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Preoperative analysis of 11q loss using circulating tumor-released DNA in serum: A novel diagnostic tool for therapy stratification of neuroblastoma

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ABSTRACT

Allelic deletion of the long arm of chromosome 11 (11q loss) is closely associated with the prognosis of neuroblastoma (NB). Here we examined 11q loss using tumor-released DNA fragments in the sera of 24 cases. The allelic intensity score of a panel of polymorphic markers in 11q23 in serum DNA was significantly different between the 11q loss-positive group and the11q loss-negative group. The 11q loss-positive and -negative groups did not overlap when a cut-off value of 0.5 was chosen for the allelic intensity score. Our serum-based 11q loss analysis could predict the allelic status of 11q in tumors.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of early childhood. Genomic changes in the tumor correlate with the behavior and outcome of NB patients. Amplification of the *MYCN* gene (MNA), located on 2p24, was detected in about 22% of the patients with NB, and is considered the strongest prognostic factor [1–3]. The non-MNA NBs fall into 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% even though they are given dose-intensive, multimodal therapy. In the high-risk non-MNA group, development of NB depends on factors other than MNA, such as genetic expression profiles [4–6], aberrant hypermethylation of tumor suppressor gene [7–11], and chromosomal loss of heterozygosity (LOH) [12–14].

Non-MNA NB patients frequently have allelic deletion of the long arm of chromosome 11 (11q loss) [15,16], and have poor outcomes [12,17]. Accordingly, routine assessment of 11q loss status as well as MNA is required for therapy stratification of NB in the INRG staging system [18]. However, patients with localized NB and non-MNA, who were categorized as having low-risk NB, sometimes were given reduced therapy and were advised to accept a wait-and-see strategy [19,20]. Therefore, an early and non-invasive detection system for 11q loss that does not require any surgical procedure is needed to help select the appropriate therapy for these patients. We previously developed a test that uses tumor-released DNA that is

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present in the serum of NB patients [21,22] to decide which therapeutic strategy to use for treating NB before any invasive intervention. Preoperative combined assessments of MNA and 11q loss using serum DNA will make it possible to safely perform risk-adapted therapy. Here we describe a system for evaluating the allelic status of chromosome 11 using the sera of NB patients.

2. Patients and methods

2.1. Subjects

Twenty-four children diagnosed with NB at the Hospital of Kyoto Prefectural University of Medicine and Kyoto City Hospital were enrolled onto this study with the informed consent of their parents. The only inclusion criteria was the availability of outcome data and DNA samples from three tissues: serum obtained at the onset of NB, the tumor, and non-tumor tissue. At the time of diagnosis, 12 patients were younger than 1 year, and 12 were between 1 and 5 years of age. Seven of the patients had MNA, and 17 patients did not have MNA. According to the International Neuroblastoma Staging System [23], the 24 children included 9 in stage 1, 1 in stage 2A, 1 in stage 4S, 1 in stage 3, and 12 in stage 4. The serum and tumor samples were linked to clinical and biological information and the laboratory investigators were blinded to these data (Table 1).

2.2. Tumor and serum DNA preparation

DNA was extracted with a QIAamp DNA Mini kit (Qiagen) as per the manufacturer's protocol. Patients' sera were obtained before any therapy and surgery, and stored at −20 °C. Serum DNA was extracted as described previously [21,22]. For serum DNA isolation, serum was centrifuged at 15,000 rpm for 10 min to remove leukocytes, and then we used 100 μL of serum supernatant, which contained 1 μg of salmon testes DNA (Sigma) as a carrier DNA.

2.3. Allelic status of 11q by microsatellite analysis

Previous studies have revealed the smallest region of deletion overlap (SRO), which is a region of deletion that is shared by all cases of NB with 11q loss, and polymorphic markers involved in that region [12,15,16]. Thus, we chose three highly informative polymorphic markers from the SRO of 11q for microsatellite analysis (Supplementary Table S1). Sense primers were labeled with FAM, VIC, or NED fluorescent dyes respectively, and antisense primers were modified with a reverse tail to promote single nucleotide overhangs (Applied Biosystems, Foster City, CA, USA). To discriminate whole chromosome loss, we also chose three informative polymorphic markers that were located in the short arm of chromosome 11, and performed the microsatellite analysis simultaneously. When possible, the markers were combined in multiplex fluorescence screening panels. Samples that gave equivocal results were rescreened in a conventional uniplex polymerase chain reaction (PCR).

