Detection of N-myc Oncogene Expression in Human Neuroblastoma by in Situ Hybridization and Blot Analysis: Relationship to Clinical Outcome

Eileen F. Grady-Leopardi, Manfred Schwab, Arthur R. Ablin, and Werner Rosenau

Departments of Pathology [E. F. G.-L., W. R.J, Pediatrics [A. R. A.], and the Hooper Foundation (M. S.), School of Medicine, University of California, San Francisco, California 94143

**ABSTRACT**

We studied N-myc oncogene expression in 13 human neuroectodermal tumors and one teratoma by in situ hybridization. In four of six neuroblastomas, there was increased N-myc expression (15 to 49% of the cells). Many of the primitive neuroblastic cells had an increase of N-myc RNA not observed in the larger, more differentiated cells. Two neuroblastomas matured to ganglioneuroblastomas; no biopsies performed during this progression expressed increased N-myc RNA. Three ganglioneuroblastosmas, two tumors presenting as ganglioneuromas, a cerebral neuroectodermal tumor, a neurofibrosarcoma, and the teratoma did not have increased N-myc expression. The data obtained by in situ hybridization correlated well with data obtained by blot analysis. Neuroblastomas/ganglioneuroblastomas with a favorable course did not have appreciable elevation of N-myc expression over 10 to 77 mo of follow-up; thus N-myc may not be involved in the maintenance of the neoplastic state. However, such tumors with a fatal outcome 2 to 14 mo after diagnosis usually had elevated N-myc expression. These findings suggest a relationship between elevated levels of N-myc RNA and poor prognosis.

**INTRODUCTION**

Amplification and translocation of the N-myc oncogene have been detected as a frequent feature of human neuroblastoma cells within double minutes or homogeneously staining regions within macrochromosomes (1–4). Amplification and elevated expression of N-myc have also been reported in 2 additional neuroectodermal tumors, retinoblastoma (5) and small cell (oat cell) carcinoma of the lung (6). Previously, we found elevated N-myc expression in 5 of 6 neuroblastoma cell lines and 2 of 2 neuroblastomas (4).

To determine the relationship between expression of N-myc and prognosis, we studied the expression of N-myc RNA in all recent neuroblastic tumors from this institution. Tumors were studied by in situ hybridization using a 125I-labeled N-myc probe hybridized to tissue sections, followed by autoradiography, as well as by blot analysis to investigate N-myc DNA amplification and RNA elevation. These findings on N-myc were related to the clinical course and outcome. Increased levels of N-myc RNA were associated with a fatal outcome, and low levels were found in tumors with a more favorable prognosis.

**MATERIALS AND METHODS**

**Tissues**

The tumors studied were consecutive neuroectodermal tumors and a teratoma (except for Case 6). For in situ hybridization, fresh tissue samples were divided, and a portion was frozen immediately in liquid nitrogen. The remainder of the tissue was fixed in 10% formalin and embedded in paraffin. In Case 6 (a neuroblastoma, Stage WS) and in Tissues cell) carcinoma of the lung (6). Previously, we found elevated N-myc levels in 5 of 6 neuroblastoma cell lines and 2 of 2 neuroblastomas (4).

**RESULTS**

Four neuroblastomas (Patients 1 to 4) hybridized in situ with a 125I-labeled N-myc probe had tumor cells with clusters of autoradiographic grains (>6 grains/cell) present over many, but not all, of the small undifferentiated tumor cells, indicating increased N-myc RNA (Fig. 1; Table 1). Thus, N-myc expression among cells of the same level of differentiation was heterogeneous. Larger cells in these neuroblastomas with early differentiation did not have an increase in N-myc RNA. All 4 patients with N-myc RNA elevation died of disseminated disease 2 to 14 mo after diagnosis, despite treatment. Snap-frozen or paraffin-embedded tissues were both positive for increased expression of N-myc, but the results obtained by in situ hybridization with snap-frozen tissue were more striking. Control duplicate sections treated with RNase prior to hybridization analysis, fresh or frozen tissue was used. Blot analysis results on Patients 2 (J. T.) and 3 (A. R.) were previously reported (4).

