Loss of Heterozygosity for Loci on Chromosome 17p in Human Malignant Astrocytoma

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ABSTRACT

Loss of constitutional heterozygosity for specific chromosomal loci, when found consistently in a particular tumor type, suggests that a recessive oncogene important in the genesis of that tumor may be present within the involved chromosomal loci. DNA markers that detect restriction fragment length polymorphisms are powerful tools that have been used to detect loss of chromosomal loci in a growing number of human malignancies. The human brain tumor astrocytoma is usually malignant and virtually incurable. Two types of malignant astrocytomas are recognized histopathologically: anaplastic astrocytoma and glioblastoma multiforme. We carried out a restriction fragment length polymorphism analysis of tumors from 15 patients with anaplastic astrocytoma and 20 patients with glioblastoma using polymorphic DNA markers for loci on chromosome 17. Loss of constitutional heterozygosity for loci on chromosome 17 was found in both anaplastic astrocytoma and glioblastoma patients with equal frequency (40% of cases). Our mapping data revealed a region of loss on chromosome 17p between physical loci pi1.2 and pter that was common to both patient groups. Taken together with the previously reported finding of loss of heterozygosity for loci on chromosome 10 in glioblastoma, these results indicate that tumorigenesis in the astrocyte lineage may involve recessive oncogenes on two different chromosomes.

INTRODUCTION

Malignant astrocytoma is the most common brain tumor in humans. Infiltration of surrounding normal brain tissue by malignant astrocytoma cells makes complete surgical excision of these tumors impossible. Despite the use of adjuvant radiation and chemotherapy, tumor recurrence is the rule. One histopathological classification recognizes two grades of malignant astrocytomas: anaplastic astrocytoma and glioblastoma multiforme. Classification of patients with malignant astrocytomas into these two groups correlates with clinical prognosis. Glioblastoma, the most malignant form of astrocytoma, is uniformly fatal. Although anaplastic astrocytomas show a variable response to treatment they may progress to glioblastoma.

A major challenge in modern cancer research is to identify genes that are targets for tumor-causing mutations. Recessive oncogenes comprise a class of genes that are believed to function normally in the cell to suppress cellular proliferation. Inactivation of both copies of a recessive oncogene may contribute to neoplastic transformation by removing a normal constraint to cell growth. The best studied example of a recessive oncogene is found in retinoblastoma, a malignant ocular tumor that affects children. Retinoblastoma is associated with loss of both alleles at a single genetic locus on chromosome 13 and the recessive oncogene at that locus has been cloned (3, 4). Loss of a specific chromosomal locus, when found consistently in a particular type of tumor, suggests that a recessive oncogene important in the genesis of that tumor resides at the missing chromosomal locus. One approach to identifying loss of chromosomal loci in tumors involves the use of DNA markers that detect RFLPs at specific chromosomal loci. These markers are used in Southern transfer analysis to compare the genotype of somatic (constitutional) DNA and tumor DNA from affected individuals. Loss of constitutional heterozygosity in tumor DNA indicates that the tumor cells have lost one allele at the chromosomal locus identified by the marker. This type of RFLP analysis has been used successfully to detect loss of alleles that signal the presence of recessive oncogenes in a growing number of malignancies afflicting both children and adults (for review see Ref. 5). We and others have recently reported loss of heterozygosity for loci on chromosome 10 in a majority of patients with glioblastoma multiforme (6, 7). Chromosome 10 loss was not found, however, in tumor DNA from patients with less malignant astrocytomas.

We report here that loss of constitutional heterozygosity for loci on the p arm of chromosome 17 was found with equally high frequency among patients with the two histopathological types of malignant astrocytoma: anaplastic astrocytoma and glioblastoma multiforme. Taken together with the previously reported finding of allele loss on chromosome 10 in glioblastoma, these results suggest that recessive oncogenes important in the genesis of malignant astrocytomas may be present at loci on two different chromosomes.

MATERIALS AND METHODS

Human Tumor Samples. All tumor samples were surgically removed from the brain prior to radiation or chemotherapy except in four cases (A3, U7, U18, and U20) of tumors that recurred following radiotherapy. Histopathological grading of astrocytomas was based on the classification scheme of Burger and Vogel (1). All tumor samples were reviewed by a single neuropathologist and were judged to contain greater than 75% neoplastic cells.

