Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: Correlation with N-myc amplification

(suppressor gene/oncogene/restriction fragment length polymorphism/neural-crest tumors)

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Communicated by Donald C. Shreffler, February 21, 1989

ABSTRACT Partial monosomy of the short arm of chromosome 1 is the most consistent cytogenetic abnormality found in human neuroblastomas, but its overall frequency and significance are unclear. Using a panel of chromosome-1-specific DNA probes that identify restriction fragment length polymorphisms, we demonstrate that 13 of 47 human neuroblastomas (28%) have somatic loss of heterozygosity (LOH) at one or more loci on the distal short arm of chromosome 1. The chromosomal region that shows LOH most consistently is between 1p36.1 and 1p36.3; loss of a gene or genes in this region may be critical for the development or progression of neuroblastomas. The region of LOH in human neuroblastoma may resemble that described for pheochromocytoma, medulillary thyroid carcinoma, and melanoma, which are also tumors of neural-crest origin. Although LOH for distal chromosome 1p can occur in early stages of neuroblastoma, the loss usually occurs in tumors of advanced clinical stages. LOH for the short arm of chromosome 1 correlates significantly with N-myc amplification, suggesting that these two genetic events are related. Indeed, these two lesions appear to characterize a genetically distinct subset of particularly aggressive neuroblastomas.

Cytogenetic and molecular studies of human cancer cells have revealed characteristic genetic lesions, usually consisting of deletions, translocations, and gene amplification (1, 2). Although the latter two abnormalities have been associated primarily with oncogene activation, deletions are thought to identify the location of putative cancer suppressor genes or "antioncogenes," the loss or inactivation of which may play a role in tumor development or progression. The locations for a number of these putative suppressor genes have been identified, based on consistent chromosome deletions or allelic loss in specific malignant diseases (1, 2). However, only one candidate member of this class of cancer-related genes has been cloned—the retinoblastoma gene located at chromosomal region 13q14 (3–5).

Cytogenetic analysis of near-diploid neuroblastomas and tumor-derived cell lines has revealed a consistent deletion of the distal short arm of chromosome 1 in >70% of cases (6–8). Indeed, this finding is confirmed by a recent statistical analysis of 60 near-diploid neuroblastoma karyotypes, which indicates that deletion of chromosome 1p is the only numerical or structural abnormality that occurs with increased frequency (P < 0.001) (9). Unfortunately, most tumors analyzed have come from patients with advanced stages of disease or from established neuroblastoma cell lines. In addition, cytogenetic analysis of primary-tumor tissue is not always successful and may be difficult to interpret.

Therefore, we have taken a molecular approach by examining restriction fragment length polymorphisms (RFLPs) in both normal and tumor tissues from individual patients to identify loss of heterozygosity (LOH) on chromosome 1 in a large number of unselected neuroblastomas. We intended to determine the frequency of this lesion in an unselected series of neuroblastomas, to further define the region that is consistently deleted in these tumors, and to determine the relationship of this abnormality to N-myc amplification and clinical variables.

MATERIALS AND METHODS

Tumors and Cell Lines. We studied pairs of human neuroblastoma DNA and constitutional DNA from 47 individual patients. Of the 47 tumor samples, there were 45 primary neuroblastomas and 2 tumor-derived cell lines. The 47 corresponding constitutional DNA samples were derived from Epstein–Barr-virus-transformed lymphoblastoid cell lines (34 cases), untransformed leukocytes (8 cases), and other somatic tissues (e.g., kidney, liver; 5 cases).

DNA Probes. We used a panel of 19 chromosome-1-specific DNA probes (10–22), and the order of these probes was determined by genetic linkage in the Centre d'Etude du Polymorphisme Humain reference panel families (10). Of these probes, eight define known gene loci—proatriadiatin (PND) (11), a-fucosidase (FUCAI) (12), the protooncogene c-fgr (FGR) (13), the protooncogene L-myc (MYCL) (14), a-amylase (AMY1) (15), β nerve growth factor (NGFB) (16), anti-thrombin III (AT3) (17), and renin (REN) (18); two probes define chromosome-1-specific repetitive sequences—D1Z2 (19) and D1S57 (20); seven probes were isolated from a flow-sorted chromosome 1 library (D1S15, D1S16, D1S17, D1S18, D1S19, D1S21, and D1S22) (10), and two probes define anonymous DNA sequences—CRI-L336 (D1S47) (21) and D1S2 (22). A locus is considered informative for a particular patient when the constitutional DNA from that patient displays two different alleles (i.e., heterozygosity at that locus).

DNA Methods. DNA was isolated from tumor tissue and a normal DNA source from each patient and quantitated using fluorometric methods as described (23, 24). Five micrograms of DNA was digested with a restriction enzyme, electrophoresed on 0.8% agarose gels, and transferred to Zeta-probe nylon membranes (Bio-Rad) according to a standard protocol (23, 24). Probes were labeled with dCT32P using the random-primer technique (25). Southern hybridization and autorad-

Abbreviations: LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism.

