myc Gene Amplification and Expression in Primary Human Neuroblastoma

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ABSTRACT

Although N-myc amplification in neuroblastomas correlates with poor prognosis, not all neuroblastomas which fail to respond to therapy have N-myc amplification. To determine whether other modes of myc gene activation underlie progression of some neuroblastomas, 45 were analyzed for amplification of N-myc, c-myc and L-myc and 26 were studied for transcription of these oncogenes. N-myc amplification was found in 6 of 45 tumors; no tumor had amplification of c-myc or L-myc. Transcription of both N-myc and c-myc occurred in 21 of 26 neuroblastomas. No tumor without N-myc amplification had a level of N-myc expression near that of a tumor or cell line with amplification. One tumor with N-myc amplification was the only specimen with N-myc but not c-myc expression. Five samples had c-myc but not N-myc expression; all had histological features of ganglioneuroma. DNA index did not correlate with myc gene amplification or expression. It is concluded that N-myc and c-myc are commonly expressed in primary untreated neuroblastomas, but in the absence of N-myc amplification, expression of these genes does not appear to correlate with disease progression.

INTRODUCTION

Amplification of the protooncogene N-myc occurs in approximately one-third of neuroblastomas and correlates with poor prognosis (1, 2). Yet the absence of N-myc amplification does not necessarily predict a favorable outcome since a high proportion of children over 1 year of age with stage III or IV neuroblastomas eventually die from their disease (3, 4). Although nonaneuploid DNA content (5–7) and deletion of the short arm of chromosome 1 (8) have been found to correlate with advanced disease, no molecular genetic markers have been identified in the advanced neuroblastomas lacking N-myc amplification which might explain their poor outcome.

The association of N-myc amplification with poor prognosis suggests two hypotheses to explain the progression of tumors without amplification. First, N-myc is one member of a “family” of nuclear oncogenes (9), which also includes c-myc, L-myc, B-myc etc. In small cell lung carcinomas, amplification of N-myc, c-myc, or L-myc occurs in various tumors (10, 11), and c-myc amplification has been found in other neoplasms (12–15). It is therefore possible that some neuroblastomas might have amplification of one of these other myc family oncogenes. A second possibility is that some tumors might achieve high levels of myc gene expression by means other than amplification. This phenomenon has been observed in some Wilms’ tumors, for example, which do not have N-myc amplification but may have levels of N-myc expression approximating those seen in neuroblastomas with amplification (16).

In this paper we present results of the study of 45 primary untreated neuroblastomas for amplification of N-myc, c-myc, and L-myc. In addition, 26 tumors were analyzed for expression of these myc family oncogenes. The data are compared with clinical outcome of the patients, histological features of the tumors, and DNA histograms obtained by flow cytometry.

MATERIALS AND METHODS

Tumor Specimens. Tumor samples were obtained either in the operating room or the pathology laboratory, immediately frozen in liquid nitrogen, and stored at ~70°C. The neuroblastoma cell lines LAN-1 and IMR-32 were used as controls for N-myc amplification.

Pathological Examination. Portions of tumors were fixed and stained by conventional histopathological means. Samples studied for oncogene expression were examined by a pathologist for areas of differentiation and classified according to the criteria of Shimada (17). The histological analysis was performed without knowledge of the results of RNA studies.

Flow Cytometry. Nuclei were isolated and harvested from 50-μm-thick sections of formalin-fixed paraffin blocks by deparaffinization, rehydration, digestion in pepsin, disaggregation by vortex, digestion in RNase, and filtration through 50-μm nylon mesh (18). Papanicolaou-stained smears were used to assess proportion of tumor nuclei and adequacy of disaggregation. The nuclei were stained with propidium iodide and processed through a Becton-Dickinson fluorescence-activated cell sorter flow cytometer. Data on 5000 nuclei were analyzed with an EPICS multimeter data acquisition and display system single parameter computer. The proliferative compartment of the cells of the tumor samples was estimated as the percentage of cells in S phase plus G2; plus M phase by cytographic pattern.

