Expression of N-myc and c-src Protooncogenes Correlating to the Undifferentiated Phenotype and Prognosis of Primary Neuroblastomas

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ABSTRACT

Genomic amplification of the N-myc protooncogene in neuroblastomas correctly predicts poor outcome for the patients. However, the prognosis for neuroblastomas with a single copy of N-myc is also poor in cases diagnosed after 1 year of age but good in infantile cases. To elucidate the different prognoses depending upon the age of the patients with neuroblastoma, we performed an analysis of the expression of protooncogenes related to neural differentiation. We examined the genomic amplification of N-myc in 26 specimens of neuroblastomas and further analyzed 22 of the 26 cases for expression of N-myc, c-src, c-Ha-ras, and c-fos. Consequently, we observed frequent overexpression of N-myc in undifferentiated neuroblastomas and enhanced expression of c-src and c-Ha-ras in infantile neuroblastomas with favorable prognosis and in neuroblastomas differentiated by chemotherapy. These findings suggest that c-src and c-Ha-ras play important roles in the neural differentiation of infantile neuroblastomas.

INTRODUCTION

Neuroblastoma, the origin of which is the neural crest, is the most common neoplasm in solid cancers of children. The prognosis of patients with neuroblastoma widely depends on the age of the patient at the time of diagnosis (1). Regardless of the spread of the disease, neuroblastomas diagnosed before 1 year of age are expected to show good response to treatments. Although the reason for this phenomenon has not been clarified sufficiently, it is possible to speculate that predisposition of infantile neuroblastomas to mature into benign phenotypes might account for a favorable prognosis. Neuroblastomas are known to differentiate in response to chemotherapy or spontaneously (2). Ganglioneuromas and well-differentiated ganglioneuroblastomas, which are mature phenotypes of neuroblastomas, are incidentally found in older children and are often gross tumors beyond the midline of the body. These observations strongly suggest the ability of neuroblastomas to differentiate in vitro.

N-myc protooncogene plays important roles in the development of the neural tissues (3–5). The genomic amplification of the N-myc gene correlates with the rapid progression of the disease and the poor diagnosis (6, 7). A single copy of N-myc, however, does not result in a good clinical course in advanced neuroblastomas diagnosed after 1 year of age. Protooncogenes other than N-myc are also known to be involved in maturation of the neural tissues. The protooncogenes, c-src and c-Ha-ras, are known to associate with neural differentiation (8–11). Augmented expression of c-Ha-ras p21 was observed in neuroblastomas with favorable prognosis (12). In various cell lines including neuroblastoma cell lines, c-fos protooncogene is activated rapidly and transiently when cells are induced to differentiation (13–18).

Although these protooncogenes have been shown to play important roles in the differentiation of neuroblastoma cell lines in vitro, their expression in primary neuroblastoma tissues has not yet been documented. If neuroblastomas diagnosed at less than 1 year of age have an ability to differentiate, these genes may be expressed differently in accordance with the age of the patients or the degree of maturation of tumors. We therefore examined the genomic amplification of N-myc in 26 specimens of neuroblastomas and further analyzed 22 of the 26 cases for the mRNA expression of N-myc, c-src, c-Ha-ras, and c-fos.

MATERIALS AND METHODS

Tumor Specimens. All patients with neuroblastoma analyzed were treated at the Chiba University Hospital from 1985 to 1989 and were followed up for over 1 year. High-molecular-weight cellular DNAs were obtained from 26 cases of neuroblastomas. Undegraded and sufficient total RNAs were extracted from 22 of the 26 cases. These 22 cases consist of 3 groups: (a) 5 cases of naturally differentiated neuroblastomas, 3 ganglioneuromas, and 2 well-differentiated ganglioneuroblastomas; (b) 8 cases of neuroblastomas less than 1 year old, 7 of them not treated with chemotherapy prior to analysis; (c) 9 cases of neuroblastomas over 1 year of age, 7 of them treated with chemotherapy prior to analysis. Data on the age, sex, and outcome of the patients and stage and histology of the tumors are summarized in Table 1. The tumor tissues were obtained at the time of operation and were immediately frozen and stored in a liquid nitrogen container until tested. These specimens were confirmed to consist of tumor cells by pathological examinations before gene analyses.

Cell Lines. The neuroblastoma cell line IMR32 (19) was used as a control for N-myc amplification. The neuroblastoma cell lines SK-N-NSH (20) and c-NBI1 and cervical carcinoma cell line HeLa S3 were used as controls for the mRNA expression of N-myc, c-src, c-Ha-ras, and c-fos.

