Enhanced Expression of N-myc Messenger RNA in Neuroblastomas Found by Mass Screening

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ABSTRACT

A substantial fraction of neuroblastomas found by mass screening have been suggested to regress spontaneously because of the high incidence of infantile neuroblastomas in the screening population. In this study, 70 neuroblastomas were analyzed for expression of proto-oncogenes related to neuronal differentiation to clarify the biological significance of proto-oncogene expression in the screening-positive and -negative tumors. The tumors consisted of 39 neuroblastomas found by screening (group 1), 16 non-N-myc-amplified neuroblastomas found by clinical symptom(s) (group 2), and 15 N-myc-amplified neuroblastomas found by clinical symptom(s) (group 3). The expression of c-src, trk A, and N-myc in tumor tissues was analyzed by quantitative RNA PCR. Neuronal c-srcN2 expression varied significantly in the following order: group 1 > group 2 > group 3. The level of expression of trk A was markedly reduced in group 3 but did not differ in groups 1 and 2. Most tumors in group 3 overexpressed N-myc. However, N-myc expression in group 1 was significantly higher than that in group 2. Thus, the characteristics of proto-oncogene expression in screening-positive tumors included enhanced expression of c-srcN2 and N-myc mRNA, regardless of nonamplification of N-myc. Our results suggest that the role of N-myc differs in neuroblastomas detected by screening and in N-myc-amplified tumors.

INTRODUCTION

The prognosis of patients with neuroblastomas, the most common malignant solid cancers in early childhood, is strongly affected by heterogeneous expression of the proto-oncogenes related to neuronal differentiation. Highly aggressive tumors often exhibit amplification of the N-myc gene and are usually found as advanced disease after 1 year of age (1, 2). In contrast, infantile neuroblastomas identified at an age of <1 year frequently express NGF receptor trk A and its signal-mediating molecules Ha-ras and src proto-oncogene (3–8). A complete cure can be expected in most of the localized neuroblastomas diagnosed before 1 year of age, particularly in tumors found through mass screening.

A mass screening program to detect aggressive neuroblastomas early was launched in Japan in 1985 (9). However, the incidence of infantile neuroblastoma has been reported to be on the increase in the screening population, whereas unfavorable biological markers were rarely found in the screening-positive tumors (9–11). Therefore, it has been suggested that the mass screening system tends to identify additional tumors that would regress spontaneously or mature. The detailed biological characteristics of screened tumors in relation to proto-oncogene expression are not yet fully understood.

The expression of N-myc is developmentally regulated and associated with the undifferentiated phenotype of neuronal cells (12–14). In clinical neuroblastomas, naturally or chemically maturated tumors express reduced levels of N-myc mRNA (15). Genomic amplification of N-myc is characteristic of aggressive neuroblastomas and is frequently accompanied by overexpression of N-myc mRNA (16, 17). However, infantile neuroblastomas have been reported to express relatively high levels of N-myc mRNA, and the clinical significance of N-myc mRNA expression is thus controversial (3, 15, 18–21). The expression of N-myc mRNA in infantile neuroblastomas found by mass screening remains to be analyzed.

In this study, to examine the biological characteristics of screening-positive neuroblastomas, we analyzed expression of neuronal c-src, trk A, and N-myc mRNA by quantitative RNA PCR (22) in 39 neuroblastomas identified through mass screening and 31 neuroblastomas found clinically.

