Significance of Chromosome 1p Loss of Heterozygosity in Neuroblastoma


ABSTRACT

We analyzed 156 primary neuroblastoma tumor samples for loss of heterozygosity at the distal short arm of chromosome 1 (1p LOH). We also compared 1p LOH with clinical and genetic prognostic features as well as patient outcome. 1p LOH was detected in 30 of 156 tumors (19%) and was strongly associated with adverse clinical and biological features. 1p LOH was also strongly predictive of a poor outcome in univariate analyses (estimated 4-year survival, 32 ± 10% SE versus 76 ± 5% SE; P < 0.001). However, the prognostic value of 1p LOH was equivocal when stratified for amplification of the MYCN oncogene (P = 0.16). We conclude that 1p LOH is an important component of a pattern of genetic abnormalities in neuroblastoma associated with an aggressive clinical course.

INTRODUCTION

Neuroblastoma is a common childhood tumor arising from the post-ganglionic sympathetic nervous system. One of the most notable features of this tumor is striking clinical heterogeneity. Metastatic disease in older children is nearly always fatal despite aggressive, combined modality treatment, whereas metastatic disease in an infant may respond very well to chemotherapy or even regress spontaneously (1). Clinical features of the patients and histopathological features of the tumor specimen have both been used to predict outcome (2). Age greater than 1 year, advanced tumor stage (large, unresectable primary tumor and/or metastatic disease), and an adrenal primary site all have been associated with a worse prognosis (1, 2). Similarly, an elevated serum LDH3 or ferritin at presentation, and an unfavorable histopathological grading are all associated with decreased survival (3–8). However, attempts to stratify therapy based on these clinical and laboratory variables has led only to modest improvements in overall cure rates.

Characterization of the genetic alterations in neuroblastomas has been performed in an attempt to predict clinical outcome and stratify therapy. Amplification of MYCN was the first example of oncogene activation proven to be of clinical relevance (9). MYCN amplification reliably predicts an aggressive clinical course and poor outcome in patients of essentially any age or stage (10). Amplification is currently used in virtually all pediatric group trials to assign newly diagnosed patients to more intensive treatment. However, the vast majority of patients with MYCN amplification have other high risk features.

Tumor cell DNA content (ploidy) has been shown to predict responsiveness to therapy in infants with advanced stages of disease (11). Infants with a hyperdiploid modal chromosome number respond well to conventional therapy, whereas infants with an advanced stage and a diploid DNA content often fail conventional therapy (12). Diploid tumors occur more frequently with MYCN amplification, and it has been suggested that ploidy independently predicts a poor outcome (13). However, this does not hold for children over 24 months of age, probably because the hyperdiploid tumors in older children have structural cytogenetic abnormalities, as opposed to primarily whole chromosome gains in the infants (12, 14).

Deletions or rearrangements of the short arm of chromosome 1 (1p) are the most common cytogenetic abnormality in neuroblastoma tissue and cell lines (15–17). LOH studies have confirmed that partial 1p monosomy is a common feature of primary neuroblastomas (18, 19). Transfection of 1p into a neuroblastoma cell line restores a differentiated phenotype and abrogates tumorigenicity (20). Taken together, these experiments suggest the existence of at least one tumor suppressor gene located on 1p, the functional inactivation of which presumably would be important in neuroblastoma initiation and/or progression. 1p allelic loss has been reported to occur in 20–89% of primary neuroblastoma specimens in various series, with a higher percentage reported in studies restricted to near-diploid or advanced stage tumors (18, 21–30). Although most deletions are large, a common region of LOH has been localized to subbands 1p36.2–36.3 (29). It is postulated that this region contains at least one tumor suppressor gene involved in neuroblastoma tumorigenesis and/or progression.

Allelic loss on 1p is correlated with MYCN amplification (18, 23, 29), but studies of the independent predictive power of 1p LOH have been inconclusive. Cytogenetic studies suggested decreased survival for the subset of patients with partial 1p monosomy (31, 32). However, molecular genetic studies have been contradictory regarding the independent role of 1p LOH in identifying poor prognosis patients (23, 26, 28, 30, 33). The majority of these studies analyzed small groups of selected patients. We therefore sought to determine the biological and clinical importance of 1p LOH in a large group of newly diagnosed neuroblastoma patients.

