Evidence for the Involvement of a Potential Second Tumor Suppressor Gene on Chromosome 17 Distinct from p53 in Malignant Astrocytomas

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Abstract

Molecular analysis of malignant astrocytomas demonstrated three distinct groups of tumors with chromosome 17p abnormalities, which include (a) deletion of the p53 locus (17p13.1) and mutations in the remaining allele, (b) deletion of the p53 locus but no detectable mutations in the remaining allele, and (c) deletions not including the p53 locus but mutations in one of the alleles. Furthermore, deletion mapping analysis demonstrated allelic loss of genes distal to D17S28/D17S5 markers in group C tumors. The loss of heterozygosity of genes on chromosome 17 without detectable mutation (group B) or deletion (group C) in the p53 gene implies the presence of a second tumor suppressor gene in the telomeric region of 17p, the homologous functional inactivation of which may play a role, either alone or in conjunction with p53, in the initiation and/or progression of astrocytic neoplasms.

Introduction

Deregulated cell growth associated with neoplasia is a consequence of multiple genetic aberrations accumulated over time. Mutations in protooncogenes, which facilitate cell proliferation, as well as in tumor suppressor genes, which prevent or suppress cell proliferation, contribute to the emergence of a malignant phenotype. There is evidence for the functional inactivation of tumor suppressor genes in many human neoplasms. Homologous functional inactivation of a tumor suppressor gene is often accomplished by mutations in one copy of the gene combined with physical loss of the chromosomal region harboring the remaining allele of the gene. Comparison of the constitutional and tumor genotypes by RFLP analysis has demonstrated allelic deletions on specific chromosomes in many human malignancies, suggesting shared chromosomal lesions in diverse cancers. For example, deletion of sequences on chromosome 17 occurs in a broad spectrum of human neoplasms, including cancers of the colon, lung, breast, bone, ovary, and brain. It is widely believed that the target of these deletions is the gene and mutations of the p53 gene in the telomeric region of 17p, the homologous functional inactivation of which may play a role in the malignant form of the disease, glioblastoma multiforme (20, 29-31), whereas allelic losses of genes on chromosome 17 and mutations of the p53 gene have been reported to be associated with low- and high-grade astrocytomas (32-36). Our analysis of 40 glial tumors for gene losses on chromosome 17 and for mutations of the p53 gene suggests a crucial role for the p53 gene in the evolution of glioblastomas. Furthermore, in addition to p53, our data indicate the presence of at least one other target gene in the telomeric region of the short arm of chromosome 17.

Materials and Methods

Tumor Specimens and Histopathology. At surgery tumors were carefully separated from the surrounding normal brain, and the central part of the tumor was quickly frozen in liquid nitrogen and stored at −80°C. The tumors were graded according to the guidelines of the WHO (38). Of the 40 tumors analyzed in this study, 34 were grade IV glioblastoma multiforme (24 primary and 10 recurrent), three were grade III anaplastic astrocytoma (2 primary and 1 recurrent), and three were oligoastrocytoma. Blood samples, obtained at surgery, were used to isolate lymphocyte DNA and served as normal controls.

RFLP Analysis. DNA was isolated from the peripheral blood lymphocytes and frozen tumor tissues by sodium dodecyl sulfate-proteinate K digestion followed by phenol/chloroform extraction according to established methods (39). Ten μg of DNAs from matching lymphocytes and tumors were digested with appropriate restriction enzymes, electrophoretically separated in 0.8% agarose gels, and transferred to Nytran membranes. The following probes (obtained from the American Type Culture Collection, Rockville, MD) were used for the RFLP analysis: D17S34 (p13.3); D17S28 (p13.3); D17S5 (p13.3); D17S31 (p13.1-p11.2); p53 (p13.1); D17S71 (p11.2); and D17S4 (q23-25.3). Southern hybridization was performed using random primed radiolabeled insert probes (specific activity, >10⁶ cpm/μg of DNA). Hybridization, subsequent washing of the filters, and autoradiography were performed as previously described (40). In some cases where the patients were constitutionally homozygous for BamHI and Scal polymorphism at the p53 locus, PCR-based amplification of a 250-base pair genomic fragment containing the Thal polymorphism (41) at codon 72 of exon 4 of the p53 gene was used.