MATERIALS AND METHODS

Patients and Tumor Specimens. Seventy patients with neuroblastomas who were treated at Chiba University Hospital, Chiba Children’s Hospital, or Matsudo Municipal Hospital (all in Chiba, Japan) between 1987 and 1998 were studied. The median follow-up period after diagnosis for the surviving children was 75 months (range, 13–150 months). The neuroblasto-
mas were staged according to the International Neuroblastoma Staging System. Of the 70 cases, 22 were diagnosed at 1 year of age, whereas the remaining 48 were diagnosed at <1 year of age, and 39 of the 48 infantile patients were identified by a neuroblastoma mass screening system. All of the tumors found by screening contained a single copy of N-myc. The tumors were categorized into three groups according to clinical presentation: (a) 39 tumors were found by mass screening (group 1); (b) 16 non-N-myc-amplified tumors were found by clinical symptom(s) (group 2); and (c) 15 N-myc-amplified tumors were found by clinical symptom(s) [group 3 (Table 1)]. High molecular weight cellular DNAs and undegraded total RNAs were extracted from the neuroblastoma tissue obtained by biopsy or surgery before chemotherapy. The specimens were confirmed to consist of tumor cells by pathological examination before the gene analyses.

**RNA PCR.** Total cytoplasmic RNA (5 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random hexanucleotide primers, essentially as described previously (23). Target N-myc and control β2-microglobulin gene sequences were cloned in the same reaction using the following gene-specific oligonucleotide primers: (a) 5'-GACCCACAGGCCCTAGTC-3' (N-myc, forward primer); (b) 5'-GTGAGAAGGACATCGTT-3' (N-myc, reverse primer); (c) 5'-AAGGATCTCGGACCTACCT-3' (BIN1, forward primer); (d) 5'-CGGTGTGGCAATGCGTGACTT-3' (BIN1, reverse primer); (e) 5'-ACCCCACTGAAAGATGA-3' (β2-microglobulin, forward primer); and (f) 5'-ATCTTCACACTCATGATG-3' (β2-microglobulin, reverse primer).

The expected sizes of the PCR products amplified using these sets of primers are 240 (N-myc), 242 (BIN1), and 120 (β2-microglobulin) bp. Aliquots of cDNA corresponding to 50 ng of RNA were subjected to PCR in a final volume of 25 μl using 1 unit of AmpliTaq Gold Polymerase (Perkin-Elmer, Norwalk, CT). An initial denaturation of 9 min at 94°C was followed by 32 cycles (N-myc) or 36 cycles (BIN1) of a 30-s denaturing step at 94°C, a 30-s annealing step at 57°C and a 30-s extension step at 72°C. Finally, an additional 7-min extension at 72°C was performed. The method of choosing PCR conditions has been described previously (8). All PCR assays of the neuroblastoma clinical samples were performed simultaneously with cDNA from neuroblastoma cell line RT-BM-1 (24) and its chemically differentiated cells as references. After the PCR, 8-μl aliquots of the PCR reaction mixture were subjected to electrophoresis on 2.5% agarose gels. Analyses of c-src and trk A expression by quantitative RNA-PCR were performed as described elsewhere (8).

**Estimation of Gene Amplification and Expression.** To determine N-myc gene copy number in each tumor, Southern blot hybridizations using a N-myc gene probe and an internal marker Udh gene were performed (15). Human placental DNA or DNA extracted from IMR32 neuroblastoma cells was used to detect a single copy or amplified copies of N-myc, respectively. Signals from Southern blottings were measured using a Bio-imaging Analyzer (BAS 2000; Fujix, Tokyo, Japan). Signals of the Udh gene were used to normalize DNA concentration. The PCR products in gels containing a DNA-staining solution, SYBR Green, were visualized by UV transillumination and recorded as digital images using a Kodak Digital Science DC40 camera, and the intensity of each band was measured using the 1D Image Analysis Application program (Eastman Kodak, Rochester, NY). The ratio of the N-myc or trk A PCR band intensity to the β2-microglobulin PCR band intensity (N-myc or trk A PCR ratio) and the ratio of c-srcN2 PCR band intensity relative to that of all three c-src species (c-srcN2 PCR ratio) were calculated for all tumors. For the cumulative event-free survival analyses, the N-myc PCR ratio of each individual tumor was categorized as high or low by subdividing around the median PCR ratio obtained from the tumor samples analyzed for survival. For multivariate analyses of proto-oncogene expression and N-myc gene amplification, the absolute value of the PCR ratio was used.