Electrophoresis was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems) and analyzed with Genescan software (Applied Biosystems). Allelic deletion of 11q23 was considered to have occurred at an individual marker when a comparison of the allelic intensity score

Table 1
Characteristics and results of 11q loss of the patients.

No.	INSS stage	Age of onset (mo.)	*MYCN* status	Mass screening	Outcome	11q loss in serum (representative allelic intensity score of STS marker on 11q23)	11q loss (MLPA)
1	4	13	Non-amp.		Died of disease	+(0.15)	+
2	4	48	Non-amp.		Died of disease	−(1.04)	−
3	4	8	Amp		Died of disease	−(1.08)	−
4	3	11	Amp.	+	Died of disease	−(0.99)	−
5	2	11	Non-amp.	+	Die of disease	+(0.30)	+
6	1	10	Amp.		Alive	−(1.14)	−
7	4	13	Amp.		Die of disease	−(1.00)	−
8	4	58	Gain		Alive with disease	−(0.65)	−
9	4	36	Non-amp.		Die of disease	−(1.17)	−
10	4S	2	Non-amp.		Alive	−(1.04)	−
11	1	4	Non-amp.		Alive	−(0.65)	−
12	4	60	Non-amp.		Alive with disease	+(0.38)	+
13	1	18	Non-amp.	+	Alive	−(1.01)	−
14	1	36	Non-amp.	+	Alive	−(1.09)	−
15	1	9	Non-amp.	+	Alive	−(1.00)	−
16	1	11	Non-amp.	+	Alive	−(0.67)	−
17	1	8	Non-amp.	+	Alive	−(1.05)	−
18	1	10	Non-amp.	+	Alive	−(0.67)	−
19	4	49	Amp.		Died of disease	−(0.93)	−
20	4	36	Non-amp.		Alive	−(0.99)	−
21	1	11	Non-amp.	+	Alive	−(1.00)	−
22	4	1	Non-amp.		Alive	−(0.97)	−
23	4	60	Non-amp.		Died of disease	+(0.20)	+
24	4	12	Amp.		Alive	+(0.10)	+

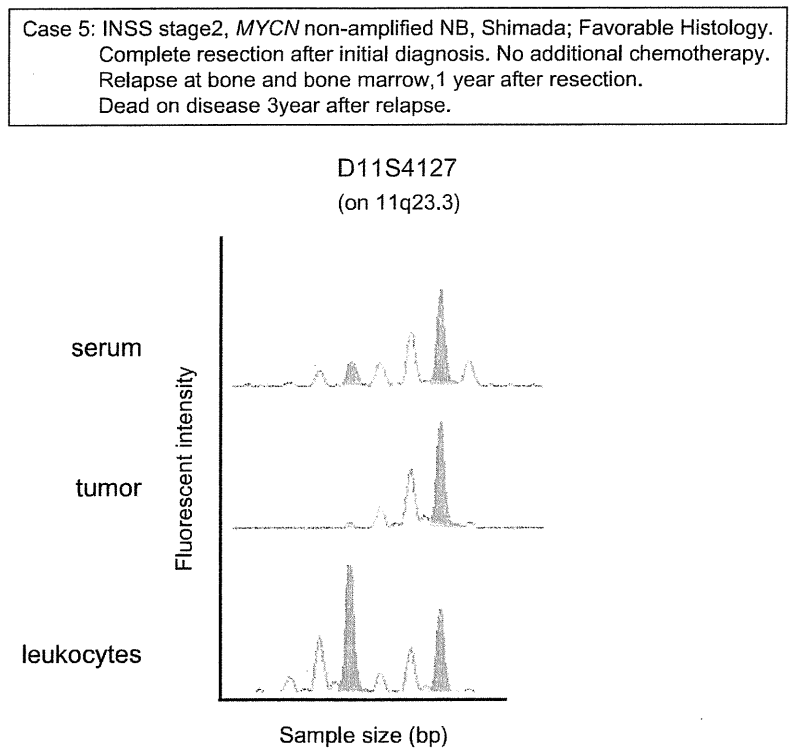

Fig. 1. Representative case of 11q loss. Microsatellite analysis using STS marker D11S4127 located on 11q 23.3. Non-tumor DNA purified from leukocytes has two fluorescent peaks (shown in blue), whereas in tumor and serum DNA, one of the peaks is reduced, suggesting the existence of 11q loss. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

calculated by a fluorescence electrophoretogram yielded a score of less than 0.5 (indicating a 50% reduction in intensity of one tumor allele), as previously described [12,13]. A sample was considered to have allelic deletion of 11q23 if the locus had at least two informative markers showing. Whole chromosome loss was assumed to have occurred when the allelic intensity of every microsatellite marker showed a decrease of over 50%. During the assessment of the allelic status of a patient, investigators were blinded to the patient's data.