DNA Extraction. Tumor tissues excised at surgery were immediately frozen in liquid nitrogen for genomic DNA extraction. Tissue samples were Dounce homogenized in lysis buffer containing 0.5% SDS, 0.1 M NaCl, 40 mM Tris-Cl (pH 8.0), and 20 mM EDTA. The viscous cell lysate was digested with proteinase K at 37°C overnight. The resulting solution was extracted with phenol and chloroform and then precipitated with sodium acetate and ethanol. Clumps of precipitated DNA were removed and dissolved in TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA). DNA extraction was performed by a single neuropathologist and were judged to contain greater than 75% neoplastic cells.

Received 5/14/89; revised 8/11/89; accepted 8/30/89.

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1. This work was supported by NIH Clinical Investigator Award CA01049 (to D. F.), American Cancer Society Research Grant CD-404 (to D. F.), and American Cancer Society Clinical Oncology Career Development Award 89-183 (to G. A. T.).

2. To whom requests for reprints should be addressed, at Division of Neurosurgery, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, Utah 84132.

3. Investigator of the Howard Hughes Medical Institute.

4. The abbreviations used are: RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M sodium chloride: 0.015 M sodium citrate, pH 7.4).
Southern Transfer Analysis. Genomic DNA (5 μg) from peripheral blood leukocytes and tumor tissue was digested to completion with restriction enzymes and separated by electrophoresis through 1.0% agarose gels. The DNA fragments were alkali-denatured in the gels and transferred to nylon filters (Genelman Biotrace) with ammonium acetate (9). DNA probes (200 ng) were labeled to high specific radioactivity (greater than 10⁶ cpm/μg) with 3²P using the random oligonucleotide priming technique (1). Radiolabeled probes were applied to prehydrated filters in hybridization solution containing 50% formamide, 4x SSC, 5% Dextran sulfate, 100 μg/ml denatured human placental DNA, 1x Denhardt's solution, and 20 mm sodium phosphate (pH 7.5) at 42°C (1x SSC = 150 mM NaCl/15 mM sodium citrate at pH 7.0). After 48 h of hybridization under these conditions the filters were washed twice at room temperature in 2x SSC/0.5% SDS for 20 min and then once at 62°C for 45 min in 0.1x SSC/0.1% SDS. Filters were exposed to X-ray film with intensifying screens at −70°C for autoradiography.

In cases where tumor DNA showed loss of constitutional heterozygosity, quantitative densitometric scanning was carried out to measure any residual hybridization signal corresponding to the deleted allele due to non-tumor cell DNA contamination. All cases of loss of heterozygosity described in "Results" showed greater than 50% reduction in signal intensity in tumor DNA compared to leukocyte DNA.

DNA Markers. The following DNA markers and restriction enzymes were used in the following RFLP analyses: pYNZ22.1 (D17S30), TaqI, RsaI, or MspI (11); pYNH37.3 (D17S29), TaqI (11); pHHH202 (D17S33), HindIII (13); pYNM67 (D17S29), TaqI (11); pHHH202 (D17S33), HindIII (13); pYNZ22.1 (D17S30), TaqI (11); YNM3 (D8S38), HindIII (14); pJCZ67 (D7S396), RsaI (15); p213-205Ed, MspI (16); pYNZ22.1 (D17S30), TaqI (11); pYNZ22.1 (D17S30), TaqI (11); pYNZ132 (D6S40), TaqI (11); pTHH59 (D15S27), Mspl (21); p79-2-23 (D16S7), MspI (22); pEFZ10 (D18S22), PvuII (23); pCZ3.1 (D19S20), BglII (24); pDZH2 (D20S56), TagI (25); pMCT15 (D21S113), MspI (26); pEFZ23 (D22S32), MspI (27).

The physical location of the chromosome 17 markers is based on previously reported mapping studies (11, 28, 29).