**Probe Preparation**

The N-myc DNA is a cloned 2.0-kilobase pair EcoRI fragment (Nb-6) derived from a human neuroblastoma cell line (1, 4). The probes were synthesized by reverse transcription utilizing random primer prepared from salmon sperm DNA (7) with 125I-labeled dCTP or [32P]-dCTP.

**In Situ Hybridization**

Snap-frozen tissues were sectioned, fixed immediately in methyl alcohol, and pretreated as described by Haas (8, 9). Formalin-fixed, paraffin-embedded tissue sections were pretreated as described by Blum (10). Tissue sections were coated with 5 μl of single-stranded 125I-labeled N-myc DNA (1 ng/μl; 1 to 5 x 106 dpm/μg), and hybridization and autoradiography were conducted as previously described (8–10). Neuroblastomas with N-myc amplification or RNA elevation known from blot analysis yielded many cells with clusters of more than 6 grains/cell. After RNase digestion, only scattered grains (<3 grains/cell) were evident, and these were considered as background. Tissues known to be negative by blot analysis for N-myc expression had a similar pattern of widely scattered grains without clustering. Most cells had no grains, a few cells had 1 or 2 grains, and 3 grains per cell were extremely exceptional. Therefore, cells with more than 6 grains were recognized as being positive for increased N-myc RNA expression. These known positive and negative controls were routinely included in the determinate experiments where 200 cell in coded slides were counted, and the percentage of cells with greater than 6 grains was recorded. Tissues were studied in 3 separate experiments using different lots of N-myc probe.

**Biochemical Analysis**

RNA. Total RNA was isolated by the guanidine-isothiocyanate procedure (11) followed by cesium chloride centrifugation (12). Polyadenylated RNA was purified by oligo(dT)-cellulose chromatography, transferred to nitrocellulose filter paper, and hybridized to 32P-labeled N-myc (7).

DNA. DNA was isolated, digested with restriction endonuclease EcoRI, fractionated on agarose gels, and transferred to nitrocellulose filter paper to which 32P-labeled N-myc was then hybridized (7).
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Fig. 1. Human undifferentiated neuroblastoma (Patient 1) with numerous clusters of autoradiographic grains, indicating increased levels of N-myc RNA. Note heterogeneity of numbers of grains per cell. In situ hybridization with N-myc probe of snap-frozen tissue. H & E, x 750.

Fig. 2. A replicate section of the neuroblastoma, Patient 1, treated with RNase followed by N-myc probe. There are scattered grains at background level, similar to those obtained with blank slides. Thus, these random grains are not indicative of increased levels of N-myc RNA, and no tight clusters of grains are seen. In situ hybridization. H & E, x 750.

Fig. 3. Neuroblastoma of adrenal gland (Patient 1). Most of the cells are primitive neuroblastic elements, but a few (arrow) have features of slight differentiation with the appearance of distinct nucleoli. These latter cells do not express N-myc when examined by in situ hybridization (not shown). Formalin-fixed, paraffin-embedded, H & E, x 750.

Fig. 4. Human ganglioneuroma with scattered grains at background level. The cell in the center is a mature neoplastic ganglion cell, with shrinkage artifact encountered in frozen sections. The other elements are mature neoplastic Schwann cells. The ganglioneuroma matured from a biopsy-proven neuroblastoma. Patient 5, Biopsy 2, in situ hybridization with N-myc probe. H & E, x 750.

showed few grains scattered randomly over the tissue (Fig. 2). Tissue known to be negative for N-myc elevation (normal intestine, normal lymph node) showed the same random scattering of grains over the sections with or without RNase treatment, as did tumor sections treated with unrelated probes (H/ras, fos, src, c-myc), with buffer only, or a blank slide treated with N-myc probe. Blot analysis of N-myc RNA in Patients 1 to 3 revealed good correlation with in situ hybridization (Table 1). A paraffin section of a neuroblastoma stained with hematoxylin-eosin is illustrated (Fig. 3).

