p<0.001$) higher in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) than in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test). Adopted from Ref. [17]

Combaret et al., who first reported the existence of the *MYCN* gene in the sera of NB patients confirmed our data using a European cohort and the same method that we used. Also, they

indicated that the sensitivity of serum-based *MYCN* status depends on the stage of NB and is lower for patients with locoregional NB [22]. Indeed, they analyzed 10 serum samples obtained at diagnosis from MNA-NB patients with INSS stages 1 and 2, and 16 serum samples from MNA-NB patients with INSS stage 3 disease, and revealed that only one of 10 patients with stage 1 and 2 disease, and 12 of the 16 INSS patients with stage 3 disease showed high levels of circulating *MYCN* DNA sequences (10% and 75% sensitivity, respectively). In contrast, significant levels of circulating *MYCN* DNA sequences were detected in the patients with stage 4 disease and MNA (85% sensitivity).

We also confirmed the sensitivity and specificity of serum MNA analysis using 148 samples obtained from NB patients with various INSS stages in two Japanese cohorts other than ours and a cohort from the Children Oncology Group. A sub-group analysis according to INSS stages revealed that the sensitivities and specificities were not statistically different (67% and 95%, respectively, in stages 1 and 2, 92% and 86% in stage 3, and 87% and 97% in stage 4), although the number of each group was not statistically sufficient ($p=0.48$ for sensitivity, $p=0.68$ for specificity, chi-squared test) (our unpublished data). Accordingly, the serum *MYCN*/*NAGK* ratio would be a specific tool for the prediction of tumor *MYCN* amplification in patients with NB regardless of tumor stage, even though the sensitivity of serum MNA analysis showed a tendency to be low among the patients with INSS stages 1 and 2. Indeed, for most therapeutic regimens, primary radical resection is recommended for localized non-MNA NB. Moreover, up-front surgical resection is not indicated for advanced localized NB, including tumors with MNA. Therefore, knowing the preoperative serum-based *MYCN* status should result in better decision-making, especially for localized NB. As mentioned above, circulating tumor-derived cell-free DNA could be detected in the serum of cancer patients regardless of the tumor stage. Therefore, it was of interest to determine whether the serum-based *MYCN* status was also useful to determine the risk classification for localized NB. Being able to determine the *MYCN* status of NB patients from a blood sample would be very useful for cases who cannot provide tumor samples for a molecular analysis.

2.2. Detection of methylated DNA fragments in the sera of NB patients

It is important to have additional biomarkers with prognostic value for the management of non-MNA cases of NB because some cases without MNA also have a poor prognosis. Recent studies have revealed that epigenetic alterations, such as silencing of tumor suppressor genes by aberrant hypermethylation of the promoter, often play important roles in the pathogenesis and progression of NB. A positive correlation has been found between the hypermethylation of the promoters of these genes and a poor prognosis, thus suggesting that hypermethylation influences the phenotype of neuroblastoma.

We previously revealed that the aberrant hypermethylation in the promoter region of the *DCR2* gene in serum is a potent prognostic factor especially in non-MNA NB [18]. *DCR2* (decoy receptor 2) is a tumor necrosis factor alpha receptor superfamily gene, and is negatively associated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, because it lacks an intracellular death domain [23]. In NB tumors, the methylation profile of *DCR2* has been found to be drastically different and independent of the *MYCN* status

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[10, 24, 25]. Moreover, *DCR2* methylation was found to be associated with rapidly progressing tumors and a reduced overall survival [10]. Using the methylation-specific real-time PCR analysis we established [18], the aberrant hypermethylation in the promoter region of *DCR2* gene could also be detected in the serum DNA, and strongly correlated with the expression in the tumor. The 5-year event-free survival and overall survival of NB patients with methylated *DCR2*, as detected by a serum-based assay, were significantly poorer than those of NB patients with unmethylated *DCR2* [18] (Figure 2). These observations indicated that the serum-based *DCR2* methylation status could help predict the prognosis of NB patients, especially those without MNA. Additionally, the serum-based *DCR2* methylation status can distinguish patients with a poor outcome within the non-MNA group, and may allow for a new type of risk stratification for patients with non-MNA NB in future trials.

