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

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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, D17S254. 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 [i(17q)], which is present in approximately one-third of cases (2–5). There have been several tumors in which an i(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 i(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.

Investigations of genetic changes in these tumors have indicated the presence of a tumor suppressor gene on chromosome 17 as a putative target of genetic events. In several pediatric tumors and its map location, p53 became a candidate gene for the smallest area of allelic loss has been localized to 17p11.2. Pertinent data are 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 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 reported cytogenetic findings, several investigators have analyzed the LOH of chromosome 17p loci in CNS PNET. The smallest area of allelic loss has been localized to 17p11.2. Pertinent data are 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 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.

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

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2 To whom requests for reprints should be addressed, at Division of Human Genetics and Molecular Biology, The Children's Hospital of Philadelphia, 34th and Civic Center Blvd., Philadelphia, PA 19104.

3 The abbreviations used are: PNET, primitive neuroectodermal tumors; CNS, central nervous system; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TEN solution, 10 mM Tris, pH 7.5-10 mM EDTA, pH 8.0-150 mM NaCl.

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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 proteinase 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 methyl-acceptor acid fixative (31). Nuclei were pelleted for 5 min, rinsed once in Hanks’ salt solution, rinsed once in TEN solution, then resuspended in 2–3 µl 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 (Immobilon; 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 65°C for 15 min. The membranes were analyzed on an automated stringency washes (twice in 2 x standard saline citrate-1% 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 radiographic detector (Ambis Systems) before being exposed to Kodak XAR autoradiographic 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:

\[ \frac{\text{Test 17}}{\text{control for normal}} \]

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.

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 (88-139) 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 17 chromosome 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.t(16;20)(q12-22;q33.3),i(17q)46,XY,del(21)(q22),t(16;20)(q12-22;q33.3),i(17q)</td>
</tr>
<tr>
<td>87-21</td>
<td>2</td>
<td>F</td>
<td>PNET, glial differentiation</td>
<td>84,XX,-X,-X,-3,-8,-10,-(13)-15,—21,1,i(17q),+der(21)(q21;21)(q22;22)</td>
</tr>
<tr>
<td>90-41</td>
<td>5</td>
<td>F</td>
<td>PNET, neuronal differentiation</td>
<td>45,XY,-8,-10,-18,+der(10)(t(10;7)(17q))</td>
</tr>
<tr>
<td>88-23</td>
<td>6</td>
<td>M</td>
<td>PNET, NOS*</td>
<td>83-89,XXXYY,3-5,7,7,7,7,8,8,10,-11,-11,-13,-20,+der(7)(7q);11p1q11),del(16)(1q12,1q178,i(17q),i(17q),+mar,+dnim</td>
</tr>
<tr>
<td>88-93</td>
<td>9</td>
<td>M</td>
<td>PNET, NOS (recurrent)</td>
<td>46,XY,-4,-5,-6,-6,-12,-12,-16,-17,+18,+19,del(1)(p21),+del(1)(q31),3p+q+,+der(4)(q33;35),+der(5)(5;7)(q35;7),+der(16)(16;12;21;?),+der(17)(17p;17p),del(22)(q11;4</td>
</tr>
<tr>
<td>88-79</td>
<td>3</td>
<td>F</td>
<td>PNET, NOS</td>
<td>near triploid,-7,7,11,-14,-16,+der(11)(11q23;23),+der(16)(16q11;12;7)</td>
</tr>
<tr>
<td>88-57</td>
<td>11</td>
<td>F</td>
<td>PNET, NOS, keratin positivity</td>
<td>89,XX,,-X,-4,-5,5,-7,8,8,-11,-14,-16,-17,-18,-19,-19,+,+del(1)(p13</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>
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<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,,-X,-1,-6,-6</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>89-101a</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.

Table 1 Clinical and cytogenetic data for PNET cases

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is shown in Fig. 1B. The decreased signal for the 4.3-kilobase allele of locus D17S28 in this case indicates a quantitative allelic loss and is consistent with the cytogenetic finding of three normal copies of 17 and an i(17q). Therefore, instead of a complete loss for an allele, this case demonstrates an altered allelic ratio of 1:2 for the 4.3-kilobase to the 4.0-kilobase allele in the tumor. As shown in Fig. 2, each of these four tumors also showed gain of alleles for each probe tested on 17q, consistent with the presence of an i(17q).

Two of the three PNET with more complex cytogenetic abnormalities of chromosome 17 (cases 88-93 and 88-79) demonstrated allelic loss at only the most distal 17p locus, D17S34. The third tumor, which had a complex karyotype, and the 4 tumors with cytogenetically normal chromosomes 17 did not demonstrate a loss or gain at any of the chromosome 17 loci examined. Of the three PNET for which tumor karyotypes were not obtained, one case (89-139) demonstrated allelic loss at the most distal 17p locus, D17S34.

The tumor suppressor gene p53 was one of the loci examined in this series of pediatric tumors. No rearrangement of p53 on Southern blots was detected for the 11 tumors analyzed. As a preliminary screen to determine if mutations in the p53 gene were frequent in PNET, we undertook a limited sequence analysis of tumors that demonstrated loss of alleles on 17p. Genomic DNA from the 7 PNET was analyzed for sequence 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 cytogenetically characterized. This series of tumors included cases in which frequent cytogenetic 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 cytogenetic results. Formation of an isochromosome results in a relative loss of chromosome 17p 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
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 Ogaki 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, 88-119, 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 mapping of the medulloblastoma locus on chromosome 17p. Genomics, 1: 255-260, 1989.


Therefore a larger series of PNET must be examined with combined molecular and cytogenetic techniques. The mapping of the boundaries of the deletions in 17p in the tumors with loss at D17S34, coupled with a search for smaller deletions in additional tumors, should provide a means to isolate a PNET tumor related locus.

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17p TUMOR RELATED LOCUS DISTINCT FROM pS3 IN PEDIATRIC PNET


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