A neuroblastoma diagnosed in a 16-mo-old child (Patient 5), which after treatment matured to ganglioneuroma, showed no increase in N-myc RNA by in situ hybridization in either the early neuroblastomatous or the later ganglioneuromatous nodules from 4 biopsies obtained over 3 yr (Table 1). The first biopsy (1 wk of age) and the second (6 mo of age) had neuroblastomatous patterns with an increase in the number and size of the nodules during the interval. The third biopsy (18 mo of age) and the fourth (3 yr of age) showed maturation of the tumor to ganglioneuroma with a marked decrease in the number and size of the nodules. The left adrenal, removed at 3 yr of age, had a calcified scar, the presumed involuted primary site. Patient 5 is alive without tumor, while Patient 6 is well but has a few stationary nodules, 2 of which proved to be ganglioneuromas on biopsy. These patients have done well 37 and 77 mo after treatment.

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A ganglioneuroblastoma (Patient 7) did not show elevation of N-myc DNA copy number or N-myc RNA in one metastatic nodule available for examination (Table 1). This patient subsequently succumbed to the disease. By in situ hybridization, 2 additional ganglioneuroblastomas (Patients 8 and 9) had few cells (<2%) with clusters of more than 6 grains, indicative of N-myc RNA increase. Blot analysis showed no increase in N-myc DNA copy number in Patients 8 and 9 and no elevation of N-myc RNA in Patient 8. Two cases presenting initially as ganglioneuromas (Patients 10 and 11) did not have an increase in N-myc RNA by in situ hybridization or in DNA copy number by blot analysis (Table 1).

As controls, a primitive cerebral neuroectodermal tumor (Patient 12) and a neurofibrosarcoma (Patient 13) were studied, neither of which showed increased N-myc RNA or DNA (Table 1). Patient 12 died of the brain tumor, while Patient 13 is alive and without known tumor 18 mo after surgical excision. Patient 14, with a sacral teratoma removed shortly after birth without subsequent recurrence, did not exhibit elevation of N-myc RNA (Table 1).

**DISCUSSION**

These studies show that N-myc DNA copy number or the level of N-myc RNA is elevated in certain cases of neuroblastoma. There is good correlation between results obtained by in situ hybridization in tissue sections and those determined by blot analysis on tissue extracts (Patients 1 to 3). In one biopsy (Patient 4), 15% of the cells had increased levels of N-myc RNA as determined by in situ hybridization, but blot analysis did not reveal DNA amplification. Here, increased N-myc RNA may be due to increased transcription alone, a phenomenon described for N-myc retinoblastoma (5). While few cells (<2%) seen in Cases 8 and 9 had elevated N-myc RNA by in situ hybridization, no elevation of DNA copy number and/or RNA elevation was demonstrated by blot analysis. RNA or DNA elevation in such a small percentage of tumor cells would not be detectable by blot analysis. Thus, in situ hybridization supplements blot analysis and detects small numbers of cells with increased gene expression in a largely negative population.

Furthermore, only small samples are required for study by this method, and it can identify particular cells that express a gene in a heterogeneous tissue population. In addition, it permits retrospective studies of gene expression on paraffin tissue blocks of cases where the clinical course and response to treatment are known; paraffin blocks stored for up to 10 yr have been used (13).

In 4 of 5 cases of neuroblastoma/ganglioneuroblastoma with a fatal outcome, increased N-myc expression was demonstrated by in situ hybridization. One case (Patient 5) did not have increased levels of N-myc RNA; this finding is based on one sample from a single metastasis, and there may be variable expression of N-myc in different metastatic sites. Alternately, increased N-myc expression may not be found in all tumors with a fatal outcome. Undifferentiated neuroblastoma cells within a tumor nodule show different levels of N-myc RNA. This variation may be related to the cell cycle, clonal variation, or other factors. Increased levels of N-myc RNA were not observed in neuroblastomas showing early differentiation within the same tumor or in mature ganglioneuromas. Brodeur et al. found amplification of N-myc in 24 of 48 patients with Stage III or IV neuroblastoma, but none in patients with Stage I or II neuroblastoma using blot analysis (3). Using the same method, Seeger et al. detected N-myc genomic amplification in 2 of 16 patients with Stage II neuroblastoma, 13 of 20 with Stage III disease, and 19 of 40 patients with Stage IV disease, and they suggested that amplification has a key role in determining aggressiveness of the neoplasm. In our studies, all tumors were examined by in situ hybridization and results were correlated with blot analysis in 12 of 14 cases. Our results by blot analysis for DNA copy number agreed essentially with the findings of Brodeur et al. and Seeger et al. in that amplified N-myc was obtained from Stage IV tumors.