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iography were performed as described (23, 24). These tumor/normal DNA pairs were analyzed with probe and enzyme combinations known to show RFLPs in a substantial percentage of cases.

RESULTS

The percentage of patients informative at any one locus ranged from 18% to almost 100%. Of the 47 patients, all were informative at 3 or more loci (median 7 loci, range 3–11 loci) for a total of 311 informative loci in the 47 patients. All patients were informative for at least 2 loci at or distal to D1S57 at 1p32. LOH at two or more alleles on the distal short arm of chromosome 1 was detected in 13 of the 47 cases (28%). Representative autoradiograms of selected loci are shown in Fig. 1.

Twelve of the 13 tumors reveal a pattern of LOH consistent with terminal deletions of the short arm of chromosome 1 (cases 1–4 and cases 6–13, Fig. 2), whereas the remaining tumor (case 5) may have an interstitial deletion. Thus, the common region of LOH in these 13 tumors lies between the distal end of the DNA locus defined by probe FGR and the proximal end of the locus defined by probe D1Z2, corre-

![Fig. 1. LOH on chromosome 1p in human neuroblastomas. Odd lanes represent tumor DNA (T), and even lanes represent normal somatic DNA (N). Restriction enzymes known to show RFLP with the various probes were used to digest the DNA. A tumor/somatic DNA pair is uninformative when the somatic DNA shows only one of two (or more) possible RFLP alleles (e.g., case 3 for probes D1S47 and FGR, case 5 for probes D1S57 and D1S21, and case 10 for probe FGR). The pair is informative when two RFLP alleles are seen in the somatic DNA. LOH in the informative cases is defined as loss of one of the two RFLP alleles in the tumor compared to normal somatic DNA (e.g., case 3 for probes D1Z2, D1S57, and D1S21; case 4 for probes D1Z2, D1S47, FGR, and D1S57; case 5 for probes D1S47 and FGR; and case 10 for probes D1Z2 and D1S47). There is no LOH in the informative cases when the two alleles seen in the somatic DNA are preserved in the tumor DNA (case 4 for probe D1S21, case 5 for probe D1Z2, case 10 for probes D1S57 and D1S21).]

*Buroker et al. (19) performed Southern hybridization studies on 27 unrelated individuals using the D1Z2 probe and Taq I. They found no two individuals with identical banding patterns due to the high degree of polymorphism. This observation is supported by our study of >50 unrelated patients (results not shown). Constitutional homozygosity at this locus is inferred to be extremely unlikely. Individuals showing no change in banding patterns between tumor and normal DNA are considered to have shown no LOH; the possibility remains that LOH is not detected in this case because of superimposition of bands.
The possible regions of overlap included in all deletions appears to be those gene loci probed between FGR and D122.

LOH has been detected to date on any of these other chromosomes. These findings agree with the very low incidence of random chromosome deletion or loss for chromosomes other than chromosome 1 seen in the cytogenetic analysis of 60 near-diploid tumors (9). Thus, the background of nonspecific LOH in neuroblastomas was extremely low for the other chromosomes tested. Although not every chromosome or chromosome arm was tested, our findings show a highly significant and nonrandom involvement of chromosome 1p in human neuroblastomas.

The other genetic lesion consistently associated with neuroblastoma is amplification of the protooncogene N-myc (23, 24). The presence of N-myc amplification has been shown to correlate positively with advanced clinical stage and poor prognosis in human neuroblastomas (23, 24). Table 1 shows the correlation between the clinical staging and either the LOH for chromosome 1p or the degree of N-myc amplification (or both) for the 47 patients in this study. Although there is a trend toward association with advanced stages of disease (stages 3 and 4), it is not yet statistically significant ($\chi^2 = 2.86; P > 0.05$). However, there was a very strong correlation between N-myc amplification and chromosome 1p LOH ($\chi^2 = 17.24; P < 0.001$): amplification was found in 8 of 13 cases (62%) with 1p LOH, compared with only 1 of 34 (3%) without 1p LOH. Moreover, both N-myc amplification and deletion of chromosome 1p (as detected by cytogenetic analysis) appear strongly correlated with a poor clinical outcome (23, 24, 26, 27).

**DISCUSSION**

Most previous cytogenetic studies of neuroblastomas suffer from the inherent bias that only ~20% of the primary tumors
Table 1. Correlation of chromosome 1p LOH and N-myc amplification with stage

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Total</th>
<th>LOH for 1p</th>
<th>N-myc amplification</th>
<th>Both LOH and N-myc amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
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<tr>
<td>4S</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>13</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