PCR Amplification and Sequencing of the p53 Gene. A 1.9-kilobase fragment of the p53 gene covering exons 5-9 was amplified using 200 ng of the genomic DNA and 1 μM concentration of the 5'-CATCGAATTCGTAGGAATTCACTTGTGCCCTGACTT-3' (sense) and 5'-CATCGAATTCGAAAACATTCCACCTGAGT-3' (antisense) primers (42), which contained EcoRI restriction sites at the 5' ends to facilitate cloning of the PCR products. The amplified double-stranded DNAs were digested with EcoRI and ligated into the EcoRI site of the plasmid pUC19. The plasmids were transformed into competent XL1-blue cells, and 50 colonies were randomly selected for DNA sequencing validation.

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The abbreviations used are: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

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fragments were spin-dialyzed through Centricon-100 columns (Amicon, Danvers, MA) and either cloned into Bluescript plasmid vector or used as templates in an asymmetric PCR reaction using a 1:100 dilution of the limiting primer. Sequencing of exons 5–9 of most tumors were carried out by two independent methods: (a) using the cloned DNA from pooled plasmids as the template for sequencing and (b) direct sequencing of the asymmetric PCR products was carried out for the remaining exons 1–4 and 10 and 11 using the sequencing primers described by Lehman et al. (43). Sequencing was performed by the dideoxy chain termination method using Sequenase kit reagents (United States Biochemicals).

Results

Deletion Mapping of Genes on Chromosome 17 and Sequencing of the p53 Gene. Twelve of the 40 (30%) tumors analyzed had loss of heterozygosity of one or more markers on chromosome 17, whereas heterozygosity of informative markers was maintained in 28 (70%) tumors. The 12 tumors with loss of heterozygosity of chromosome 17 were further distinguished into three groups based upon the results of deletion mapping and p53 gene mutation analyses (Table 1); representative examples are shown in Fig. 1. Group A consisted mostly of tumors with loss of heterozygosity of several markers on the short arm of chromosome 17, including the p53 locus, and a missense mutation in the other copy of the p53 gene (Table 1). One example of these tumors (tumor from patient 14) is shown in Fig. 1A. Reduction to homozygosity was detected at multiple markers on 17p including the p53 gene; the remaining copy of the p53 gene contained a mutation in codon 273 of exon 8. In the tumor DNA from patient 37, compared to other tumors in this group, a relatively small region of homozygosity was detected which was centered at the p53 locus; among the six 17p markers analyzed, reduction to homozygosity occurred only at the D17S31 marker, whereas heterozygosity was maintained at all other informative markers. Although the p53 locus was not informative in patient 37, sequencing analysis showed presence of only the mutated copy of the p53 gene in the tumor, suggesting that p53 was included in the deleted region. The C to T transition in codon 306 of exon 8 produced a premature termination codon that would generate a truncated protein (Fig. 1A).

The two tumors (tumors 22 and 32) in group B, like those in group A, had deletion of multiple markers on chromosome 17p. Fig. 1B shows an example of the tumor from patient 32 in which somatic loss of heterozygosity of all six 17p markers occurred. Sequencing of exons 5–9 in tumors from patients 22 and 32 revealed no mutation in this region. Since mutations in exons other than 5–8 or in introns have occasionally been reported (33, 44), the remaining exons (exons 1–4, 10, and 11) were sequenced in these two tumors. No mutation was detected in the entire coding region or the splice junctions between all 11 exons of the p53 gene.

Fig. 1C displays RFLP analysis of the 17p markers in the lymphocyte and tumor DNAs from the three patients of group C. In the tumors from all three patients the telomeric region of chromosome 17p13.3 was the target of deletion. In patient 10, a reduction to homozygosity was observed in the tumor DNA at all three markers, D17S5, D17S28, and D17S34, mapped to 17p13.3. In tumors from patients 4 and 7, however, allelic loss was seen only at the D17S34 locus, whereas constitutional heterozygosity was maintained at the D17S5 and D17S28 markers. Although the p53 gene was not included in the allele loss region of any of these three tumors, sequence analysis demonstrated mutations in the p53 gene (codon 273 in exon 8, codon 194 in exon 6, and codon 151 in exon 5 in patients 4, 7, and 10, respectively) and the presence of one wild-type and one mutated copy of the p53 gene in all three tumors (Fig. 1C).