An untreated case of neuroblastoma, Stage IVS (Patient 6), was followed by serial biopsy from age 1 wk to 3 yr. At the time of birth, the patient had many s.c. metastases with an immature neuroblastoma pattern. Later, many of the nodules spontaneously disappeared, and biopsy of others showed maturation to ganglioneuroma. No elevation of N-myc expression was found in any of the biopsies. The lack of elevation of N-myc RNA may be a prognostic indicator in Stage IVS neuroblastoma.

**Table 1. N-myc expression and clinical outcome in neuroectodermal tumors and a teratoma**

Frozen sections of tumor tissue were hybridized to 125I-labeled N-myc probe and subjected to autoradiography. RNA and/or DNA was isolated from fresh or frozen tumor tissue, run on an agarose gel, blotted on nitrocellulose paper, and hybridized to a 32P-labeled N-myc probe.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor Description</th>
<th>Stage at diagnosis</th>
<th>In situ hybridization, % of cells positive</th>
<th>Blot analysis</th>
<th>DNA copy no.</th>
<th>RNA expression</th>
<th>Outcome</th>
<th>Age at diagnosis (mo)</th>
<th>Follow-up (mo)</th>
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<td>1</td>
<td>Neuroblastoma</td>
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<td>24</td>
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<td>2</td>
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<td>ND</td>
<td>ND</td>
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<td>16</td>
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<td>ND*</td>
<td>ND</td>
<td>Alive, rare stationaty nodules</td>
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<td>1</td>
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<td>Alive without tumor</td>
<td>58</td>
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<td>1</td>
<td>Dead</td>
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<td>Teratoma</td>
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<td>ND</td>
<td>Alive</td>
<td>Birth</td>
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</table>

* ND, not done.
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mas, which have a favorable outcome in 80% of the cases. Our finding of a lack of N-myc elevation in a Stage IVS neuroblastoma by in situ hybridization agrees with the results of Seeger et al. obtained by blot analysis of 5 such neoplasms (14).

Neoplastic transformation is a multistep process in which several oncogenes may act in sequence, leading to cell proliferation and then to malignant conversion (15–17). Modifications of cell growth after malignant conversion may be responsible for the considerable differences observed in the characteristics of a given type of malignant neoplasm. A highly variable clinical course and outcome of neuroblastomas are seen in different patients. Our observation, that cases of neuroblastoma/ganglioneuroblastoma with a favorable outcome usually do not show demonstrable elevation of N-myc RNA, would suggest that N-myc is not involved in the maintenance of neoplastic transformation, but that its elevation is related to a more aggressive variant of the neoplasm with a poorer prognosis. We examined 2 other neuroectodermal tumors, a highly undifferentiated cerebral neuroectodermal tumor that proved to be fatal and a neurofibrosarcoma arising from a peripheral nerve. Neither tumor had increased levels of N-myc RNA. Similar results were obtained from a pluri-potential sacral teratoma. None of the other oncogenes that we tested for by in situ hybridization (i.e., Ha-ras, fos, src, and c-myc) was expressed in any of the tumor tissues, but they were detected in other neoplasms by others with analysis of nucleic acids in tumor extracts or by immunoperoxidase staining for the oncogene product (6, 18–21).

Elevated N-myc expression is observed in many primitive human neuroblastoma cell lines (1, 2, 4). Expression of N-myc is greatly decreased prior to morphological evidence of differentiation when such lines are induced to differentiate in vitro by the addition of retinoic acid (22, 23). Arrest of cells in G1 by metabolic starvation does not depress N-myc expression. Thus, elevation of N-myc appears to be linked to an undifferentiated state associated in vivo with a more aggressive behavior. Agents that cause a decrease in N-myc expression and result in differentiation may lead to new successful therapeutic approaches to the treatment of neuroblastoma.

ACKNOWLEDGMENTS

We wish to acknowledge the helpful suggestions of Linda Stowring, Hubert Blum, and Michael Busch, and the editorial assistance of David Geller.

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