*Clinical stages are defined as follows. Stage 1, localized tumor confined to the area of origin; complete gross excision with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative microscopically. Stage 2, unilateral tumor with complete gross excision and/or with ipsilateral regional nodes positive for tumor; identifiable contralateral lymph nodes negative microscopically. Stage 3, tumor infiltrating across the midline with or without regional lymph node involvement, or unilateral tumor with contralateral regional lymph node involvement, or midline tumor with bilateral regional lymph node involvement. Stage 4, dissemination of tumor to distant lymph nodes, bone, bone marrow, liver and/or other organs (except as defined in stage 4S). Stage 4S, localized primary tumor as defined for stage 1 or 2 with dissemination limited to liver, skin, and/or bone marrow (modified from ref. 57). There was an apparent correlation between LOH for chromosome 1p and advanced stage, but it did not reach statistical significance (two-by-two test, \( \chi^2 = 2.86; P > 0.05 \)). However, LOH for chromosome 1p and N-myc amplification are highly correlated (\( \chi^2 = 17.24; P < 0.001 \)).

can be karyotyped. Thus, the findings seen from these cytogenetic studies may not be representative of all neuroblastomas, but rather of a select subset. On the other hand, most molecular studies to date present only information on N-myc amplification and do not address LOH on chromosome 1p in the same patients. Therefore, our study of these two important genetic lesions in a large series of unselected patients provided the most accurate genotypic analysis of neuroblastomas to date.

Our studies also provide a more precise localization of the putative neuroblastoma locus on chromosome 1 to the region 1p36.1-1p36.3. Our finding of LOH for chromosome 1p in ~30% of patients contrasts with the frequency of >70% seen in an analysis of near-diploid tumors and cell lines (9). However, if one includes only primary tumors and does not exclude those with a hyperdiploid or triploid karyotype (>57 chromosomes), the frequency of 1p deletions drops to ~40% (9), which agrees more closely with our studies.

Thus, several explanations are possible for why LOH was not seen in a higher proportion of neuroblastomas: (i) most cytogenetic analyses are derived from advanced-stage tumors with near-diploid karyotypes, in which the incidence of large chromosome 1p deletions may be more common; (ii) mutational events at the critical region in some neuroblastomas may be too small (e.g., point mutations or small deletions) to be detectable by LOH analyses with currently available probes; (iii) LOH for chromosome 1p may play a role in malignant transformation in only a subset of neuroblastomas (such as those with advanced stages of disease); and (iv) LOH for chromosome 1p may be a secondary event that occurs in some tumors during the course of clonal evolution. Although we did find LOH for chromosome 1p in an early-stage neuroblastoma, our data predominantly showed LOH for 1p in advanced disease stages (see below) and thus supports the latter two possibilities.

LOH at specific chromosomal regions has been seen in a variety of other human neoplasms (3–5, 28–51). In retino-

blasta, deletion of a specific gene on chromosome 13, thought to be critical in tumorogenesis, has been identified (3–5, 51), and its RNA and protein product have been initially characterized (52–54). However, even in this prototypic example of a recessive cancer gene and even with probes that are within the actual gene that is frequently deleted, abnormalities of the gene structure and/or expression are in the range of 12–40% of the cases (3–5, 51), similar to our findings.

In some melanomas, medullary thyroid carcinomas, and pheochromocytomas, deletion or somatic loss of heterozygosity in the tumor tissue has been demonstrated at loci on distal chromosome 1p (28, 55). Because these neoplasms also are embryologically derived from neural-crest cells, our studies of neuroblastomas raise the possibility that a common mechanism may underlie the formation or progression of these embryologically related tumors.

In summary, at least two genetic events in the course of tumor evolution in neuroblastoma have been identified—loss of a critical region on the short arm of chromosome 1 and activation (usually by amplification) of the N-myc protooncogene. Our studies suggest that the two genetic events are related and that one may precede or predispose to the other. The functional relationship between the loss of a gene or genes from this critical region of chromosome 1p and other genetic events related to neuroblastoma development or progression remains to be determined. However, these data, as well as the consistency with which normal or increased N-myc copy number is seen in tumors from individual patients over time (56), suggest that deletion of chromosome 1p as well as N-myc amplification are features of a genetically distinct subset of particularly aggressive neuroblastomas.

We are very grateful to Drs. Webster K. Cavenee and Alex Koufos for sharing their data on LOH for other chromosomes in their series of neuroblastomas. We thank the following individuals or institutions for providing us with recombinant probes used in the present study: M. Litt for D12Z2, C. Helms (Collaborative Research) for CRI-L356 or DIS47, P. Fossard for PND, J. S. O'Brien for FCU2A1, R. White for DIS57, J. Minna for MYCL, P. Pearson for DIS52, A. Ulrich (Genentech) for NGFB, K. Ishizaki for AMY1, E. Prochownik for AT3, and J. Chirgwin for REN. We acknowledge the technical assistance of J. Wasson, D. Norman, and H. Gordon throughout the course of the study. Patient and tumor samples were kindly provided by members of the Pediatric Oncology Group. This work was supported by grants from the National Institutes of Health—CA39771 and CA60127 (G.M.B.), CA44176 (N.C.D.), CA40842 (D.E.H.); the National Cancer Center (C.-T.F.); the American Cancer Society—IN-36-29-4 (C.-T.F.); the Children's United Research Effort (G.M.B.); the Joshua Macy, Jr. Foundation (P.S.W.); and the Fern Waldman Memorial Fund for Cancer Research.