DNA and RNA Analysis. DNA and RNA were prepared as described previously (19). DNA samples were digested with EcoRI and quantified by DAPI (4'-6-diamidino-2-phenylindole) fluorescence. Three-μg samples were loaded onto a 0.7% agarose gel, subjected to electrophoresis, and blotted onto Hybond-N (Amersham). Total cellular RNA was quantified by spectrophotometry and 25-μg samples were electrophoresed in formaldehyde-agarose gels and then blotted onto Biotrans (ICN) membranes.

Hybridization was carried out sequentially using N-myc (20), c-myc (21), and L-myc (10) DNA probes labeled with 32P by the random primer method (22). RNA blots were also hybridized with a chick α-tubulin sequence (23) as a control for amount of RNA loaded into the gel. Hybridizations were carried out in 50% formamide at 42°C and washed at high stringency in 0.1 x standard (1 x = 0.15 m NaCl-0.015 m sodium citrate) saline-citrate at 65°C. Autoradiography was done using X-Omat AR film and an intensifying screen.

Hybridization intensity was measured using an Ultrascan XL laser densitometer. N-myc copy number was determined by comparison of the hybridization intensity of the N-myc band between the tumor, a normal control sample, and DNA from the neuroblastoma cell line IMR-32, which has 25-fold N-myc amplification (24). A single copy sequence (D13S28) hybridized to the same bands was used to correct for amount of DNA loaded into each lane. For RNA analysis, in order to compare intensity of hybridization between samples on the same blot, and between samples on different blots, densitometric readings were normalized to the intensity of the α-tubulin hybridization for each sample.

Statistical Analysis. Levels of N-myc and c-myc expression were tested for correlation with age at diagnosis, stage, site of tumor, Shimada classification, degree of differentiation, and DNA index using the Mann-Whitney nonparametric method (25). Correlation of levels of expression with proliferative compartment was tested using the Spearman rank correlation coefficient (25).
RESULTS

Gene Amplification. A total of 45 untreated primary neuroblastomas were examined for amplification (Table 1). No tumor had amplification of c-myc or L-myc, and six neuroblastomas had N-myc amplification. All tumors with amplification arose from the adrenal. Two were stage III, three were stage IV, and one was stage IVS.

Clinical profiles of tumors with N-myc amplification are shown in Table 2. The three patients with stage IV disease and N-myc amplification have died. Of the two with stage III disease and amplification, both are currently being treated for relapse. Among the 9 patients with adrenal tumors and stage III or IV disease without N-myc amplification, 4 have died and 2 are alive with active disease. One IVS tumor had 5-fold N-myc amplification. The tumor in this child disappeared without treatment and the patient remains alive 3.5 years after diagnosis.

Flow cytometric studies of 4 tumors with N-myc amplification revealed that 2 were nonaneuploid and 2 aneuploid. Proliferative compartments of these tumors ranged from 37 to 65%.

Oncogene Expression. The results of analysis of N-myc and c-myc expression are presented in Table 3. High levels of N-myc hybridization were seen in LAN-1, a neuroblastoma cell line with approximately 100-fold N-myc amplification (24), and in tumor 8503, which had 50-fold amplification (Fig. 1a). No c-myc hybridization could be seen in either of these samples. RNA prepared from an adrenal gland from a 16-week-old human fetus and from a 3-year-old child hybridized to c-myc but not N-myc (Fig. 1b). All stage I and II tumors and the stage III tumors without N-myc amplification had hybridization to both N-myc and c-myc (Fig. 1c). The same was found in the stage IV tumors without N-myc amplification that were untreated prior to analysis. Three of the four treated stage IV tumors had hybridization to c-myc but not N-myc (Fig. 1d). The fourth had no detectable c-myc or N-myc but did have readily visible hybridization to α-tubulin.

Relative levels of N-myc and c-myc hybridization are shown in Table 3 and Fig. 2. The values listed are units of hybridization intensity measured on autoradiograms by densitometry, corrected for the amount of RNA loaded into each lane (see "Materials and Methods"). Considering only stage I to IV untreated tumors without N-myc amplification, levels of hybridization varied widely but did not correlate with patient age, sex, clinical stage, Shimada classification, degree of differentiation, DNA index, or proliferative compartment (all P values >0.05). Two untreated tumors without N-myc amplification, 8411 and 8709, did not respond to therapy. These tumors had the highest proliferative compartments of any stage I to IV neuroblastoma without N-myc amplification but could not be distinguished from the others in terms of levels of c-myc or N-myc expression. No tumor without amplification had a level of c-myc or N-myc hybridization equal to that of the tumor or cell line with amplification.

