Evidence for a 17p Tumor Related Locus Distinct from p53 in Pediatric Primitive Neuroectodermal Tumors

Jaclyn A. Biegel, Carol D. Burk, Frederic G. Barr, and Beverly S. Emanuel

Division of Human Genetics and Molecular Biology, The Children's Hospital of Philadelphia [J. A. B., C. D. B., B. S. E.]; Department of Pediatrics, University of Pennsylvania School of Medicine [J. A. B., B. S. E.]; and Departments of Human Genetics [B. S. E.] and Pathology and Laboratory Medicine [F. G. B.], Hospital of the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT

Primitive neuroectodermal tumors of the central nervous system are the most common malignant brain tumors in children. Cytogenetic analysis of these tumors has demonstrated alterations of chromosome 17, in particular isochromosome 17q, as the most frequent chromosomal abnormality detected. Since the consistent loss of a specific chromosomal region in a given tumor type most likely indicates the presence of a tumor related gene in that region, we undertook a combined molecular and cytogenetic approach to examine alterations of chromosome 17 in primitive neuroectodermal tumors. Seven of 14 tumors analyzed demonstrated loss of alleles for loci on 17p. In three of the seven tumors tested, a loss in copy number was observed for only the most telomeric locus on 17p13-3, D17S54. Limited sequence analysis of the same seven tumors did not reveal mutations in four highly conserved coding regions of the p53 gene. These data suggest a new tumor associated locus on 17p distinct from and distal to TP53, which is involved in the initiation or progression of at least a subset of primitive neuroectodermal tumors.

INTRODUCTION

PNET, or medulloblastomas, are the most common malignant CNS tumors of childhood, accounting for approximately 25% of all pediatric brain tumors. While advances in chemotherapy and radiation therapy have improved long-term survival (1), a significant number of patients suffer late relapses which are usually fatal. Relatively little is known about the pathogenesis of the disease and no consistent marker of biological behavior has been identified as a reliable predictor of clinical outcome. Genetic changes, known to underlie the development of numerous pediatric malignancies, have not been identified.

Toward that end, we have begun to characterize the cytogenetic changes in CNS PNET and have found several chromosomes to be nonrandomly involved. The most common abnormality seen in these tumors is an isochromosome 17q [(17q)] which is present in approximately one-third of cases (2–5). There have been several tumors in which an (17q) was the only structural abnormality seen (2). These findings implicate the loss of sequences on 17p or gain of copies of 17q as a primary genetic event. Numerical changes and structural abnormalities of chromosome 17 other than (17q) have also been observed in PNET, demonstrating alterations of chromosome 17 in approximately 50% of tumors. Deletions and nonreciprocal translocations involving various other chromosomes, such as 6, 11, and 16, have also been seen in a number of PNET but are not as consistent or frequent as the chromosome 17 abnormalities. In contrast to many of the other solid tumors of childhood, most notably the sarcomas, specific translocations do not appear to be characteristic of this disease. The cytogenetic data suggest that loss or gain of sequences may be involved in initiation or progression of these tumors. Loss of a specific chromosomal region has been observed in a variety of solid tumors of adults as well as the other embryonal tumors of childhood, such as retinoblastoma, Wilms' tumor, and neuroblastoma. Such chromosomal losses or deletions suggest the existence of a tumor suppressor gene within the deleted region.

Approaches to the molecular study of solid tumors have used LOH for polymorphic loci or DNA dosage analysis to identify allele loss and thereby pinpoint and narrow areas of the genome that potentially contain tumor suppressor loci. A variety of solid tumors demonstrate LOH for 17p, including colorectal carcinomas (6), lung cancers (7), breast tumors (8–10), osteosarcomas (11), and ovarian (12) and brain tumors (13–21). On the basis of reported cytogenetic findings, several investigators have analyzed the LOH of chromosome 17p loci in CNS PNET. The smallest area of allele loss has been localized to 17p11.2–pter in 6 of 23 tumors analyzed by Thomas and Raffel (17), to 17p12–13.1 in 4 of 9 patients studied by Cogen et al. (19), and to 17p in 5 of 11 medulloblastomas analyzed by James et al. (20). Although there were no cytogenetic studies reported for these tumors, the mechanism of LOH in these studies was thought to be consistent with chromosomal loss.