**Statistical Analysis.** The Mann-Whitney U test was used to evaluate the significance of the expression of the proto-oncogenes in the tumors of the three groups. The probability of the event-free survival of the patients was calculated by the product limit method of Kaplan and Meier and compared using the log-rank test. The multivariate analyses were performed using Cox’s proportional hazards regression model.

**RESULTS**

The event-free survival of the patients in each group is shown by Kaplan and Meier curves in Fig. 1. The expression of
c-src, trk A, and N-myc in the 70 primary neuroblastomas was analyzed by quantitative RNA PCR using β2-microglobulin as an internal control. Representative results are shown in Fig. 2.

The PCR ratio of N-myc to β2-microglobulin was calculated for all tumors, and the expression levels were plotted (Fig. 3). Overexpression of N-myc was observed frequently in N-myc-amplified tumors (group 3), but not all tumors in group 3 expressed enhanced levels of N-myc. In group 3, 12 of 15 patients died of disease or had recurrent disease, and 3 patients with tumors expressing N-myc at relatively high levels remained event-free for 49–115 months after diagnosis. Unexpectedly, N-myc expression of the tumors in group 1 was generally higher than that in group 2, where an unfavorable outcome was observed in six patients with tumors expressing relatively low levels of N-myc. One screening-positive tumor in group 1 overexpressed N-myc at a level comparable to the mean level of N-myc expression in N-myc-amplified tumors.

The PCR ratio of c-srcN2 expression relative to that of all three c-src species and the PCR ratio of trk A to β2-microglobulin were also examined. Fig. 4 shows the mean levels of expression of the three proto-oncogenes in each group. c-srcN2 expression differed significantly among the groups in the following order: group 1 (ratio  = 0.25 ± 0.01) > group 2 (ratio  = 0.17 ± 0.02) > group 3 [ratio  = 0.07 ± 0.01 (groups 1 and 2,  = 0.0034; groups 2 and 3,  = 0.0022; groups 1 and 3;  < 0.0001)]. trk A expression was nearly identical in groups 1 (ratio  = 0.76 ± 0.04) and 2 (ratio  = 0.69 ± 0.10) and was significantly reduced in group 3 [ratio  = 0.05 ± 0.02 (groups 1 and 3,  < 0.0001; groups 2 and 3,  < 0.0001)]. N-myc expression in group 1 (ratio  = 0.47 ± 0.04) was significantly higher than that in group 2 [ratio  = 0.24 ± 0.04 (groups 1 and 2,  = 0.0035), and N-myc expression in group 3 (1.42 ± 0.21) was even higher (groups 1 and 3,  < 0.0001; groups 2 and 3,  < 0.0001).

Multivariate analyses of the prognostic value of c-srcN2 and trk A were performed to examine the influence of N-myc gene amplification. High levels of expression of c-srcN2 and trk A were significantly correlated with the event-free survival independent of N-myc amplification (Table 2).

The level of expression of N-myc in each individual tumor was classified as high or low in relation to the mean PCR ratio calculated for all tumors (Fig. 5). The cumulative event-free survival data obtained by the method of Kaplan and Meier
indicated a significantly better prognosis for patients with tumors expressing low levels of \( N\text{-myc} \) \((\chi^2 = 4.694; P = 0.0303)\). However, when multivariate analysis was performed, the prognostic value of \( N\text{-myc} \) expression proved to be dependent on \( N\text{-myc} \) gene amplification (Table 2). In fact, when the \( N\text{-myc} \)-amplified tumors were excluded from the analysis, the patients with tumors expressing \( N\text{-myc} \) at high levels had an excellent outcome due to a high population of screening-positive tumors expressing higher levels of \( N\text{-myc} \) [high-level \( N\text{-myc} \) expression \((n = 21)\), event-free survival at 7 years = 100%; low-level \( N\text{-myc} \) expression \((n = 34)\), event-free survival at 7 years = 79.7%; data not shown]. When only the tumors found by clinical symptom(s) were analyzed for the prognostic value of \( N\text{-myc} \) expression, the Kaplan and Meier curve of event-free survival indicated that the outcome of the patients with tumors expressing high levels of \( N\text{-myc} \) tended to be worse than that of patients with tumors expressing low levels of \( N\text{-myc} \). However, this relationship was not significant [high-level \( N\text{-myc} \) expression \((n = 12)\), event-free survival at 7 years = 25.0%; low-level \( N\text{-myc} \) expression \((n = 19)\), event-free survival at 7 years = 48.1%; \( \chi^2 = 3.779; P = 0.0519 \)].