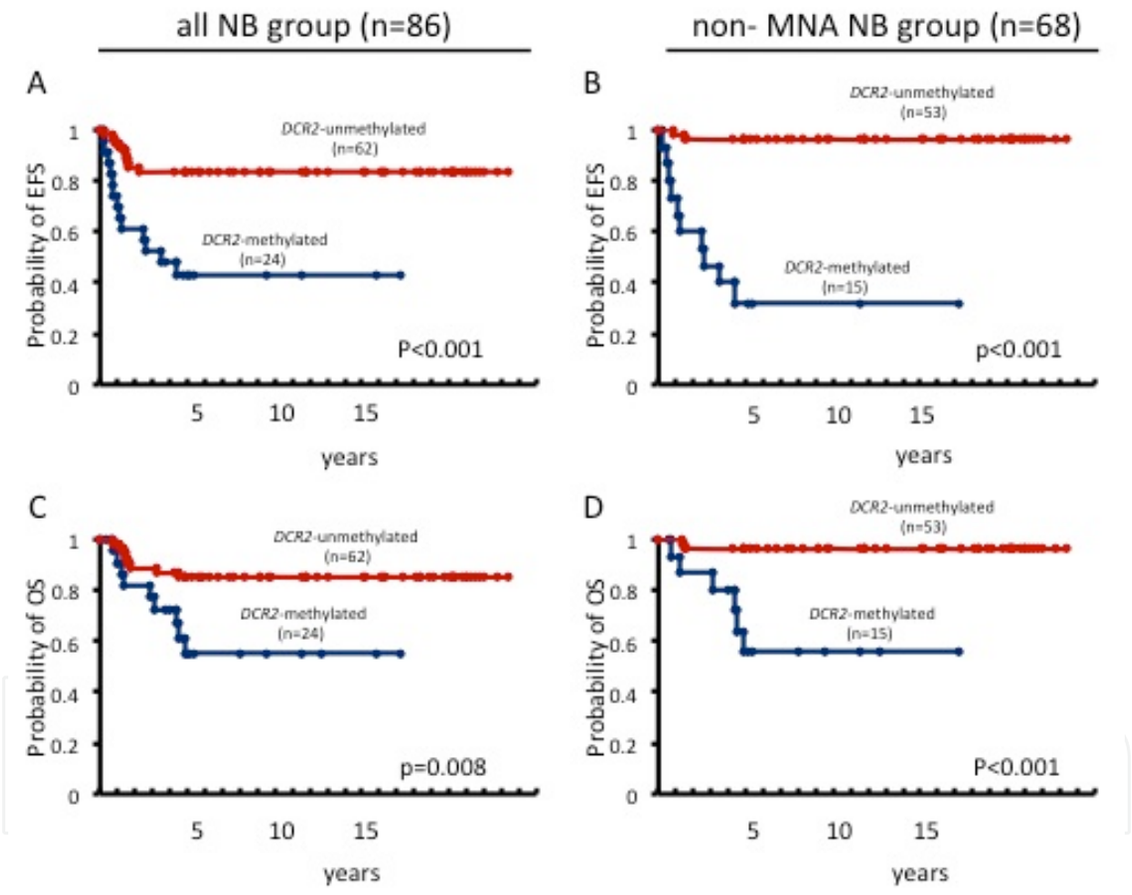

Figure 2. Event-free and overall survival for patients with neuroblastoma according to the serum-based *DCR2* methylation status. A: Event-free survival in all patients with neuroblastoma: methylated (n = 24) and unmethylated (n = 62); P < 0.001. B: Event-free survival in patients with non-MNA neuroblastoma: 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. Adopted from Ref. [18]

RASSF1A is hypermethylated in 93-94% of NB primary tumors and in 100% of relapsed tumors [19, 26]. *RASSF1A* hypermethylation in tumors appears to be a relatively early event in NB

tumorigenesis, as it is usually detectable in early stage tumors. On the other hand, *RASSF1A* methylation was detected in the serum from only 25% of NB patients, and methylated *RASSF1A* was more frequently detectable in the serum of advanced NB patients than in those with early stage NB, although the *RASSF1A* methylation status in NB tumors was not significantly different between patients with advanced and early stage NB. These discrepancies in the sensitivities between serum-based and tumor-based methylation assays could be explained by the smaller quantity of DNA released from a small tumor burden. Nevertheless, this serum-based *RASSF1A* methylation assay should help to determine the appropriate risk classification.

2.3. Detection of chromosomal loss and gain in the serum DNA of NB patients

Chromosomal gain and/or loss are frequently observed in NB as mentioned above. Among the various unbalanced chromosomal aberrations, 17q gain is the most frequent chromosomal aberration, and correlates with a poor outcome [27]. The loss of 11q is also a strong prognostic factor that can be used in addition to MNA [11], and routine assessment of the 11q status, as well as MNA, is required for therapeutic stratification of NB in the INRG staging system [8].

Some groups, including ours, have developed serum-based assays to detect chromosomal gain and/or loss in NB using various techniques. Combaret et al. reported a serum-based detection system for 17q gain [20]. They simultaneously quantified the gene dose of MPO (17q.23.1) and survivin (17q25) as targets, and p53 as a reference, by quantitative real-time PCR using 142 serum samples. They revealed that the serum-based determination of 17q gain had good specificity (94.4%) and sensitivity (58.8%) in patients who were less than 18 months old ($p<0.001$), while this approach showed moderate specificity (71.4%) and sensitivity (51.2%) in patients over 18 months of age. In a subset analysis according to the stage of NB, the sensitivity of serum-based 17q gain determination tended to increase with the stage of the disease. On the other hand, for metastatic NB, the sensitivity of the test never exceeded 60%, which is lower than the results achieved by the analysis of the serum-based *MYCN* status.