RESULTS

We examined pairs of somatic DNA (peripheral blood leukocytes) and tumor DNA (surgical specimens) from 15 anaplastic astrocytoma patients using a panel of eight polymorphic DNA markers for loci on chromosome 17. Six of the 15 patients (40%) showed loss of constitutional heterozygosity for loci on the p arm of chromosome 17 spanned by the following six markers: YNZ22.1, YNH37.3, MCT35.1, BHP53, 10.5, and YNM67. The number of patients showing loss of constitutional heterozygosity with these markers expressed as a percentage of informative cases ranged from 55% with marker YNZ22.1 to 11% with YNM67 (Table 1). None of the 15 patients showed loss of heterozygosity for loci identified by markers HHH202 and THH59, both of which map to 17q. Fig. 1A shows representative autoradiograms from one anaplastic astrocytoma patient (A4) demonstrating loss of constitutional heterozygosity at the locus identified by marker YNH37.3 but conservation of marker locus YNM67. These results indicate that alleles for several loci on chromosome 17p were lost in a subset of patients with anaplastic astrocytoma.

To determine whether loss of heterozygosity for loci on chromosome 17 was found in both types of malignant astrocytomas, we examined pairs of leukocyte DNA and tumor DNA from 20 patients with glioblastoma multiforme with the same panel of chromosome 17 markers used to study the anaplastic astrocytoma patients. Loss of constitutional heterozygosity for loci on chromosome 17 was found in eight of the 20 glioblastoma patients (40%). The number of patients showing loss of heterozygosity in tumor DNA expressed as a percentage of informative cases ranged from 50% with markers MCT35.1 and 10.5 to 7% with YNM67 (Table 2). Fig. 1B shows representative autoradiograms from one glioblastoma patient (U7) demonstrating loss of constitutional heterozygosity for marker locus YNZ22.1. Conservation of marker locus THH59. We concluded from these results that loss of constitutional heterozygosity for loci on chromosome 17 was found in both anaplastic astrocytoma and glioblastoma multiforme with equal frequency.

To identify specific loci on chromosome 17 that were lost in malignant astrocytomas, we examined the pattern of allele loss among the 14 patients that showed loss of constitutional heterozygosity for loci on chromosome 17 (Fig. 2). In tumor DNA from five patients with anaplastic astrocytoma (A1, A4, A5, A3, and A15) and four with glioblastoma (U4, U7, U8, and U20), loci on 17p were lost while loci on 17q were conserved, indicating that regions on the p arm of chromosome 17 were lost and that loss of sequences on 17p was common to both types of malignant astrocytomas. The subchromosomal location of a candidate recessive oncogene on 17p is indicated by the set of markers that define the smallest common region of chromosome loss. In tumor DNA from three anaplastic astrocytoma patients (A1, A4, and A5) and four glioblastoma patients (U4, U7, U8, and U14), marker YNM67, which has been mapped previously to physical locus p11.2, was conserved and markers located more distally on 17p were lost. In one glioblastoma patient (U12), marker YNZ22.1 which maps to locus p13.3, was conserved while marker THH59 on 17q was lost. Although the pattern of allele loss in patient U12 suggests that YNZ22.1 defines the upper limit of the common region of loss on 17p, lack of informative markers between YNZ22.1 and THH59 in that patient raises the possibility that sequences lost were only lost from the q arm through mechanisms distinct from those causing loss of sequences on 17p.

In two glioblastoma patients (U15 and U22), constitutional heterozygosity was lost with every informative marker spanning both p and q arms of chromosome 17. The patterns of allele loss observed in these two patients indicated loss of one entire copy of chromosome 17 in each patient's tumor cells rather than a subchromosomal deletion. Taken together, these results indicated that the smallest common region of loss on chromosome 17p is defined by the set of markers distal to YNM67 (vertical bar in Fig. 2) and the location of the candidate recessive oncogene lies between physical loci p11.2 and pter.

To ascertain that the chromosome 17 losses that we observed in the malignant astrocytomas were specific genetic events rather than manifestations of a generalized process of chromosome loss we examined pairs of leukocyte DNA and tumor

Table 1. Loss of constitutional heterozygosity for loci on chromosome 17 in 15 patients with anaplastic astrocytoma