Recent studies suggest that the p53 gene on 17p13 is a tumor suppressor locus (22–24). Allelic loss on 17p, resulting in the unmasking of a mutated p53 allele on the remaining chromosome 17, was first demonstrated in colorectal tumors (6) but has now been seen in numerous tumor types, including brain tumors (21, 25–29). In addition, germline mutations in p53 have recently been observed in families with the Li-Fraumeni cancer syndrome (30), suggesting that abnormalities of p53 may account for their genetic predisposition to a variety of pediatric and adult malignancies. By virtue of its involvement in other tumors and its map location, p53 became a candidate gene for the 17p locus involved in PNET.

On the basis of our cytogenetic findings in CNS PNET of childhood, we sought to further define the region of chromosome 17 involved in the initiation or progression of CNS PNET of childhood and to examine the involvement of p53 in this disease.

MATERIALS AND METHODS

Specimens. A clinical description and the complete karyotypes for nine of the cases have previously been reported (2). The molecular analysis presented here of cases 88-124, 87-21, 88-23, 88-93, 88-79, 88-57, 88-49, 88-60, and 87-40 refer to patients 20, 4, 12, 18, 19, 17, 14, 15, and 7, respectively, from that report. Five additional PNET were processed as described (2).

At the time of the initial procurement of the specimens, the portion of tumor tissue not needed for cytogenetic studies was immediately
frozen at −70°C. Peripheral blood lymphocytes from the patients were transformed with Epstein-Barr virus and served as the normal control.

DNA Extraction. Tumor samples were taken from −70°C and immediately frozen in liquid nitrogen. The frozen tissue was then pulverized in a frozen mortar and pestle. The resultant powder was incubated overnight at 37°C in 2–3 ml of TEN solution with 400 μg/ml protease K and 1% sodium dodecyl sulfate. After two phenol-chloroform extractions, the DNA was precipitated in 2 volumes of ethanol. The pellet was washed twice in 70% ethanol, dried, and resuspended in 200–800 μl of 10 mM Tris, pH 7.5–1 mM EDTA buffer. DNA was also isolated from lymphoblast cells and from cytogenetic pellets preserved in methanol-acetic acid fixative (31). Nuclei were pelleted for 5 min, rinsed once in Hank’s salt solution, rinsed once in TEN solution, then resuspended in 2–3 ml TEN, and treated as described above.

Southern Blot Analysis. Five μg of DNA, quantitated by spectrophotometry and electrophoretic gel analysis, from matched normal and tumor tissue were digested to completion with the restriction enzymes TaqI or BamHI. The digested DNA was size fractionated on 0.8% agarose gels and transferred according to the manufacturer’s protocol to transfer membranes (Immbobilon; Stratagene). The membrane was exposed to UV light (Stratallinker; Stratagene) and hybridized with 32P-labeled probes in 50% formamide-1% SDS-10% dextran-1 M NaCl at 42°C for 16–20 h. For each hybridization, 1 x 10^7 cpm of the control probe were used simultaneously. After high stringency washes (twice in 2 x standard saline citrate-16 SDS at room temperature for 10 min and once in 0.1 x standard saline citrate-0.1% SDS at 65°C for 15 min) the membranes were analyzed on an automated radioanalytic detector (Ambis Systems) before being exposed to Kodak Biomax X-ray film. The ratio of signal for the chromosome 17 test probe to the control probe was calculated for normal tissue and tumor tissue in each case (15). A ratio of tumor to normal dosage data was then calculated as:

\[
\text{Test 17:control for tumor} = \frac{\text{signal of genomic DNA from normal tissue}}{\text{signal of genomic DNA from tumor tissue}}
\]

Test 17:control for normal

A loss or gain of at least 35% in the ratio was necessary for it to be scored as a loss or gain of copy number.