MATERIALS AND METHODS

Patients and Specimens. One hundred fifty-six children with newly diagnosed neuroblastoma and with treatment initiated between 1984 and 1992 on a POG protocol were included in this study. All patients had tumors and matched blood specimens submitted to a reference laboratory at the time of diagnosis. Patients were selected for study based solely on the availability of an adequate amount of tumor and constitutional DNA. DNA from tumor and blood specimens were isolated as described (23).

Tumor staging is by the POG classification (12) because we were unable to retrospectively assign patients according to the International Neuroblastoma Staging System (34). For the purpose of this report, unfavorable tumors are defined as POG stages C and D (distant lymphatic or hematogenous dissemination), and favorable tumors are defined as stages A, B, and DS. All children were treated on a POG clinical trial determined by their age, stage, and year of diagnosis (POG protocol numbers 8104, 8441, 8741, 8742, 8743, 9047, and 9243). Serum LDH and ferritin levels were measured in standard fashion. Serum ferritin values were available in only 92 patients.

Detection of 1p LOH. Thirty DNA polymorphisms mapping to 28 distinct loci on distal 1p were used to detect LOH (Table 2). LOH was demonstrated either by RFLP, SSCP, or STRP analysis. The RFLP and SSCP assays were
performed as described previously (23, 24). The STRP assays were performed in the following standardized fashion except for D1505, which was as described by Budowle et al. (35). PCR was used to separately amplify 10 ng of genomic DNA from the tumor and blood specimens of an individual patient. This was performed in a 25-μl reaction containing 0.2 μM each primer (one primer end-labeled with [γ-32P]dATP by T4 polynucleotide kinase; Promega, Madison, WI), 70 μM each dNTP, 1.5 mM MgCl2, and 1X PCR buffer II with 1 unit of Taq polymerase (Perkin Elmer Cetus, Branchburg, NJ). Amplification was 3 per haploid genome, although most cases had at least 50–100-fold amplification.

The modal DNA content of neuroblasts was determined on propidium iodide-stained tumor cells by flow cytometry as described previously (11). The DNA content was converted to a DNA index by comparison to the normal diploid DNA content of 1.0. A tumor DNA sample was considered diploid if the DNA index was ≤1.0 and hyperdiploid when >1.0.

### RESULTS

#### 1p LOH in Neuroblastoma Patients

Patient characteristics are listed in Table 1. The 156 patients analyzed for 1p LOH in this study are generally representative of the known epidemiological distributions for children with neuroblastoma (40, 41). 1p LOH was detected in 30 of 156 tumor specimens (19%) with a panel of 30 highly informative polymorphisms (Table 2). Twenty-six of these DNA markers mapped to 24 separate loci on 1p36, providing an average of denaturation at 94°C, annealing at either 55 or 60°C, and elongation at 72°C. Five μl of the PCR product was denatured, and the alleles were separated through an 8% polyacrylamide sequencing gel. A tumor specimen was considered to have LOH when there was diminution of one tumor allele intensity by at least 60% compared to the corresponding heterozygous constitutional DNA specimen. Interpretation of LOH results were carried out by individuals blinded to patient characteristics and outcome.

### Other Biological Studies

MYCN copy number was determined by Southern hybridization with serially diluted tumor DNA samples as described previously (9). A tumor was considered amplified if the MYCN copy number was ≥3 per haploid genome, although most cases had at least 50–100-fold amplification.
density of one marker per Mb for this subband. All patients were constitutionally heterozygous at a minimum of six loci. Each tumor demonstrating LOH had a deletion documented at a minimum of three separate loci (median, 6.1; range, 3–11). Whereas most tumors with LOH had allelic loss for 1p34–1pter, all deletions involved 1p36.2–36.3 (Fig. 1). The common region of deletion in these tumors is defined distally by D1Z2 and proximally by D1S228. We recently published details of the deletion mapping on a subset of the patients reported here (29). The smallest region of overlap was not narrowed with the additional patients analyzed in this study.

**MYCN Amplification and Ploidy.** Amplification of the MYCN oncogene was demonstrated by Southern blotting in 27 of 153 tumors (18%). In addition, a diploid DNA index (unfavorable) was detected by flow cytometry in 39 of 141 specimens (28%).