To examine whether the higher expression of \( N\text{-myc} \) in group 1 was a consequence of early detection of the screened tumors or a biological characteristic of the tumors themselves, we analyzed the relationship between the level of \( N\text{-myc} \) expression and the patient’s age at diagnosis in groups 1 and 2. The early detected tumors had a tendency to express higher levels of \( N\text{-myc} \), but this tendency was not significant \((r = -0.243; P = 0.0734; \text{data not shown})\).

\( \text{BIN1} \), a novel putative tumor suppressor gene interacting with the \( \text{myc} \) oncprotein, was expressed in all 70 tumors analyzed. The PCR ratio of \( \text{BIN1} \) was \( 1.19 \pm 0.03 \) in group 1, \( 1.15 \pm 0.08 \) in group 2, and \( 1.11 \pm 0.10 \) in group 3. There was no relationship between the level of expression of \( \text{BIN1} \) and biological or clinical feature of the tumors.

Finally, the relationship between the PCR ratios of \( c\text{-srcN2} \) expression and \( N\text{-myc} \) expression for each sample in group 1 was analyzed because enhanced expression of \( c\text{-srcN2} \) and \( N\text{-myc} \) was characteristic of screening-positive tumors. However, no significant correlation between the expression of these two genes was observed \((r = 0.261; P = 0.1090; \text{data not shown})\).

DISCUSSION

Neuroblastomas are biologically heterogeneous tumors, and the clinical features of the disease generally depend on the age of the patient at diagnosis (25). A mass screening system for neuroblastomas was started in Japan in 1985, with the aim of detecting unfavorable tumors early and increasing their likelihood of cure. However, the incidence of neuroblastoma increased after the introduction of the mass screening system, as compared with that in the control population. Furthermore, unfavorable genetic changes such as \( N\text{-myc} \) amplification, deletion of the short arm of chromosome 1, and diploid DNA content were rarely found in screening-positive tumors (26). These observations could mean that a large number of the screened tumors have the ability to regress spontaneously or mature and would not have been found clinically. In fact, it was recently documented that 11 of 12 screened tumors that were observed without any therapeutic intervention decreased in size (27).

In this study, we analyzed the expression of proto-oncogenes related to neuronal differentiation (\( c\text{-src}, \text{trk A}, \text{and N-myc} \)) in 39 screening-positive tumors and 31 screening-negative tumors to distinguish biological features between these subgroups. The \( \text{trk A} \) gene encodes a transmembrane tyrosine-specific protein kinase that is an essential component of the high-affinity NGF receptor and is necessary for functional NGF signal transduction (28–31). The \( \text{src} \) gene encodes a membrane-bound tyrosine-specific protein kinase that acts as a signal transduction mediator in NGF and other signal transduction pathways (32–34). Neuron-specific \( \text{src} \) isoforms have been postulated to play an important role in the functions of the \( \text{src} \) protein involved in neuronal differentiation (35, 36).