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of informative patients</th>
<th>Patients with loss of constitutional heterozygosity</th>
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</thead>
<tbody>
<tr>
<td>YNZ22.1</td>
<td>11</td>
<td>6 55</td>
</tr>
<tr>
<td>YNH37.3</td>
<td>11</td>
<td>4 36</td>
</tr>
<tr>
<td>MCT35.1</td>
<td>9</td>
<td>2 22</td>
</tr>
<tr>
<td>BHP53</td>
<td>6</td>
<td>2 33</td>
</tr>
<tr>
<td>10.5</td>
<td>8</td>
<td>2 25</td>
</tr>
<tr>
<td>YNM67</td>
<td>9</td>
<td>1 11</td>
</tr>
<tr>
<td>HHH202</td>
<td>9</td>
<td>0 0</td>
</tr>
<tr>
<td>THH59</td>
<td>9</td>
<td>0 0</td>
</tr>
</tbody>
</table>

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Fig. 1. RFLP analysis of chromosome 17 alleles in one patient (A4) with anaplastic astrocytoma (●) and one patient (U7) with glioblastoma multiforme (□). Genomic DNA (5 μg) from peripheral blood leukocytes (L) and tumor tissue (T) obtained from each patient was digested with restriction enzyme, electrophoresed through 1% agarose gels, transferred to nylon filters, and hybridized to 32P-labeled DNA probes specific for the chromosome 17 loci indicated. Dried filters were exposed to X-ray film at —70°C for 24–72 h to generate the autoradiograms shown. On each autoradiogram, the length of each polymorphic restriction fragment is expressed in kilobase pairs. The restriction enzymes used were TaqI (YNH37.3, YNM67, THH59) and RsaI (YNZ22.1).

Table 2 Loss of constitutional heterozygosity for loci on chromosome 17 in 20 patients with glioblastoma multiforme

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of informative patients</th>
<th>Patients with loss of constitutional heterozygosity</th>
<th>Number</th>
<th>% of informatics</th>
</tr>
</thead>
<tbody>
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<td>YNZ22.1</td>
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<td>YNH37.2</td>
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<td>33</td>
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<td>MCT35.1</td>
<td>8</td>
<td>4</td>
<td>50</td>
<td></td>
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<tr>
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<tr>
<td>10.5</td>
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<td>5</td>
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<tr>
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<tr>
<td>HHH202</td>
<td>8</td>
<td>1</td>
<td>13</td>
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<tr>
<td>THH59</td>
<td>14</td>
<td>3</td>
<td>21</td>
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DNA from the anaplastic astrocytoma and glioblastoma patients with a panel of 20 polymorphic DNA marker for 18 additional chromosomes (Table 3). Only one marker, TBB2 (c-H-ras) on chromosome 11p, detected loss of constitutional heterozygosity with a frequency approaching that of the chromosome 17p markers in the region of smallest common loss (29% of informatics).

DISCUSSION

We report here that loss of constitutional heterozygosity for loci on the p arm of chromosome 17 was found in a significant proportion of patients with anaplastic astrocytoma and glioblastoma multiforme; the two histopathological types of malignant astrocytoma. This finding suggests that a recessive oncogene important in the development of malignant astrocytoma may be present on 17p. The physical location of the candidate-recessive oncogene on chromosome 17 can be inferred from the smallest common region of chromosome loss among the patients showing loss of heterozygosity. Our mapping data revealed a region of loss on chromosome 17 between physical loci p11.2 and pter that was common to both types of malignant astrocytomas.

These results support the findings of a recently reported study wherein this same region of chromosome 17p was found to be the site of frequent mitotic recombinations in astrocytomas (30). Loss of heterozygosity for loci on chromosome 17p has also been found in association with neoplasms arising outside the central nervous system, namely, carcinomas of colon, lung, and breast (31–33). Since these tumors share with malignant astrocytoma a common region of loss on chromosome 17, it is possible that a single gene important in these diverse neoplasms may be present in this region.

The gene encoding the human tumor antigen p53 has been localized previously to physical locus p13.1 of chromosome 17 (12, 29). This locus is within the region of chromosome loss that we observed in the malignant astrocytomas. The p53 gene has properties of a recessive oncogene in that inactivation of
The gene's normal function may lead to neoplastic transformation (for review see Ref. 34). In a recent report, DNA from a colorectal carcinoma showed a deletion involving locus p13 on one copy of chromosome 17 and a point mutation in the p53 gene on the other copy of chromosome 17 indicating that the p53 gene is a potential target of the 17p deletions found frequently in colorectal carcinomas (35). Since p53 is implicated in the process of neoplastic transformation and since human p53 maps to the same region of chromosome 17 where alleles were lost in malignant astrocytomas, the p53 gene must be considered a candidate target for 17p deletions in malignant astrocytomas as well.

