Combined Analysis of DNA Ploidy Index and N-myc Genomic Content in Neuroblastoma


ABSTRACT

The aim of the study was to assess, in a group of nonselected patients with neuroblastoma, the prognostic value of both N-myc gene amplification and DNA ploidy index, taking into account potential confounding factors such as age and stage. Of 59 patients studied, 23 were younger than 1 year at diagnosis, 31 presented with stage IV, 10 with stage III, 5 with stage II, 8 with stage I, and 4 with stage IV-S. N-myc genomic content was analyzed by Southern blot hybridization technique and N-myc amplification (23 copies/haploid genome) was present in 6 stage IV, 2 stage III, and 1 stage IV-S. The DNA ploidy index was analyzed by flow cytometry. Of the 59 neuroblastosmas, 26 were diploid (DNA index, 1) and 33 were aneuploid (DNA index, >1). The majority of the aneuploid tumors (28 of 33) were near-triploid with DNA indexes between 1.25 and 1.68, 4 were near-diploid (DNA index up to 1.18), and 1 was hypotetraploid (DNA index, 1.85). The proportion of near-triploid tumors was significantly greater among patients under 1 year of age and among patients presenting with stages I, II, and IV-S. Interestingly, 0 of 28 near-triploid neuroblastosmas exhibited N-myc gene amplification, compared to 9 of 31 in the group of diploid, near-diploid, and hypotetraploid tumors (Fisher’s exact test, \( P < 0.001 \)).

Four factors were significantly related to a high risk of relapse in univariate analysis, i.e., age, stage, DNA index, and N-myc amplification. In multivariate analysis, only N-myc amplification and the DNA index remained significantly associated with a high risk of relapse. The 2-year disease-free survival rate was 94% (95% confidence interval, 77-98%) for patients with near-triploid neuroblastoma, compared to 45 and 11% (95% confidence interval, 32-70 and 4-23%) for patients with diploid or near-diploid tumors, without and with N-myc amplification, respectively. We concluded that the combination of N-myc and DNA index should be included in routine management of neuroblastoma.

INTRODUCTION

Among the clinical factors found to be of prognostic value in neuroblastoma, age at diagnosis and stage (I-IV) have been shown to be the most reliable. However, in some cases, the clinical findings alone are not sufficient to predict the patient’s outcome. Attempts have been made to identify other parameters which could also provide prognostic information such as histopathology (7), age linked histopathologic examination (8), cytogenetic abnormalities (9), DNA ploidy (5, 9-13), and N-myc gene amplification (14-18). Two of these parameters have emerged as reliable predictors of the clinical outcome for the individual patient: (a) N-myc gene amplification has been shown to be strongly associated with rapid tumor progression and poor prognosis (15, 16); (b) the DNA ploidy index has also been shown to be predictive of clinical outcome, since the aneuploid neuroblastoma has been generally associated with favorable evolution of the disease (9-13). However, in most series, the prognostic value of N-myc gene amplification or DNA ploidy index has been considered separately. The aim of the present study was to assess, in a group of nonselected patients with neuroblastoma, the prognostic effect of both N-myc gene amplification and DNA ploidy index taking into account potential confounding factors such as age and stage.

MATERIALS AND METHODS

Tumor Specimens, Patients, and Treatment. All the neuroblastoma samples analyzed were obtained at surgery of the primary tumor from 59 unselected patients treated at the Institut Gustave Roussy between 1985 and 1988. Normal cell samples were lymphocytes obtained from 6 patients. All specimens were frozen immediately and stored in liquid nitrogen. Patients were evaluated according to the staging system of Evans et al. (1). All patients with stage IV (31 patients) and unresectable stage III (5 patients) tumors received 4 to 6 courses of primary chemotherapy followed by surgery of the primary tumor. Any tumor with less than 5% malignant cells and/or 70% or more necrosis after chemotherapy was excluded from this study. The chemotherapeutic regimen included doxorubicin, vincristine, and cyclophosphamide, plus cis-platinum and etoposide in 18 cases, as reported previously (19). Intensification associated with autologous bone marrow transplantation was performed in most patients with stage IV tumor (20). After the chemotherapy course, evaluation of the response included reiteration of pretreatment measurements. The response of the primary tumor was measured using computed tomography scan (two-diameter measurements) while the response of metastases were assessed by \( m-[123]-\)iodobenzylguanidine scan, computed tomographic scan, multiple bone marrow aspirates, and trephine biopsies. Patients with stage I (8 patients), stage II (5 patients), resectable stage III (5 patients), and stage IV-S (4 patients) tumors did not receive chemotherapy before surgery of the primary tumor.

