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Classification of neuroblastomas based on an analysis of the expression of genes related to prognosis

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Abstract

Purpose: To select the optimal treatment according to the grade of malignancy of neuroblastoma (NB), it is essential to accurately and rapidly identify genetic abnormalities associated with the prognosis. We have identified *BIN1* and *neuronatin β* as the novel prognosis-related genes for NBs. This study aims to assess the correlation between the combination of the expression level of prognosis-related genes and the outcome of NB.

Methods: In 44 NB samples, the expression levels of *TrkA*, *BIN1*, and *neuronatin β* were determined using quantitative reverse transcriptase–polymerase chain reaction; furthermore, the correlation between the expression of these genes' expression levels and the clinical progression of NB were assessed.

Results: It was possible to classify 44 NBs into 4 groups regarding the grade of malignancy of NB. These 4 groups were all significantly associated with the clinical stages international NB staging system as well as the outcomes of the patients ($P < .001$, according to the trend test by Kruskal-Wallis exact test).

Conclusion: The combination of the expression levels of these genes using quantitative reverse transcriptase–polymerase chain reaction is indicated as the effective method to quickly and accurately evaluate the grade of malignancy of NBs.

Neuroblastoma (NB) is the most common solid tumor in children, and its development is still uncharacterized [1]. The prognosis for those with NB tends to vary greatly, and many studies have demonstrated that both clinical and biologic factors are correlated with the outcome [2]. Some of these prognostic factors are known, such as *MYCN* amplification, DNA ploidy, or *TrkA* expression [1-3]. These factors are used clinically to predict the patient's prognosis and to select the optimal therapy. It is important to select the optimal therapy according to the property of these tumors. However, the potential for malignancy in NB cannot be accurately predicted by these factors [1-3].

BIN1 (2q14) encodes multiple tissue-specific isoforms of a myc-interacting adaptor protein that has some characteristics of a tumor suppressor, including the ability to inhibit myc-mediated cell transformation and promote apoptosis [4]. Previous studies hypothesized that *BIN1* may function as a suppressor gene in NB because *BIN1* is highly expressed in neural tissues and binds myc within a region

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with 100% homology to *MYCN* [4]. Furthermore, other reported data correlate with the reduced expression of *MYCN*-interacting *BIN1* isoforms with unfavorable features in primary NB [5].

The *neuronatin* gene was first identified in 1994, and the expression of that gene was specifically localized in the fetal mouse brain [6,7]. The *neuronatin* gene has 2 expression forms, *neuronatin* α and *neuronatin* β (*Nnat* β). In the mouse, its expression appears on the 8.5th fetal day at the hindbrain and then spreads to the central and peripheral nervous system and thereafter gradually disappears after birth. It is also expressed in the sympathetic nervous system and adrenal grand on the 15th fetal day [8]. Although the specific function of this factor is still not known, it is thought to be a membrane protein based upon its structure [9]. In our previous study, the expression of *neuronatin* was analyzed in a number of NB samples, and the results confirmed the effectiveness of this gene as a prognostic factor by assessing its correlation to the known prognostic factors and outcome [10]. Specifically, the expression levels of *Nnat* β were significantly lower in all groups with unfavorable prognostic factors than in the groups with favorable prognostic factors [10].

In this study, the expression levels of *TrkA*, *BIN1*, and *Nnat* β will be determined using quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) in 44 NB primary samples. Furthermore, the correlation between the combination of these genes' expression levels and the clinical findings of NB will be assessed.

1. Materials and methods

1.1. Clinical data of patients and biologic data of NB samples

Patients with NB evaluated at the Department of Pediatric Surgery, Kyushu University, Fukuoka, Japan, were diagnosed and staged according to the international NB staging system [11]. This study is approved by our institution's institutional review board. Forty-four frozen tumor samples were obtained from untreated patients with NB. The patient population included 26 males and 18 females, and the age at diagnosis ranged from 19 days after birth to 11 years. Of the 44 cases, 19 patients were diagnosed at older than 1 year, whereas the remaining 25 were diagnosed at younger than 1 year. Twenty-three patients were identified using an NB mass screening system. Of the 44 samples, 29 were tumors from patients who were in stage 1, 2 or 4S, whereas 3 were in stage 3, and 12 were in stage 4. Thirty-one patients are still alive, of whom 3 cases are still under treatment, whereas 13 patients have died as a result of the disease. The follow-up period after treatment ranged from 2 to 12 years. In all 44 samples, the status of *MYCN* amplification was previously determined by the Southern blot method. Regarding the histologic findings, all 44 cases were classified based on the classification by Shimada et al [12]. Thirty-nine cases showed a favorable histology, whereas the remaining 15 cases showed an unfavorable histology.

1.2. RNA extraction and complementary DNA synthesis

Total RNA was extracted from the frozen samples using Isogen (Nippon Gene, Osaka, Japan), and reverse transcription was performed with a first-strand complementary DNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) using random hexanucleotide primers.

1.3. Quantitative RT-PCR

The primers and TaqMan probes for the *TrkA*, *BIN1*, and *Nnat* β were designed using the application-based primer design software Primer Express (Applied Biosystems [ABI], Foster City, Calif). The sequences of the polymerase chain reaction (PCR) primers and TaqMan probes were as follows: *TrkA*—forward primer, 5′-CAT CGT GAA GAG TGG TCT CCG-3′; reverse primer, 5′-GAG AGA GAC TCC AGA GCG TTG-3′; TaqMan probe, 5′-AGG AGT GAA ATG GAA GGC ATC TGG CG-3′; *BIN1*—forward primer, 5′-AAG GCC CAG CCC AGT GAC-3′; reverse primer, 5′-GAG CCA TCT GGA GGC GAA G-3′; TaqMan probe, 5′-CGC GCC TGC AAA AGG GAA CAA GA-3′; *Nnat* β—forward primer, 5′-TCG GCT GAA CTG CTC ATC ATC-3′; reverse primer, 5′-TTC TGC AGG GAG TAC CTG AAC A-3′; TaqMan probe, 5′-ACA TCT TCC GCG TGC TGC TGC AG-3′. *18s r*RNA was used as an internal control gene to analyze the gene expression. Polymerase chain reaction primer and TaqMan probe for *18s r*RNA were purchased from ABI as a kit of TaqMan *18s r*RNA Control Regent. Quantitative PCR was performed in a final volume 25 μL, and each sample was analyzed in duplicate. Each reaction mixture contained 0.1 pmol/μL TaqMan probe, 0.2 pmol/μL each primer, 1× TaqMan PCR master mix, and 10 to 50 ng DNA. Thermal cycling was initiated with a 2-minute incubation at 50°C followed by the first denaturation step of 10 minutes at 95°C and then 40 cycles of 2-step PCR consisting of 95°C for 5 seconds and 60°C for 1 minute. The quantification of the *MYCN* gene was achieved by means of the ABI Prism 7700 Sequence Detection System (ABI). Genomic DNA from 1 NB with 90 copies of *MYCN,* determined by Southern blotting, was serially diluted to establish the calibration curve.

1.4. Statistical analysis

The expression levels of *TrkA* (*TrkA/r*RNA), *BIN1* (*BIN1/r*RNA), and *Nnat* β (*Nnat* β*/r*RNA) in the subgroups were represented by percentile (50%). A comparison of the expression in relation to clinical and genetic parameters was made using Mann-Whitney *U* test. Kruskal-Wallis exact