**1p LOH Associations with Patient Variables and Outcome.** 1p LOH was significantly associated with most of the clinical variables analyzed that are reported to be predictive of a poor outcome (Table 1). 1p LOH was more frequent in children older than 1 year, having advanced stage disease and with an elevated serum LDH at presentation. There was no significant association with an adrenal primary site. Because children at POG institutions do not routinely have serum ferritin measured, it was available in only 62% of patients. However, 1p LOH was significantly associated with an elevated serum ferritin at presentation within this subset ($P = 0.002$). 1p LOH was also strongly associated with the adverse biological features of MYCN amplification and diploidy (Table 1).

1p LOH was strongly associated with decreased survival in univariate analyses ($P < 0.001$; Table 3 and Fig. 2a), as was MYCN amplification and diploidy (Fig. 2b; ploidy data not shown). However, 1p LOH was not an independently significant prognostic variable when stratified for MYCN amplification ($P = 0.16$ by log-rank test; Table 3 and Fig. 3). In a forward stepwise Cox analysis, with 1p LOH, MYCN amplification, ploidy (diploid versus hyperdiploid), LDH (subdivided at 1500 units/liter), and age (subdivided at 365 days) as independent variables, MYCN amplification, ploidy, and age were independently prognostic ($P < 0.05$; Table 4). After adjusting for these variables, the $P$ value for 1p LOH was $P = 0.72$.

To determine whether the extent of the 1p deletion was of prognostic importance, patients were grouped according to the size

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Fig. 1. Examples of 1p LOH in two neuroblastoma patients. Approximate genetic localization of selected 1p36 polymorphisms is shown. Representative PCR-based polymorphic assays from four separate loci are demonstrated. D1S80 is a PCR-formatted minisatellite polymorphism; D1S160, D1S489, and D1S507 are STRPs. LOH is demonstrated at each locus in both patients, except at D1S507 in patient 2. Patient 1 has LOH and patient 2 has retention of heterozygosity at all informative proximal loci assayed, including polymorphisms mapping to 1p35, 1p34, and 1p23. T, tumor DNA; C, constitutional DNA.
Table 3 Prognostic significance of 1p LOH

<table>
<thead>
<tr>
<th></th>
<th>No 1p LOH</th>
<th>1p LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Expected</td>
</tr>
<tr>
<td>All Patients</td>
<td>126</td>
<td>28</td>
</tr>
<tr>
<td>No MYCN MYCN</td>
<td>114</td>
<td>21</td>
</tr>
<tr>
<td>MYCN</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Total (stratified)</td>
<td>123</td>
<td>27</td>
</tr>
</tbody>
</table>

* Two-sided log-rank test.

of the deletion. On the basis of previous reports (25, 27), patients were subdivided according to deletions involving 1p36 only versus those also including more proximal loci. Nineteen patients were analyzable in this respect, 10 of these having large deletions. Although a trend toward worse survival in patients with a larger deletion was evident, the numbers were too small to be conclusive (9 failed with 6.1 expected for deletions of 1p34–1pter; 4 failed with 6.9 expected for deletions of 1p36 only; \( P = 0.10 \) by two-sided log-rank test).

DISCUSSION

Determining the genetic basis for the clinical heterogeneity of various childhood cancers has been a goal of pediatric oncologists. Neuroblastoma has served as a paradigm in this respect. There is marked diversity in both clinical presentation and course. Furthermore, the clinical behavior of the tumor is only partly explained by traditional clinicopathological features. It is not surprising that it is becoming increasingly evident that there is also genetic heterogeneity. This study has sought to clarify the biological and clinical significance of distal 1p LOH, one of the most common genetic abnormalities in neuroblastoma.

We found an incidence of 1p LOH of 19% in 156 unselected cases of neuroblastoma. This figure is at the lower end of the spectrum of incidence figures reported previously (18, 21–28). However, in many other series there is a selection bias toward MYCN amplified, higher stage patients. Our cohort of patients is generally representative of the observed distribution of age, sex, and stage at presentation within the POG and the Children’s Cancer Group, perhaps with a slight bias...
toward favorable patients from whom tumor tissue is more often available. The figures for our cohort of children, with a 22-month mean age, 55% greater than 12 months, and 54% with advanced stage disease at diagnosis, are similar to published POG figures of 22 months, 64 and 65%, respectively (40, 41). Therefore, the true incidence of 1p LOH, as detected by the polymorphisms used in this study, is probably no more than 25–35%.