N-myc Gene Amplification Measurements. High molecular weight DNA was extracted from frozen specimens according to the method of Maniatis et al. (21). DNA samples (6 \( \mu \)g) were digested with restriction endonuclease EcoRI and fractionated by electrophoresis through 0.8% agarose gels. The DNA was transferred onto Gene Screen Plus membrane. The filters were hybridized with \( 32P \)labeled nick-translated N-myc probe, pNbl1 [a gift of Dr. Schwab (14)]. Simultaneously, a globin pseudogene was used as a control of a single copy gene. A human neuroblastoma line, IGR-N-835, established from one of the neuroblastosmas of this series exhibited a 50-fold increase in the number of N-myc copies (22) and was used as a standard positive control. The copy number of N-myc gene was evaluated by microdensitometer tracings of autoradiograms (Joyce Loebl Chromoscan 3). N-myc gene amplification was scored when the gene copy number per haploid genome was 3 or more (15).

Flow Cytometric DNA Measurements. Suspensions of nuclei suitable for flow cytometry were prepared from frozen specimens after secondary fixation in 70% ethanol (30 cases). When there was not enough frozen material available after analysis of N-myc genomic content, the suspension of nuclei was obtained from the tumor area of the corresponding paraffin-embedded routine blocks using the method of Hedley

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et al. (23) (19 cases). Briefly, paraffin sections (50 μm) were dewaxed in xylene (2 baths, 20°C, 15 min each), rehydrated in graded ethanol, and finally rinsed in 1 bath of distilled water.

The samples were disrupted in 0.4% freshly prepared pepsin at pH 1.9 and incubated at 37°C for 45 min, with constant agitation. Clumps were removed by filtration through a 35 μm nylon mesh. The suspension of nuclei (3 to 6 × 10⁶/ml) was then centrifuged at 1500 rpm and the pellet was resuspended in 3 ml of phosphate-buffered saline containing propidium iodide at a final concentration of 20 μg/ml and 0.1% DNase-free RNase (Sigma). Analysis was performed using an Ortho flow cytometer. The 488 nm line of an argon ion laser was used for excitation. Red fluorescence (DNA) area and peak signals were collected so that doublets and triplets could be excluded. At least 10,000 events were stored in list mode. The occurrence of 2 G₀-G₁ peaks was considered as evidence of DNA aneuploidy. The DNA index was calculated from the DNA histograms as the ratio of the mean fluorescence of the G₀-G₁ channel for neuroblastoma cells over that of "normal" diploid cells (lymphocytes). S-phase fractions were fitted using the algorithm of Watson et al. (1987) (24). The mean coefficients of variation expressed in half-coefficient of variation were obtained from fresh fixed ethanol and from paraffin-embedded tissues and were found to be 4.1 (range, 2.1-6.7) and 4.5 (range, 3.1-7.2), respectively.

Statistical Methods. Results were analyzed for statistical significance by χ² and Fisher's exact test for small numbers. The χ² test for linear trends were used when appropriate. A univariate analysis was first used to determine the relative risk of relapse, followed by a multivariate analysis using Cox's proportional hazards regression (25). Age at diagnosis, stage, site of the primary, histology, ploidy, DNA index, percentage of cells in S phase, and N-myc gene amplification were taken into account. The disease free survival rates were computed by the Kaplan-Meier method (26). Follow-up from the first treatment ranged from 6 to 48 months with a mean of 20 months.