Probes. The chromosome 17 probes used in this study were p144D6 (D17S28), pYNZ22 (D17S5), pHp53B (TP53), control probe pSY2501 (IFNB1) was provided by James Sylvester (Hahnemann University). DNA was also isolated in a frozen mortar and pestle. The resultant powder was incubated overnight at 37°C in 2-3 ml of TEN solution with 400 ng/ml proteinase K and 1% sodium dodecyl sulfate. After two phenol-chloroform extractions, the DNA was amplified with Taq polymerase (Perkin-Elmer Cetus) or Taq polymerase (Perkin-Elmer Cetus) and treated as described above.

Sequencing of p53. Direct sequencing of PCR products was used to screen for mutations in the p53 gene. Primer pairs were used in exons 5, 7, and 8 based on the frequency of published mutations in other tumors (27). The genomic region containing codons 132–143 and 174–179 was amplified by PCR with oligonucleotide primers TACTCCTCTGGCTCTCAACA and CAATCGCTATCGGAGCA-GCG. A second region containing exons 236–248 and 272–281 was amplified with primers GTGGGCTCCTGACTGTAAC and TTTCCTGCAGAGATCTCT (27). For each reaction 100 ng of genomic DNA were amplified in a thermal cycler (Coy) for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. After the resultant bands were excised, the DNA was eluted by the glass powder technique (32) and resuspended in 10 mM Tris, pH 7.5-1 mM EDTA buffer. The sequencing reactions were performed using a modification of the linear PCR (33) with amplification for 20 cycles. The same oligonucleotides used to generate template were used for sequencing and 40 ng of template were amplified. Products were analyzed on 6% acrylamide gels.

RESULTS

The patient data and cytogenetic information from the 14 cases are shown in Table 1. Of the 14 cases, 4 PNET contained one or two copies of an i(17q) (cases 88-124, 87-21, 90-41, and 88-23). Three PNET demonstrated other complex changes of chromosome 17: a 17p+ chromosome in case 88-93, loss of a chromosome 17 with multiple markers in case 88-79, and loss of chromosomes 17 with marker chromosomes that possibly involved chromosome 17 in case 88-57. Two PNET (cases 88-49 and 88-60) had normal karyotypes. Cases 87-40 and 90-28 had abnormal karyotypes, but the chromosomes 17 were not detectably altered. Cytogenetic studies were not done (89-139) or were inadequate (88-107 and 89-101A) for the remaining 3 cases. All of the patients had normal constitutional karyotypes.

The 14 pairs of matched normal and tumor DNA samples were examined with a series of chromosome 17 probes by Southern blotting, hybridization, and quantitative analysis. Control hybridization probes were selected for chromosomes known to be karyotypically normal in the tumor(s). A quantitative analysis to determine copy number could therefore be performed, revealing gain or loss of copy number for every allele, regardless of whether the patient was heterozygous at a given locus.

Each of the four PNET with an i(17q) demonstrated allelic loss for every probe tested on 17p. Two examples (cases 88-23 and 90-41) are shown in Fig. 1A, each demonstrating loss of a polymorphic allele at D17S28. A second example, case 87-21,