2.4. MLPA analysis

To confirm the allelic status of 11q, we performed multiplex ligation-dependant probe amplification (MLPA), a method for detecting large deletions by simultaneously screening for the loss of up to 40 target sequences [24]. MLPA has recently been shown to be a reliable method for determining the allelic status of NB [25]. To identify chromosomal aberrations on 11q, we used a specifically designed probe set (SALSA MLPA Kit P251-B1 Neuroblastoma) (MRC Holland, Amsterdam, The Netherlands) [25]. MLPA was performed according to the manufacturer's protocol. PCR fragments generated with MLPA kit were separated by capillary electrophoresis on an ABI Prism 3130 genetic analyzer (Applied Biosystems). Peak area was measured using Genemapper software (Applied Biosystems). To identify chromosomal aberrations, relative copy numbers were calculated using reference DNA from the leukocytes of three healthy donors.

2.5. Statistics

Differences of allelic intensity score on 11q23 between the 11q loss-positive and -negative groups was calculated by Mann–Whitney's *U*-test. Descriptive statistical analyses were performed with SPSS software. $p < 0.05$ was judged as significant.

3. Results

3.1. Serum-based detection of allelic deletion at 11q23

Microsatellite analysis with three polymorphic markers on 11q23 and three markers on 11p was performed using serum DNA, tumor DNA, and non-tumor DNA, simultaneously. Fig. 1 shows a representative result of 11q loss analysis with Sequence Tagged Site (STS) marker D11S4127 located on 11q 23.3. The serum DNA lacks one peak, whereas non-tumor DNA purified from leukocytes of case 5 has two fluorescent peaks, suggesting the allelic deletion of 11q (Fig. 1). These results were confirmed by microsatellite analysis and MLPA analysis using tumor DNA (data not shown).

3.2. Serum-based microsatellite analysis as a predictor of 11q loss in NB

Of the 24 patients from whom serum DNA, tumor DNA, non-tumor DNA from leukocytes could be obtained, all the allelic intensity scores of polymorphic markers in 11q23 in serum DNA in the 11q loss-negative group were around 1.0, while those in the 11q loss-positive group were significantly lower than 0.5 (Fig. 2). In fact, the 11q loss-positive and -negative groups did not overlap when a cut-off value of 0.5 was chosen for the allelic intensity scores of STS markers on 11q23. With these cut-off values, the sensitivity and specificity of the serum-based 11q loss analysis as a diagnostic test to distinguish 11q loss-positive and -negative

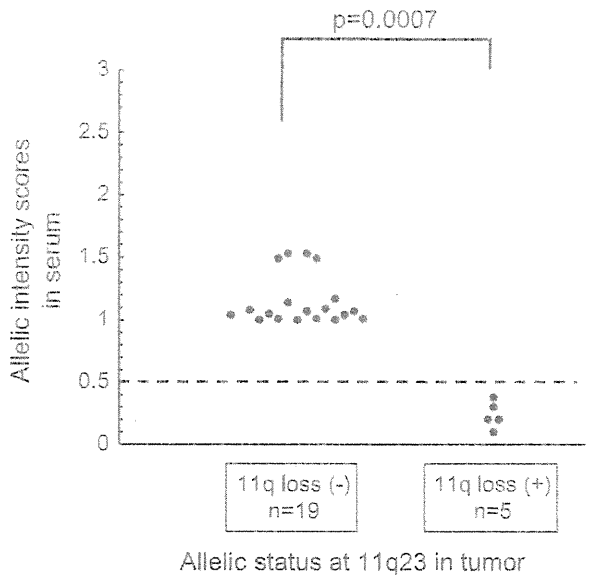

Fig. 2. A scatter plot of allelic intensity score of STS marker in 11q23 using serum DNA from patients with 11q loss-positive and -negative neuroblastomas. With cut off values of 0.5, which indicate a 50% reduction in intensity of one tumor allele, the results of 11q loss analysis in serum DNA were completely correlated with that in tumor DNA (Mann–Whitney *U* test, $p = 0.0007$).

patients were both 100% for our limited number of patients. This suggests that the serum-based microsatellite analysis can predicted allelic status of 11q23 in an NB tumor.