RESULTS

Of 71 unselected tumor specimens, 59 were suitable for both N-myc and DNA ploidy content measurements. N-myc amplification of more than 3 copies/haploid genome was found in 9 of 59 tumors including 1 stage IV-S, 2 stage III, and 6 stage IV tumors (Fig. 1; Table 1). Using the flow cytometry criteria outlined above, 26 neuroblastomas were diploid (DNA index, 1) and 33 were aneuploid (DNA index, >1) (Fig. 2). The majority of the aneuploid tumors (28 of 33) were near-triploid with DNA indexes between 1.25 and 1.68, 4 were near-diploid (DNA index up to 1.18), and 1 was hypotetraploid (DNA index, 1.85). N-myc amplification was present in 9 of 26 diploid neuroblastomas.

The relationship among age, stage, site of the primary tumor, histology, N-myc gene amplification, and ploidy is reported in Table 1. N-myc gene amplification was not significantly related to the clinical stage or age at diagnosis. In contrast, there was a statistically significant relationship between the ploidy index and N-myc amplification. Indeed, 0 of 28 near-triploid neuroblastomas exhibited N-myc gene amplification, compared to 9 of 31 in the group of diploid, near-diploid, and hypotetraploid tumors (Fisher’s exact test, P < 0.001). As reported in Table 1, the proportion of near-triploid tumors was significantly greater among patients under 1 year of age and among patients pre-
senting with stages I, II, and IV-S tumors.

The proportion of cells in S phase was analyzed only for the 26 diploid neuroblastomas. The range was from 2 to 35% with a mean of 15%. Interestingly, 8 of 13 tumors had 10% or more of cells in S phase and also exhibited N-myc gene amplification, compared to 1 tumor with N-myc amplification over 13 tumors with 10% or less of cells in S phase (Fisher's exact test, P = 0.02).

During the follow-up period, 16 children died (all of disease, except 1) and 6 were alive with tumor progression. The 2-year actuarial survival rate was 76% (95% CI, 60.5–86.5%) and the 2-year actuarial disease-free survival rate was 65% (95% CI, 49–77.1%). Four factors were significantly related to a high risk of relapse in the univariate analysis, namely age, stage, DNA ploidy, and N-myc amplification (Table 2). When the exact values of the DNA index were taken into account, the neuroblastomas could be separated into 2 groups. The first one included the near-triploid tumors (DNA index between 1.25 and 1.68), and the second group included the diploid or near-diploid (DNA index, ≤1.18) plus one hypotetraploid tumor. When both the ploidy and the “DNA index” as described above were included in the statistical model, only the “DNA index” was significantly associated with a high risk of relapse.

In multivariate analysis, only N-myc amplification and the “DNA index” remained significantly associated with a high risk of relapse (Table 2). The 2-year actuarial disease-free survival rate was 94% (95% CI, 77–98%) for patients with near-triploid neuroblastoma, compared to 45 and 11% (95% CI, 32–70 and 4–23%) for patients with diploid or near-diploid tumors, without and with N-myc amplification, respectively (P < 0.01) (Fig. 3).

In an attempt to analyze the prognostic information of N-myc amplification and ploidy on a stage-related basis, we compared the 2-year actuarial disease-free survival rates in stage IV patients versus a group including stages I, II, III, and IV-S patients. In the 32 stage IV patients the 2-year disease-free survival rate was 43% (95% CI, 17–69%) when either N-myc amplification or diploid/near-diploid was present compared to 80% (95% CI, 45–100%) when there was no N-myc amplification plus near-triploid (P = 0.03). In the 27 patients of other stages, the 2-year disease-free survival rate was 58% (95% CI, 20–97%) when either N-myc amplification or diploid/near-diploid was present compared to 100% when there was no N-myc amplification plus near-triploid (P = 0.001).