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>88-124</td>
<td>10</td>
<td>M</td>
<td>PNET, neuronal differentiation</td>
<td>46,XY,del(16)(q22)(q33),i(17q),t(18;22)(q10t10)(q17)</td>
</tr>
<tr>
<td>87-21</td>
<td>2</td>
<td>F</td>
<td>PNET, glial differentiation</td>
<td>84,XX,t(6p;22)(q31;q11),i(17q),t(18;22)(q10t10)(q17)</td>
</tr>
<tr>
<td>90-41</td>
<td>5</td>
<td>F</td>
<td>PNET, neuronal differentiation</td>
<td>45,XY,46,XX,del(17)(p13,p11)</td>
</tr>
<tr>
<td>88-23</td>
<td>6</td>
<td>M</td>
<td>PNET, NOS*</td>
<td>83,84,XY,46,XX,del(17)(p13;p11),del(16)(q12.2),i(17q),t(17;21)(q22.2;q22.1)</td>
</tr>
<tr>
<td>88-93</td>
<td>9</td>
<td>M</td>
<td>PNET, NOS (recovered)</td>
<td>46,XY,45,XY,46,XX,del(17)(p13;p11),del(16)(q12.2),i(17q),t(17;21)(q22.2;q22.1)</td>
</tr>
<tr>
<td>88-79</td>
<td>3</td>
<td>F</td>
<td>PNET, NOS</td>
<td>near triploid, t(11;17)(q23;q23),del(10)(q26),del(17)(q21.3),t(17;21)(q22.2;q22.1)</td>
</tr>
<tr>
<td>88-57</td>
<td>11</td>
<td>M</td>
<td>PNET, NOS, keratin positivity</td>
<td>89,XX,XX,t(6p;22)(q31;q11),i(17q),t(17;21)(q22.2;q22.1)</td>
</tr>
<tr>
<td>88-49</td>
<td>4</td>
<td>M</td>
<td>PNET, NOS</td>
<td>46,XY</td>
</tr>
<tr>
<td>88-60</td>
<td>&lt;1</td>
<td>F</td>
<td>PNET, neuronal differentiation</td>
<td>46,XX</td>
</tr>
<tr>
<td>87-40</td>
<td>10</td>
<td>M</td>
<td>PNET, glial and neuronal differentiation</td>
<td>46,XY,del(16)(q11.2)</td>
</tr>
<tr>
<td>90-28</td>
<td>2</td>
<td>F</td>
<td>PNET, NOS</td>
<td>89,XX,XX,t(6p;22)(q31;q11),i(17q),t(17;21)(q22.2;q22.1)</td>
</tr>
<tr>
<td>89-139</td>
<td>5</td>
<td>M</td>
<td>PNET, NOS</td>
<td>ND</td>
</tr>
<tr>
<td>88-107</td>
<td>2</td>
<td>M</td>
<td>PNET, NOS</td>
<td>ND</td>
</tr>
<tr>
<td>88-101</td>
<td>2</td>
<td>M</td>
<td>PNET, vimentin positivity</td>
<td>ND</td>
</tr>
</tbody>
</table>

* NOS, not otherwise specified; ND, no cytogenetic data.
mutations in the p53 gene from codons 132–281 using the polymerase chain reaction (34). This area includes the four highly conserved regions (codons 132–143, 174–179, 236–248, and 272–281) reported to include the majority of mutations in malignant tumors (15). None of the 7 tumors, including the 4 tumors with an i(17q) and the 3 tumors demonstrating allelic loss at D17S34, contained mutations in these regions (data not shown).

**DISCUSSION**

The aim of the present study was to examine the frequency of allelic loss for chromosome 17 loci in pediatric patients with PNET of the CNS and thus begin to define the region on 17p that is likely to contain a tumor related gene. To do so, we undertook a molecular characterization, including Southern blot analysis, hybridization, and quantitative dosage analysis of PNET that had been cyogenetically characterized. This series of tumors included cases in which frequent cyogenetic abnormalities, such as an i(17q), were present, as well as tumors in which only normal karyotypes from direct chromosome preparations from tumor biopsies were obtained.