Since p53 gene mutations sometimes occur in the absence of gene deletions on chromosome 17, seven of the 28 tumors that retained both copies of chromosome 17 were analyzed for p53 mutations. No mutation was detected in exons 5–9 (a region which harbors more than 95% of reported mutations from multiple cancers) of any of the seven tumors.

p53 Gene Mutations in Glial Tumors. Table 2 summarizes details of p53 gene mutations in the 10 tumors belonging to groups A and C. Although all 10 mutations were in exons 5–8, four of them were outside the four conserved “hot spot” domains. Of the 10 mutations, codon 273, which is one of the

Table 1 Deletion mapping analysis of polymorphic markers on chromosome 17 in glial tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>D17S34</th>
<th>D17S28</th>
<th>D17S5</th>
<th>D17S31</th>
<th>p53</th>
<th>D17S71</th>
<th>D17S4</th>
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<tr>
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<td>M/Ta</td>
<td>H/T</td>
<td>M/T</td>
<td>M/T</td>
<td>M/T</td>
<td>M/T</td>
<td>M/T</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
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<td>-</td>
</tr>
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<td>5 GMB(R)</td>
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<tr>
<td>13 AA(P)</td>
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<tr>
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<td>1-10</td>
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<td>32 GBM(R)</td>
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</tr>
</tbody>
</table>

a M, mutant allele; W, wild-type allele; B, BamHI, M, MspI, P, PvuII, S, SacI, T, TagI; NI, not informative; ND, not done; +, heterozygous; -, loss of heterozygosity; P, primary; R, recurrent; GBM, glioblastoma multiforme; AA, anaplastic astrocytoma. Deleted alleles are boxed; a noninformative marker is included in the deleted region only if loci at either side of this marker are deleted. Patient 37 is an exception; although the p53 locus in this patient was not informative, sequence analysis showed the presence of only the mutated allele (see Fig. 1 and text), implying deletion of the wild-type allele.
Fig. 1. Loss of heterozygosity at loci on chromosome 17 and point mutations in the p53 gene in glial tumors. L, lymphocyte DNA; T, tumor DNA.
A potential second tumor suppressor gene on 17p in astrocytomas

Table 2 Mutations of the p53 gene in glioblastomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Allele</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>AA changes</th>
<th>Conserved region</th>
<th>Conversion</th>
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<td>M*</td>
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<td>270</td>
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<td>Phe→Leu</td>
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<td>5</td>
<td>M</td>
<td>8</td>
<td>273</td>
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<td>Arg→His</td>
<td>Y</td>
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<tr>
<td></td>
<td>13</td>
<td>M</td>
<td>5</td>
<td>145</td>
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<td>Leu→Arg</td>
<td>Y</td>
</tr>
<tr>
<td></td>
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<td>M</td>
<td>8</td>
<td>273</td>
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<td>Arg→Cys</td>
<td>Y</td>
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<tr>
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<td>25</td>
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<td>7</td>
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<td></td>
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<td>5</td>
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<td>CTT→CGG</td>
<td>Leu→Pro</td>
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<td>M</td>
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<td>306</td>
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<td>Arg→Stop</td>
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<tr>
<td>C</td>
<td>4</td>
<td>WM</td>
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<td>Arg→Cys</td>
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<td>7</td>
<td>WM</td>
<td>6</td>
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<td>Leu→Pro</td>
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<tr>
<td></td>
<td>10</td>
<td>WM</td>
<td>5</td>
<td>151</td>
<td>CCC→GCC</td>
<td>Pro→Ala</td>
<td>N</td>
</tr>
</tbody>
</table>

* M, mutant; W, wild type; Y, yes; N, no; TS, transition; TV, transversion.

Discussion

Mutational inactivation of the p53 gene occurs in many human neoplasms. The data presented here support the crucial involvement of the p53 gene in the tumorigenic process of astrocytomas and suggest the potential contribution of another tumor suppressor gene on the short arm of chromosome 17 in glioblastomas. In 7 of 12 tumors (58%) that sustained somatic deletions on chromosome 17, we detected homozygous inactivation of the wild-type p53 gene through loss of one copy and mutation in the other (group A tumors), a pattern that conforms to the classical paradigm of tumor suppressor genes. However, the remaining 42% of tumors with loss of heterozygosity on chromosome 17 had either no detectable mutation (group B tumors) or deletion (group C tumors) of the p53 gene. In these tumors as well as in the 28 tumors without gene losses on chromosome 17 or mutation in the p53 gene, the oncogenic process may have still targeted the p53 gene by other mechanisms. Sequestering of the wild-type p53 protein, observed in the cytoplasm of a significant number of breast tumors, may represent one such mechanism (45). Amplification of the MDM2 gene, whose protein product binds to the p53 protein (46), has been observed in many sarcomas (47), suggesting that complexing of the MDM2 protein with p53 may provide yet another mechanism of its inactivation. Analysis for possible genetic alterations of the MDM2 gene in all 40 gliomas examined in this study showed no evidence of amplification or other rearrangements (data not shown). It is conceivable that in some glioblastomas the negative regulatory function of the p53 gene is compromised by similar or heretofore unidentified mechanisms. Alternatively, mechanisms other than the inactivation of the p53 gene may exist on chromosome 17 involving other tumor suppressor genes. The latter possibility has been suggested for some breast tumors that sustain gene losses on chromosome 17 and retain a single wild-type p53 allele (25).