Southern Blot Hybridization. DNA was isolated from tumor tissues by proteolytic digestion with proteinase K and phenol/chloroform extraction. Five /g of DNA were digested with restriction endonuclease EcoRI, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The BamHI-EcoRI fragment of the 2nd exon of N-myc DNA (21, 22) was labeled with [32P]dCTP by nick translation and used as a probe. Hybridization was performed under highly stringent conditions, and autoradiography was performed at -80°C, as described previously (23). The UDh probe (24), which hybridizes to a 3.3-kilobase EcoRI fragment of human genomic DNA, was used to normalize the DNA amount.

Northern Blot Hybridization. Total cellular RNA was extracted by the guanidinium isothiocyanate/CSCl method. Ten /g of RNA were separated on a 1.0% agarose-6% formaldehyde gel and transferred to a nitrocellulose filter. The BandH-EcoRI fragment of the 3rd exon of N-myc RNA (21, 22) was labeled with [32P]dCTP by nick translation and used as a probe. Hybridization was performed under highly stringent conditions, and autoradiography was performed at -80°C, as described previously (25). The KpiI-Sacl fragment of the c-Ha-ras 2-4 exon (26), or c-fos (27) under the same condition as Southern blot analyses. Finally, rehybridization with mouse β-actin complementary DNA (28) was carried out to normalize the RNA amount.

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Estimation of Gene Amplification and Expression. Autoradiograms of Southern and Northern blotting were measured quantitatively by using a densitometric scanner (Hoefer Scientific Instruments; GS-300). To determine the copy number of N-myc in each sample, human placental DNA was used for the detection of a single copy of N-myc, and DNA from IMR32 for amplified copies of N-myc. IMR32 was confirmed to have 25-fold amplification of N-myc by serial dilution of its DNA (data not shown). Errors of DNA amount loaded onto gels and transfer processes were corrected by the signal intensity of the UDh probe. In Northern blot analysis, all the samples of RNA were analyzed simultaneously with RNA from HeLa S3, SK-N-SH, and c-NBI. The SK-N-SH cells contain a single copy of N-myc and express a low level of N-myc mRNA (29, 30). We carried out autoradiography until the signal of N-myc for SK-N-SH appeared. The c-NBI cells were confirmed to contain 150-fold amplified N-myc and express high levels of N-myc mRNA equal to that of IMR32 (data not shown). The RNA expression of the protooncogenes in each sample was determined by comparison with the signal intensity of SK-N-SH as a value of 1. The ß-actin probe was also used as an internal marker.

RESULTS

Genomic Amplification of N-myc. A total of 26 samples of neuroblastomas was examined for N-myc amplification. Amplification of N-myc of more than 10 copies was found in 4 tumors, and 3-fold amplification of N-myc was found in 1 tumor (Fig. 1). Amplifications of N-myc of more than 10 copies were found in cases of stage IV, and the patients with these tumors died within 25 months. The tumor with 3-fold amplification of N-myc was at stage II, and the patient with this tumor is alive well over 3 years after the operation. The correlation between N-myc amplification and clinical profiles is shown in Fig. 2. This figure indicates that the prognosis of advanced neuroblastoma over 1 year of age is not favorable even if the tumor has a single copy of N-myc and that the prognosis of infantile neuroblastoma under 1 year of age is good even if the tumor is at the advanced stage. Expression of Protooncogenes. Representative results of Northern blottings for N-myc, c-src, c-Ha-ras, and c-fos protooncogenes in 22 specimens are shown in Fig. 3, and clinical profiles and results of gene analysis are summarized in Table 1.

Cases 1 to 5 were diagnosed histologically as ganglioneuroma (1 to 3) and well-differentiated ganglioneuroblastoma (4 and 5), which are naturally differentiated phenotypes of neuroblastomas and are thought to be clinically benign tumors. In these tumors, mRNA of N-myc was not detectable, and c-src and c-Ha-ras expression was at low levels equivalent to those of the control cell lines.

Ha-ras expression was at low levels equivalent to those of the control cell lines.

DISCUSSION

Expression of N-myc gene is regulated during the development of the brain, retina, lung, and kidney of the embryo (3–5). During organogenesis of the fetal brain, N-myc is expressed only in undifferentiated neuronal cells which have already completed cell division, and its expression declines with the onset of neural differentiation (5). The neuroblasts leave the neural crest, migrate while proliferating, and invade the fetal adrenal or sympathetic ganglion. To retain an undifferentiated form in this process, the expression of N-myc is thought to be essential. The neuroblasts in the adrenal no longer express N-myc (31), or sympathetic ganglion. To retain an undifferentiated form in this process, the expression of N-myc is thought to be essential.
Fig. 3. Representative results of Northern blot analysis for expression of N-myc [2.9 kilobases (Kb)], c-src [4.0 kilobases], c-Ha-ras [1.4 kilobases], and c-fos [2.2 kilobases]. The number at the top of each lane indicates the case number of the patients shown in Table 1. All the samples were analyzed simultaneously with RNA from HeLa S3, SK-N-SH, and c-NBl. β-Actin probe was used as an internal marker.