LOH at distal 1p is strongly associated with adverse clinical features. Like MYCN amplification, this suggests that 1p LOH is a genetic alteration occurring in more aggressive disease states. However, because 1p LOH is only a gross reflection of the inactivation of a tumor suppressor gene, it is yet to be determined whether the gene is important in neuroblastoma initiation, progression, or both.

The majority of patients with neuroblastoma older than 12 months of age with disseminated disease die with current therapy. Although MYCN amplification has been a reliable indicator of poor outcome (9, 10), most of these patients have other high-risk features. It will be important to determine other genetic alterations in primary tumors that predict response to therapy. Ploidy appears to have independent predictive power in children less than 12 months of age, especially those with advanced stages of disease, but it does not have predictive value in older children (12). Our current analysis was undertaken with the intention of defining subsets of patients with intermediate survival risks.

Multivariate analyses were carried out to explore the prognostic significance of 1p LOH in relation to age, stage, LDH, MYCN amplification, and ploidy in 156 patients treated on recent POG protocols. Each of the genetic alterations was highly significant in predicting decreased survival in univariate analyses, confirming their association with aggressive disease. Only MYCN amplification retained clear prognostic significance when stratified for the other biologic variables as well as age, stage, and LDH. Although there is a trend toward an intermediate prognosis in patients with one genetic abnormality but not the other, there are not enough discordant patients in this study to reach statistical significance. A multicenter study, in which all newly diagnosed patients have both 1p LOH and MYCN amplification assayed by fluorescence in situ hybridization, has been proposed (34).

Recent studies have suggested that there are two distinct regions on 1p implicated in neuroblastomas, and that tumors with smaller, distal deletion are associated with favorable biological and clinical features (25, 27). Our data are more consistent with one commonly deleted region located within 1p36.2–36.3. Although the majority of our polymorphic probes mapped to 1p36, we saw no evidence of a separate region of LOH at a more proximal location. It also appears unlikely that we missed small interstitial 1p deletions because of the 1 Mb density of polymorphic markers used within this region. Furthermore, we saw only equivocal evidence that larger (versus smaller) deletions correlated with MYCN-amplified tumors and poor patient survival. It remains possible that there is more than one suppressor gene on 1p involved in neuroblastoma. However, our data are most consistent with a common region that is always involved when 1p deletions occur.

In conclusion, we have demonstrated that approximately 20% of primary neuroblastomas have 1p LOH detectable with a highly informative panel of polymorphisms distributed at 1 Mb density on 1p36. Our evidence suggests that a single tumor suppressor gene located at 1p36.2–36.3 is inactivated in every neuroblastoma with 1p

### Table 4 Forward Cox analysisa

<table>
<thead>
<tr>
<th>Order of entry</th>
<th>Adverse factor</th>
<th>Estimated hazard ratiob</th>
<th>95% confidence limits for hazard ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MYCN</td>
<td>581</td>
<td>293–1150</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>Age &gt;1.00</td>
<td>610</td>
<td>251–1479</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>Diploid</td>
<td>198</td>
<td>105–375</td>
<td>0.033</td>
</tr>
<tr>
<td>NEc LDH &gt;1500</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEc 1p LOH</td>
<td></td>
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a No. = 124 patients with complete data (40 deaths).
b An estimated hazard ratio of 581% for MYCN would mean that the instantaneous death rate for amplified patients is 581% (5.81 times) that of a nonamplified patient.
c NE, not entered.
d P values adjusted for the three variables entering the model.

Fig. 3. Kaplan-Meier overall survival estimates for patients with and without LOH stratified for MYCN amplification. Patients grouped according to presence (+) or absence (−) of 1p LOH and MYCN amplification. N, number of patients.

- 1p LOH; MYCN Amp (N = 109)
- 1p LOH; MYCN Amp (N = 12)
+ 1p LOH; MYCN Amp (N = 8)
+ 1p LOH; MYCN Amp (N = 18)

0 1 2 3 4 5 6 7 8 9 10
Years

% Survival
allelic loss. It remains to be determined whether a second suppressor locus is involved in a subset of these cases. Inactivation of a neuroblastoma tumor suppressor gene, manifested by 1p LOH, is associated with adverse clinicobiological features and decreased survival. Cloning of the responsible gene (or genes) will be the next critical step in understanding the clinical and biological importance of 1p LOH in neuroblastomas.

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REFERENCES

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