The molecular data for chromosome 17 confirmed, in part, what was expected from the cyogenetic results. Formation of an isochromosome results in a relative loss of chromosome 17 alleles, with concomitant gain in copy number for the 17q loci. The four tumors with an i(17q) demonstrated loss at all of the 17p loci examined, with a concomitant gain in copy number at the 17q loci studied. In contrast, tumors with normal chromosomes 17 did not demonstrate loss of chromosome 17 alleles, although the density of 17p probes examined was not sufficient to definitively rule out loss of alleles by mitotic recombination or deletion of distal 17p13.3 in these tumors.

Although mutations in the hot spots in p53, which maps to 17p13.1, have been detected in the majority of tumor types that

---

**Fig. 1.** Southern blot analysis of normal (N) and tumor (T) DNA from cases 90-41 and 88-23 (A) and case 87-21 (B). DNA samples were digested with TaqI, electrophoresed in 0.8% agarose gels, and blotted. The blots were hybridized with chromosome 17p probe pYNH37.3 (D17S28) and chromosome 2 control probe hFS-110G (INHA). Left ordinate, fragment sizes in kilobases (kb).

---

**Fig. 2.** Iodogram of chromosome 17 showing the loci examined and the results for each of the 14 PNET. Four tumors contained an i(17q), 3 had complex karyotypes involving chromosome 17, 4 had normal chromosomes 17, and there were no cyogenetic data (ND) for 3 cases. O, maintenance of heterozygosity or copy number; •, loss of heterozygosity or copy number; •, gain in copy number.
have been examined, pediatric PNET may be an exception. We did not detect mutations in the established hot spots in the seven tumors demonstrating loss of alleles for 17p; however, this finding does not preclude the possibility that there were mutations in the remaining portions of the gene or that there is some other mechanism of inactivation of p53 in these tumors. In the series of medulloblastomas examined by Saylors et al. (35), a mutation in p53 was found in only 1 of 3 cell lines and in none of the 12 primary tumors analyzed. Only 2 of 11 medulloblastomas screened by Ohgaki et al. (36) were found to contain mutations in p53. Thus, mutations in p53 are infrequent in medulloblastomas and PNET, supporting the hypothesis that there is another locus, on 17p or elsewhere, that is involved in their development.

In our study, there were three tumors that only showed allelic loss at the most distal locus, D17S34. These data suggest that a locus on distal 17p13.3 is involved in the development of PNET, thus narrowing the critical region for development of these tumors to a region not previously implicated by other studies of pediatric PNET. Further evidence for the hypothesis that there is a distal locus on 17p13.3 involved in tumorigenesis has been provided by studies of loss of heterozygosity in breast cancer (9, 10). In a study of 168 breast tumors, Coles et al. found two common areas of deletion on 17p, both distal to p53. Sato et al. (10) have obtained similar results in their studies on 79 primary breast tumor patients. These findings suggest that there are genes on 17p, other than p53, that may be involved in breast tumorigenesis.

Our quantitation of allele dosage in PNET suggests that in several cases (88-124, 87-21, 88-93, 88-79, and 88-139) there is only partial loss of one 17p allele. The finding of residual signal may be explained by contamination of some tumor specimens by normal cells. However, the presence of true partial loss is best demonstrated in case 87-21 in which the cytogenetic specimens by normal cells. Therefore a larger series of PNET must be examined with this finding does not preclude the possibility that there were some other mechanism of inactivation of p53 in these tumors.

ACKNOWLEDGMENTS

The authors wish to thank Dr. D. Johnson at Children's National Medical Center for contributing specimen T-89-139; A. Parmiter and J. Preston for technical assistance; K. Mayo, G. Rovera, and J. Sylvester for providing probes; and R. Harvey for typing the manuscript.

REFERENCES


17p TUMOR RELATED LOCUS DISTINCT FROM p53 IN PEDIATRIC PNET


Evidence for a 17p Tumor Related Locus Distinct from p53 in Pediatric Primitive Neuroectodermal Tumors

Jaclyn A. Biegel, Carol D. Burk, Frederic G. Barr, et al.