Table 1 Clinical profiles and amplification and expression of protooncogenes

<table>
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<th>Sex</th>
<th>Stage</th>
<th>Origin</th>
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<th>c-src</th>
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<td>IV</td>
<td>Med</td>
<td>2b</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>NED (47)</td>
</tr>
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</table>

* Origin of the tumor: Med, mediastinum; Ad, adrenal gland; Ret, retroperitoneum.
* Histology of the tumor: 1, ganglioneuroma; 2a, ganglioneuroblastoma (GNB), well differentiated type; 2b, GNB, composite type; 2c, GNB, poorly differentiated type; 3a, neuroblastoma, rosette-fibrillary type; 3b, neuroblastoma, round cell type.
* Clinical course: NED, no evidence of disease; REC, recurrence; DOD, death of disease. Numbers in parentheses, months after diagnosis.
* Detected by mass screening test.
* Chemotherapy was given before the analysis.

The overexpression of the N-myc was observed frequently in undifferentiated neuroblastomas, and naturally or chemically differentiated neuroblastomas were shown not to express N-myc at all. This result concurs with observations previously reported by others (36, 37) and leads us to speculate that neuroblastoma arises from immature cells which have been arrested at the developmentally undifferentiated stage and that the overexpressed N-myc might play a critical role in maintaining the malignant phenotype.

The genomic amplification of N-myc in neuroblastomas correlates with the rapid progression of the disease and its poor prognosis (6, 7). This correlation has been sustained by other reports (38, 39), as well as our results. However, the biological mechanism of this phenomenon remains to be elucidated. Our results and other reports (36, 37) indicate that tumors with N-myc amplification generally express a greater amount of N-myc transcripts than those with unamplified N-myc, but the degree of the expression does not correlate with the extent of the amplification. The infantile neuroblastomas in this study expressed high levels of N-myc and showed a good clinical course in all cases, except case 12 with N-myc amplification. Thus, it is unlikely that enhanced expression of N-myc due to gene amplification solely accounts for the poor prognosis for neuroblastomas with N-myc amplification. In the specimens obtained after aggressive chemotherapy, N-myc amplified tumors were found to express high levels of N-myc. We analyzed additional samples taken after administration of anticancer drugs from cases 12 and 14, and persisting expressions of N-myc at high levels were observed (data not shown). These results may be interpreted as indicating that the genomic amplification of N-myc might be implicated in the uncontrolled regulation of transcription which is never turned off even though aggressive chemotherapy is given. The difference in prognosis between neuroblastomas diagnosed before 1 year of age and those after 1 year of age is not explained by the degree of N-myc expression.

bryonal carcinoma cell lines has also been reported (32–35). In this study, overexpression of the N-myc was observed frequently in undifferentiated neuroblastomas, and naturally or chemically differentiated neuroblastomas were shown not to express N-myc at all. This result concurs with observations previously reported by others (36, 37) and leads us to speculate that neuroblastoma arises from immature cells which have been arrested at the developmentally undifferentiated stage and that the overexpressed N-myc might play a critical role in maintaining the malignant phenotype.

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were significantly higher than those in patients with poor prognosis (P < 0.01) with favorable prognosis. These observations strongly suggest that c-src and c-Ha-ras p21 in neuroblastomas and high expression of c-src in the fetal brain and retina (8-11). The expression of c-src in neuroblastoma has been reported by restriction fragment length polymorphism analysis (51). The c-fos is the putative gene which is involved in this gene aberration because c-fos locates on 14q21-31 (52). However, our observation of frequent overexpression of the c-fos may indicate that there is no gross deletion or rearrangement of the c-fos gene in neuroblastomas.

We found frequent overexpression of N-myc in undifferentiated neuroblastomas and enhanced expression of c-src and c-Ha-ras in infantile neuroblastomas with favorable prognosis. It seems that expression of N-myc plays the critical role in the undifferentiated appearance of neuroblastomas. With regard to the different prognoses depending upon age, we interpret our results as indicating that infantile neuroblastomas continue intrinsic expression of genes inducing neural differentiation, such as c-src and c-Ha-ras, by which regression of the tumors is prompted. The poor prognosis of neuroblastomas after 1 year of age could be explained by the decreased expression of these genes in the tumors of patients over 1 year of age.

This study was designed to assess expression of the protooncogenes related to neural differentiation. Recent studies on c-src in a neuroblastoma cell line indicated elevated levels of tyrosine kinase activity of c-src pp60 during neural differentiation (41). It is also reported that c-src protooncogenes are expressed as at least three distinct forms due to alternative splicing in neuronal tissues, and this alternative splicing of the c-src gene is suggested to be implicated in the regulation of neural differentiation (53). Further analysis is necessary to understand the biological properties of neuroblastoma, such as the tendency to differentiate particularly in infantile cases.

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