### DISCUSSION

Age at diagnosis and clinical stage are strong prognostic indicators in neuroblastoma (1–6). When considered separately, N-myc genomic content and ploidy also appear to be related to the clinical outcome (5, 9–18). The aim of the present study was to assess the prognostic value of these 2 parameters in a multivariate analysis taking into account potential confounding factors such as site of the primary, age, and stage. In our series, age >1 year, stage IV, N-myc gene copy number per haploid genome >3, diploid, and near-diploid were significantly associated in univariate analysis with poorer prognosis, extending observations of many others (1–6, 10–17). However, the striking point of this study was that only N-myc genomic content and the ploidy index remained associated with a high risk of relapse when a Cox’s regression model was performed.

The rates of N-myc gene amplification were 15% for all the neuroblastomas of this series and 19% in stage IV tumors. This latter incidence is lower than that of 30 to 40% frequently reported in untreated stage IV neuroblastomas (15). However, in this series, all patients with stage IV and 5 patients with stage III had received chemotherapy prior to surgical removal of the primary tumor. Any tumor sample with more than 70% necrosis and/or less than 5% malignant cells after chemotherapy was excluded from this study. It is conceivable that efficient chemotherapy reduces the quantity of malignant cells showing N-myc amplification in residual tumor, and this decline might explain that it was difficult in some cases to detect N-myc amplification by Southern blot analysis (18). Currently, this hypothesis is, however, a subject of controversy since Brodeur et al. (16) and other workers (27) have reported that the number of copies of N-myc generally does not vary during the course of treatment. In addition, it is noteworthy that incidence of N-myc amplification could be lower in European patients compared to those from United States and Japan as reported in a recent Italian study (28).

Among the diploid tumors, N-myc gene amplification was significantly associated with a high percentage of cells in S phase (≥10%). Although the function of the N-myc gene product is not yet known, our data suggest that the N-myc product could promote neuroblast into S phase, leading to increased cell proliferation, as suggested for other oncogene products of the myc gene family (29).
All the neuroblastomas with N-myc gene amplification were diploid. This has previously been suggested by Hayashi et al. (9) and Kaneko et al. (12) who reported respectively that 4 and 3 neuroblastomas with N-myc gene amplification were diploid or near-diploid.

The distribution of the DNA indexes among aneuploid neuroblastomas did not occur at random since most of the DNA indexes were between 1.25 and 1.68 (near-triploid). Interestingly, the distribution of the DNA indexes was also strongly dependent of age and stage: indeed most of the near-triploid neuroblastomas were found in patients under 1 year of age at diagnosis and/or with stages I, II, and IV-S tumors. Similar observations have been reported in independent series carried out either using flow cytometry or cytogenetic studies (5, 9-13). Several authors have also shown that the aneuploid (5, 9-11, 13), and especially near-triploid neuroblastomas (9, 13) were associated with a good clinical outcome. Our data are in agreement with those results since the 2-year disease-free survival rate was 94% for the near triploid tumors, compared to 29% for the diploid and near-diploid tumors. Using flow cytometry, Look et al. (11) have reported that aneuploid neuroblastoma were associated with a better tumor response to chemotherapy. We could not address this question since most of the aneuploid tumors were stages I, II, and IV-S and thus were not treated with primary chemotherapy.

In conclusion, the results of this multivariate analysis extend those of preliminary studies carried out with smaller tumor series (30, 31). They show that the combined analysis of N-myc genomic content and DNA index is a powerful indicator of tumor progression in neuroblastoma (Fig. 3). The combination of these 2 parameters should be included in routine management of neuroblastoma. Compared to N-myc gene analysis by Southern blot, the measurement of the ploidy index by flow cytometry may not be essential, namely the near-triploid neuroblastomas with N-myc oncogene with rapid progression of neuroblastoma. N. Engl. J. Med., 313:1111-1116, 1985.


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