High-level expression of \( c\text{-srcN2} \) or \( \text{trk A} \) mRNA was a favorable prognostic indicator of neuroblastomas, and the value of \( c\text{-srcN2} \) or \( \text{trk A} \) expression was independent of the influence of amplification of the \( N\text{-myc} \) gene. However, our results also showed that \( c\text{-srcN2} \), but not \( \text{trk A} \), was expressed predominantly in screening-positive tumors when compared with the clinically diagnosed tumors without \( N\text{-myc} \) amplification. The differential expression pattern of the genes in these tumors may be due to the involvement of some ligand other than NGF in the differentiation of neuroblastoma cells. \( \text{src} \) has been suggested to transduce not only the NGF signal but other signals related to neural differentiation as well (33, 34). Actually, we reported previously that neuroblastoma cells in primary culture undergo neuronal differentiation in response to glial cell line-derived neurotrophic factor (37). The signal of \( \text{ret} \), the tyrosine kinase receptor of glial cell line-derived neurotrophic factor, was demonstrated to be mediated by activated \( \text{src} \) (38). Although a signal specific to the cell death and maturation of screened neuroblastomas is still unknown, it is possible that \( \text{src} \) acts as a mediator in such signaling.

\( N\text{-myc} \) has been thought to be profoundly involved in oncogenesis and malignant progression of neuroblastomas (16, 39, 40). However, the clinical significance of \( N\text{-myc} \) mRNA expression as a prognostic indicator remains controversial (3, 15, 19–21). Our present results showed that enhanced expres-
mission of N-myc mRNA was a significantly unfavorable marker for the affected patients, but it was not independent of the influence of N-myc gene amplification. Furthermore, no significant prognostic value was observed for N-myc expression when the cases were limited to clinically detected tumors, although this observation has the limitation that the number of cases was relatively low. One unexpected observation in the present study was enhanced expression of N-myc mRNA in the screening-positive neuroblastomas compared with the clinical tumors with a single copy of N-myc.

It has been reported that infantile neuroblastomas expressed relatively high levels of N-myc mRNA (3, 15, 18). One possible explanation for higher expression of N-myc in the screening-positive tumors is that N-myc is generally expressed at high levels in neuroblastomas detected at an early age, independently of the biology of the clinically silent neuroblastomas screened. However, we found no significant correlation between the age of the patient and the expression level of N-myc. This finding indicates that high levels of expression of N-myc could be a biological characteristic of screening-positive neuroblastomas.

To test the possibility that other factors participate in N-myc-mediated cell growth and differentiation in neuroblastoma cells, we focused on BIN1 mRNA expression in neuroblastomas. BIN1 is a novel putative tumor suppressor that was identified through its interaction with myc oncoprotein (41). It was reported that the ectopic expression of BIN1 inhibited the growth of cancer cells lacking endogenous BIN1 expression, and overexpression of BIN1 induced a reduction of cell growth and apoptosis in neuroblastoma cells (42). On the assumption that BIN1 is overexpressed and suppresses the N-myc function in screened neuroblastomas, we analyzed BIN1 expression in the 70 tumors. However, expression of BIN1 was ubiquitous in the neuroblastomas, and no difference in the expression level was observed among the three groups.

Recently, a function of N-myc not related to cell proliferation or retaining the undifferentiated phenotype was documented. When ectopic expression of N-myc was enforced in neuroblastoma cells, apoptosis was induced when cells were treated with IFN-γ or with a cytotoxic drug (43, 44). Fulda et al. (44) suggested that N-myc-amplified tumors acquire treatment resistance not by overexpression of N-myc but by additional dysfunction in apoptosis signaling pathways. We also observed cell death in neuroblastoma cells without N-myc gene amplification infected with recombinant adenosine vector expressing N-myc protein at very high levels. These findings might mean that enhanced expression of N-myc could function as a death signal in concert with other biological factors in a subset of neuroblastomas. Actually, when only tumors without N-myc amplification were analyzed, all of the patients with tumors expressing N-myc at high levels survived without adverse events. We speculate that the role of N-myc in screening-positive neuroblastomas may be different from that in aggressive tumors.

The present study demonstrated that screening-positive neuroblastomas preferentially expressed c-srcN2 at a high ratio and N-myc at an enhanced level, regardless of nonamplification of the N-myc gene. The prognostic value of N-myc mRNA expression was dependent on the amplification status of the gene. The precise biological function of N-myc in screened neuroblastomas remains unclear but might be related to the death of the neuroblastoma cells.

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