The data from group B and group C tumors in our study support the presence of another tumor suppressor gene on chromosome 17 distinct from p53 in glioblastomas. In the two group B tumors, which lost one copy of the p53 gene, there was no mutation in exons 5–9, and the search for rare mutations in the rest of the exons and the intron/exon boundaries of all 11 exons was unsuccessful. Although mutations in the intronic sequences affecting the transcription of the p53 gene cannot be ruled out, in these tumors deletions may be targeting a gene other than p53 on chromosome 17.

The presence of another potential tumor suppressor gene on chromosome 17 in glioblastomas is further suggested, and its map position defined, by somatic deletions in tumors of group C. The p53 locus was not included in the region of allele loss in the three tumors (4, 7, and 10) of this group. Furthermore, retention of constitutional heterozygosity at two 17p13.3 loci, D17S5 and D17S28, but reduction to homozygosity at another 17p13.3 locus, D17S34, in tumors 4 and 7 define the proximal boundary of the deleted region harboring a potential tumor suppressor gene between the D17S5/D17S28 and D17S34 markers. The unavailability of polymorphic markers in the telomeric region distal to the D17S34 locus (13) did not allow definition of the distal boundary of the deleted region.

All three tumors of group C contained one normal and one mutated copy of the p53 gene. The hypothesis that tumors with one wild-type and one mutant allele of the p53 gene may represent an intermediate stage during tumor progression, eventually leading to the loss of the remaining wild-type copy (42), may not be relevant in the case of glioblastomas, which are very advanced and malignant tumors at presentation. Furthermore, two of these tumors, from patients 4 and 7, were recurrent tumors, whereas the tumor from patient 10 was a primary tumor. This tumor recurred 8 months after surgery, and the 17p deletions and p53 mutation profile of the recurrent tumor was identical to that of the primary tumor (data not shown).

The role of the p53 gene in tumors of group C, however, remains speculative at the present time. Several mutant forms of p53 protein have been proposed to confer growth advantage on cells and function in a dominant negative fashion (15), presumably by blocking the physiological function of the wild-type form by oligomerization (48). Normal p53, on the other hand, seems to negatively regulate the cell growth probably by virtue of its transcriptional regulatory activity (49). Furthermore, the phenotypic dominance of the normal p53 gene over its mutant form in a two-allele configuration has been demonstrated (50). The functions of wild-type and/or mutant forms of p53 may be modulated by the nature of the p53 mutation as well as by the microenvironment resulting from various patterns of genetic mutations in a particular tumor type. It is conceivable that in glioblastomas the homozygous functional loss of a gene in the telomeric region of chromosome 17 may enhance the dominant negative action of the mutant form of p53 in the presence of its normal allele. This notion is further supported by the fact that the region distal to the D17S5/D17S28 markers was always
included in chromosome 17 deletions in all of the tumors analyzed here except for one tumor (patient 37) from group A. The tumorigenic process involves many mutational steps on multiple chromosomes and considerable interactions between various mutant products. For example, in Wilms' tumor (22, 23), neurofibrosarcoma (24), breast cancer (25–26), renal cancer (27), and lung cancer (28), single chromosomes seem to be the site of multiple independent mutations, which may either cooperate with each other or provide alternative genetic mechanisms for tumor development. Here we provide evidence for the presence of another potential tumor suppressor gene distinct from p53 on chromosome 17 in glioblastomas and define its map position in the telomeric region of the short arm of chromosome 17. This gene may act alone or in conjunction with the p53 gene. Future studies aimed at the cloning and characterization of this gene should provide a better understanding of the various mutational mechanisms involved in glioblastomas.

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References


A POTENTIAL SECOND TUMOR SUPPRESSOR GENE ON 17p IN ASTROCYTOMAS


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