DISCUSSION

The present study was designed to test the hypothesis that neuroblastomas without N-myc amplification have amplification of other myc family oncogenes, specifically c-myc or L-myc, or that these tumors display high levels of expression of one or more myc family genes without amplification. No amplification of c-myc or L-myc was found. Although only 6 of 45 neuroblastomas were found to have N-myc amplification, if only tumors arising from the adrenal are counted, the proportion becomes 6 of 23 (24.8%), which, given the small numbers, is comparable to results in other published series (1, 2). Our finding that only adrenal neuroblastomas had N-myc amplification is in agreement with results of Tsuda et al. (2), who found N-myc amplification in 20 of 43 adrenal tumors but 0 of 9 tumors from other sites.

In keeping with the known correlation of N-myc amplification and poor prognosis, all of the patients in this study whose tumors had N-myc amplification have died or have recurrent disease, except for the one with IVS neuroblastoma. The IVS neuroblastoma was found to have 5-fold N-myc amplification. Unfortunately, there was not sufficient material to determine whether N-myc was expressed in this tumor. Stage IVS tumors have a distinctive pattern of distant metastasis without bone involvement, generally occur in the first year of life, and often regress spontaneously (26). Several examples of stage IVS tumors with N-myc amplification have been reported, with variable clinical outcomes (2, 27–29). In this subset of patients, therefore, N-myc amplification is not necessarily predictive of rapid progression. It is possible that N-myc is not expressed in some of these tumors in spite of amplification. Alternatively, other events may occur in non-IVS tumors in addition to N-myc amplification to cause progression, or host factors in IVS patients may somehow suppress the continued growth of the tumor.

The studies of RNA hybridization must be interpreted with recognition of the many limitations of Northern blot analysis. (a) Variable amounts of time elapsed between surgical resection and freezing of tumor specimens, which likely resulted in some degradation of RNA. (b) Densitometric analysis of band intensity is somewhat inaccurate due to the presence of broad bands and some background hybridization. Northern blots were chosen over dot blots in order to be sure that appropriately sized RNA species were identified by each probe, and the quantity of RNA obtained in most cases was sufficient for only one blot. (c) Hybridization to an α-tubulin sequence was used to correct for different amounts of RNA loaded into each lane. The validity of this is dependent on the assumption that equal levels of α-tubulin expression are seen in each sample.
myc AMPLIFICATION AND EXPRESSION IN NEUROBLASTOMA

Table 3 Clinical profiles of neuroblastoma patients studied for N-myc and c-myc expression

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<th>Case</th>
<th>Age (mo)</th>
<th>Stage</th>
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<th>DNA index</th>
<th>Status</th>
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* DIF, degree of differentiation; GN, ganglioneuroma; Med, mediastinum; Ret, retroperitoneum; Ad, adrenal; UNF, unfavorable; FAV, favorable; %SG2M, proliferative compartment (percentage of S + G2 + M); NED, no evidence of disease; REC, recurrent tumor; DOD, dead of disease.
† Number of N-myc copies per haploid genome.
‡ Expressed in corrected densitometry units (see "Materials and Methods").
§ Value may be underestimate given broad band and very intense hybridization.
¶ Treated prior to analysis.

Northern blot analysis provides information about steady-state levels of RNA. Actual rates of transcription cannot be inferred without knowledge of the stability of these transcripts in the tumors, and such studies do not demonstrate that functional N-myc protein is produced.

In spite of these limitations, a number of conclusions seem warranted. N-myc transcripts were found in all of the untreated neuroblastomas and one ganglioneuroma. None of the tumors without N-myc amplification had a level of N-myc expression near that of the cell line or tumor with amplification, however. Absolute levels of expression were at least 5- to 10-fold higher in the presence of amplification than in its absence. It was difficult to accurately measure the very high hybridization intensities of the samples with amplification on autoradiograms exposed sufficiently to detect hybridization in tumors without amplification. Therefore, levels of N-myc expression per gene copy number cannot be compared in tumors with and without amplification. Yet, given the great discrepancy in levels of expression between the tumors with and without amplification, and the wide range of clinical features in tumors without amplification irrespective of their levels of N-myc expression, it seems unlikely that N-myc expression in these tumors leads to progressive disease.