The gene for von Recklinghausen's neurofibromatosis (NF1) is also present on chromosome 17. An increased incidence of astrocytomas is found among individuals with NF1 (36). Chromosome mapping studies have indicated that the markers most tightly linked to the NF1 gene are located on the proximal q arm of chromosome 17 (28, 37). One marker that is tightly linked to the NF1 locus (HHH202) was used in this study. Marker HHH202 detected loss of heterozygosity in only one patient (U15). That patient, however, showed loss of heterozygosity for every informative marker on chromosome 17 indicating loss of one entire copy of chromosome 17 in tumor cells rather than a subchromosomal deletion. Two patients in our series had NF1. One NF1 patient (A6) with anaplastic astrocytoma showed no loss of heterozygosity with informative markers that spanned both p and q arms of chromosome 17. The other NF1 patient (U22) with glioblastoma showed a pattern of allele loss that indicated loss of one copy of chromosome 17 rather than a subchromosomal deletion (Fig. 2). These results suggest that the genetic loci for malignant astrocytoma and NF1 are distinct.

Loss of heterozygosity for specific chromosomal loci in association with a particular tumor type suggests that the involved chromosomal loci may contain genes that are targets for cancer-causing mutations. Alternatively, the observed loss of heterozygosity may represent random loss of genetic material in a population of transformed cells. The uncontrolled mitotic activity characteristic of malignant tumor cells may preclude orderly segregation of chromosomes and lead to random chromosome loss. The RFLP data in Table 3 show that a small percentage of patients with malignant astrocytomas show loss of heterozygosity for loci on various chromosomes. A background level of allele loss has been reported by others in RFLP analyses of not only malignant astrocytomas but also colorectal carcinomas (6, 38). Nevertheless, the substantially higher percentage of patients showing loss of heterozygosity for loci on chromosome 17 coupled with the loss of discrete regions on the p arm of chromosome 17 strongly suggests that the observed patterns of allele loss reflect nonrandom genetic alterations.

One marker, TBB2 (c-H-ras) on chromosome 11p, detected loss of heterozygosity in the malignant astrocytoma patients...
with a frequency approaching that of the chromosome 17p markers (Table 3). Loss of heterozygosity for loci on 11p has been reported in other human neoplasms, namely, rhabdomyosarcoma, hepatoblastoma, Wilm's tumor, and transitional cell carcinoma of the bladder (39, 40). When considered in light of these findings, the loss of heterozygosity for loci on 11p found in the malignant astrocytomas may indicate that loss of genes on chromosome 11 is important in the development of astrocytomas as well.

We did not find chromosome 17 loss in all of the malignant astrocytomas that we examined. Those tumors without gross genomic alterations, however, may still harbor small deletions on chromosome 17 that are beyond the limits of detection with the markers currently available. Alternatively, the putative recessive oncogene located on chromosome 17p may contain a point mutation which cannot be detected by RFLP analysis. In addition, we have not determined the precise molecular mechanisms involved in loss of constitutional heterozygosity. Either deletion or mitotic recombination could bring about the observed loss of sequences on chromosome 17p.

A growing body of experimental evidence indicates that multiple genes on different chromosomes are involved in the process of neoplastic transformation in the astrocyte lineage (41-44). One model that has been proposed for the genesis of glioblastoma multiforme postulates that progression of astrocytomas through stages of increasing malignancy occurs by accumulation of several genetic alterations (6). We have shown that tumor DNA from patients with anaplastic astrocytoma frequently show loss of heterozygosity for loci on chromosome 17. Furthermore, loss of heterozygosity for loci on chromosomes 10 and 17 is commonly found among patients with the more malignant glioblastoma multiforme. While these findings support a progressional model for glioblastoma, it will be important to examine tumor DNA from individual patients who have shown tumor progression from anaplastic astrocytoma to glioblastoma to determine whether histopathological progression correlates with accumulation of chromosomal deletions.

ACKNOWLEDGMENTS

The authors thank Carolyn Pedone for skillful technical assistance, Dr. M. Peter Heilbrun for critical discussions, and Dr. Jeanette Townsend for review of the neuropathology.

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