We developed an assay for detecting chromosomal aberration of 11q, using a different method that doesn't use quantitative real-time PCR. Previous studies have revealed the presence of a smallest region of overlap (SRO), which is a common region of deletion in all NB cases with 11q loss. By targeting some polymorphic markers in the SRO of 11q (most of which are located in 11q23), allelic loss could be detected using serum DNA as well as tumor DNA of NB patients [21] (Figure 3). Using this technique, the sensitivity and specificity of the results between the serum- and tumor-based 11q loss analyses were both 100%, although a further study is needed to confirm of these findings because of the limited number of cases that were analyzed.

3. Discussion

In a large-scale randomized trial of children with high-risk NBs [28], the *MYCN* status was unknown in 27% of the children. In a clinical setting, some life-threatening cases with a huge mass or hepatomegaly (hepatic metastasis in stage 4s) received chemotherapy and/or radio-

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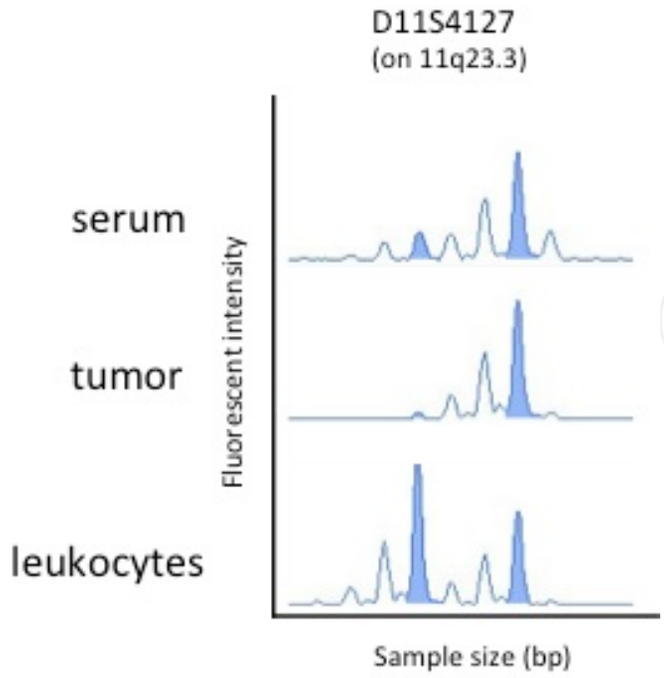

Figure 3. Representative case of 11q loss. The results of 11q microsatellite analysis were shown. This case was a neuroblastoma categorized into low-risk subgroup (INSS stage2, *MYCN* non-amplified NB, Shimada: Favorable Histology). Complete resection after initial diagnosis was performed without any additional chemotherapy. Relapse at bone and bone marrow 1 year after resection was observed, and he died of disease 3year after relapse. Microsatellite analysis were performed using his serum, tumor and leucocyte DNA, and STS marker D11S4127 located on 11q 23.3. Non-tumor DNA purified from leukocytes has two fluorescent peaks (shown in blue), whereas in tumor and serum DNA, one of the peaks is reduced, suggesting the existence of 11q loss. Adopted from Ref. [21].

therapy based on elevated tumor markers and positive MIBG scintigraphy, prior to tumor biopsy without evaluation of the *MYCN* status. The serum-based assays described above will be most useful when a primary tumor biopsy is not possible and when genetic information will influence the risk grouping and treatment allocation of the NB patients.

In Japan, infantile NB cases were formerly subclinically detected by mass-screening. Most of these cases showed a good prognosis and were recommended to undergo a reduced regimen, including the "wait-and-see" approach. However, we and others demonstrated that MNA was strongly correlated with a poor prognosis even in infantile, localized NB [7, 29]. The serum-based MNA status has considerable prognostic value for infantile NB cases. Indeed, the serum-based MNA status of NB patients has considerable prognostic value, especially in cases less than 18 months of age (our unpublished data).

On the other hand, most of the infantile, localized NB patients did not have MNA. In the Cooperative German Neuroblastoma NB95 and NB97 trials, some localized patients without MNA did not receive chemotherapy after biopsy and showed spontaneous regression [30]. Considering the clinical behavior of non-MNA NB, an early and non-invasive system for detecting genetic alterations besides MNA is needed to help select the appropriate therapy. In other words, combined preoperative assessments of MNA and 11q loss using serum DNA will make it possible to safely perform risk-adapted therapy according to the INRG staging system

[8]. Particularly, preoperative serum-based MNA and 11q loss detection can be useful for cases that are in INRG stages L2 and MS, which have a wide range of clinical outcomes and potential therapeutic strategies depending on the existence of MNA and 11q loss. Further, we may be able to select infants with NB who truly need to receive treatment including surgical treatment, intensive chemotherapy, and even radiotherapy from infants with NB who do not need to require any treatment by using our technique.

In conclusion, serum-based, less invasive molecular analysis can provide much better clinical information to determine the optimal therapeutic strategy for NB patients. Prospective validation in a large cohort will be needed to confirm the utility of these tools for assessing biological risk. Serum-based, surgery-free, rapid, sensitive, and specific genetic assessments have great potential to provide a personalized, risk-adapted therapy for patients with NB.

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