One limitation in this study is that only 2 of the 26 tumors examined failed to respond to treatment during the follow-up period. The flow cytometry data showing that all but three untreated neuroblastomas with nonaneuploid DNA indices further suggest that most of the tumors were from a favorable prognostic category. It is therefore possible that examination of a larger number of advanced neuroblastomas without N-myc amplification would reveal a subset with high level N-myc expression. Recently, Nisen et al. (30) examined 33 neuroblas-
tomas for N-myc amplification and expression. N-myc expression was found in 25 of these tumors, generally at levels less than one-half that found in tumors with amplification. Here, as well, no apparent correlation was found with disease progression.

N-myc expression has also been examined in neuroblastomas by in situ hybridization (31) and by immunohistochemical staining (32, 33). The former has detected expression only in tumors with amplification, and in one study (32) the latter identified expression in 18 of 97 nonamplified tumors. It may be that Northern blot analysis is more sensitive than either in situ hybridization or immunohistochemistry. On the other hand, detection of transcripts with Northern analysis does not prove that functional protein is produced or that the protein is stable.

Most of the neuroblastomas examined in the present study exhibited transcription of both N-myc and c-myc. The only samples which lacked c-myc expression were the tumor and cell line with N-myc amplification. Lack of c-myc expression has also been reported in other tumors with high levels of N-myc expression (16, 34). The samples with c-myc but not N-myc expression were all tumors with complete or partial differentiation to ganglioneuroma. These included a single primary ganglioneuroma and three stage IV neuroblastomas which partly converted to ganglioneuroma after treatment. Nisen et al. (30) likewise did not detect N-myc expression in three ganglioneuromas, although these tumors were not examined for c-myc expression.

Our examination of human fetal adrenal at 16 weeks of gestation revealed c-myc but not N-myc transcripts. In the fetal brain N-myc is expressed in neuroblasts up to the onset of differentiation (35), and N-myc expression in neuroblastoma cell lines with amplification is rapidly lost after induction of differentiation with retinoic acid (36). The expression of N-myc in neuroblastomas may therefore reflect normal expression of this gene in undifferentiated neural cells. It is difficult to know whether the levels of expression in the tumors are “normal” because appropriate normal cells are difficult to obtain for comparison. Nisen et al. (30) used the levels of N-myc expression in the neuroblastoma cell line SK-N-SH, which does not have N-myc amplification, as a standard. This, too, is a tumor, however, and does not necessarily have normal levels of expression.

The significance of finding both N-myc and c-myc expression in neuroblastomas is uncertain. It is not known whether N-myc and c-myc are expressed in the same cells, different subsets of tumor cells, or tumor cells versus stromal cells. Expression of both N-myc and c-myc has been seen in SK-N-SH (37) and in a number of neuroepithelioma (PNET) cell lines without N-myc amplification (34). Although N-myc and c-myc genes have regions with similar structures (38), it may be that they play substantially different roles, and their expression may not be mutually exclusive.

The DNA index of tumors determined by flow cytometry did not appear to correlate with disease progression, N-myc amplification, or myc gene expression. Other studies (5–7) have shown that tumors with a nonaneuploid DNA content occur more commonly in patients with advanced stage disease and that these patients have a poorer response to treatment than those with aneuploid tumors. In the present study only three untreated non-IVS neuroblastomas were nonaneuploid, and all three patients have shown a good response to therapy. Two of the tumors with N-myc amplification and progressive disease were aneuploid. Although the sample size is small, it seems that DNA index alone is not a reliable prognostic indicator. The proliferative compartment, on the other hand, did appear to correlate with disease progression.

In conclusion, N-myc amplification remains the best molecular genetic marker of disease progression in patients with neuroblastoma. Although we have not ruled out the possibility that other oncogenes besides N-myc are amplified in some neuroblastomas or that N-myc is expressed at high levels in some without amplification, we can at least say that other mechanisms must underlie tumor progression in some tumors. A number of other chromosomal anomalies have been described in neuroblastomas (39–41), the most common of which is deletion of the short arm of chromosome 1 (42). Further characterization of these at the molecular level may reveal genetic mechanisms of tumor progression other than myc gene activation.

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