4. Discussion

Recently, several studies have examined tumor-derived DNA fragments in serum. In cancer patients, the amount of serum DNA was much higher than that in healthy donors [26–28]. This suggests that apoptotic and/or necrotic cancer cells release DNA fragments into the blood stream, and that tumor-released DNA fragments can stably exist in a patient's serum. As a result, they are also the predominant DNA type in serum of cancer patients. Furthermore, tumor-derived DNA in serum has been used to identify various genetic abnormalities (e.g., gene amplification, aberrant hypermethylation of promoter regions, LOH, and genetic instability) that can be used as prognostic markers of many cancers. Especially, serum-based LOH analysis has recently been highlighted in head and neck cancer [29], non-small cell lung cancer [30], colorectal cancer [31], malignant melanoma [32], and breast cancer [33]. Using PCR-based microsatellite analysis, the allelic status of a chromosome can be assessed in serum DNA with high sensitivity. Serum-based genetic analysis is considered to be helpful in the management of pediatric solid tumor patients because of its non-invasiveness; however, few studies have examined the usefulness of circulating tumor-derived nucleic acid in serum for the diagnosis of pediatric solid tumors.

Our established multiplex strategy allowed for non-invasive, rapid, and high-throughput detection of allelic status of 11q using patients' sera that were routinely obtained at the onset of NB. To improve the prognosis of NB, it is important to determine the risk classification accurately and rapidly. Preoperative assessment of 11q loss can provide an appropriate risk classification according to the INRG staging system, especially in severe cases who cannot undergo tumor biopsy. Moreover, our serum-based genetic analyses can be useful for choosing the appropriate surgical procedure and therapy immediately after tumor biopsy for all NB patients.

Our serum-based 11q loss analysis can be useful for cases that are in INRG stages L2 and MS, which have a wide range of clinical outcomes, depending on the age of onset, pathological findings, and the existence of MNA and 11q loss [18]. Although most infantile localized and stage 4s NBs are categorized into the low-risk group and have good outcomes with reduced therapy, some cases are categorized into the high-risk group if they are found to have MNA and/or allelic loss at 11q23. In our cohort, case 5 was an 11-month-old boy at the onset with localized NB without MNA that was detected by the Japanese NB mass-screening system. He was categorized into the low-risk NB group (INSS stage 2) and underwent complete resection of the tumor without any other chemotherapies. However, relapse of the bone and bone marrow developed 1 year after the tumor resection, and he died of the disease despite undergoing intensive chemotherapy and hematopoietic stem cell transplantation. An additional microsatellite analysis of the patient's serum DNA obtained at the onset of NB revealed that his tumor DNA had the 11q deletion, which was confirmed by the MLPA analysis (Fig. 1). The malignant potential of localized NB without MNA, which develops at less than 1.5 years of age, can sometimes be underestimated because most of such cases, like this case, receive reduced therapy. Thus far, our established preoperative assessment technique of 11q loss using serum DNA can provide appropriate information to determine a risk-oriented therapy for such patients. Nevertheless, a large set of patients needs to be studied to verify the accuracy of this assay.

It might be difficult to interpret allelic intensity scores of STS markers when one allelic deletion has occurred in a tri- or tetrasomic chromosome. Moreover, tumor heterogeneity may result in misidentification of the allelic status of 11q. Under such situations, interphase-fluorescence in situ hybridization (I-FISH) using tumor samples would be needed to evaluate allelic status of 11q.

In conclusion, we established a serum-based assay system for detecting 11q loss. Our 11q loss analysis requires only 12 h and only 100 μL of serum, and completely matched the results of the MLPA analysis. The therapeutic modality for NB varies widely and has to be chosen immediately after the onset of NB according to its biological phenotype. Therefore, a serum-based, surgery-free, rapid, sensitive, and specific genetic assessment that can detect 11q loss should lead to better outcome of NB in the future.

Conflict of interest

The authors indicated no potential conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.05.032.

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神経芽腫における標準治療の確立と新規治療の開発に関する研究

平成23年度　総括・分担研究報告書

研究代表者　池田　均

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