Accumulation of Wild Type p53 Protein in Human Astrocytomas

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Abstract

We have previously described 10 astrocytomas with accumulation of p53 protein but no mutations in p53 exons 5–8, and we have suggested that they may represent overexpression of wild type protein or mutations in less conserved regions of the gene. To investigate these possibilities further, we studied the tumors with immunohistochemistry for wild type and mutant p53 protein and showed that all cases stained with the wild type Pab 1801 antibody but only one case stained with the mutant-specific Pab 240 antibody. To support the hypothesis that the accumulated p53 protein is wild type in most cases, we used single-strand conformation polymorphism analysis and DNA sequencing to evaluate p53 exons 4, 9, and 10 and did not detect mutations at these loci. Although the product of the MDM2 oncogene binds wild type p53 and may account for p53 accumulation, slot-blot analysis of these astrocytomas did not detect MDM2 gene amplification. Thus, evidence suggests that some astrocytomas may accumulate wild type p53 protein but not as a result of MDM2 gene amplification. Furthermore, Pab 1801 immunohistochemistry may not be an adequate method of screening astrocytomas for p53 mutations.

Introduction

Mutations of the p53 tumor suppressor gene are common in astrocytomas and may be associated with elevated levels of p53 protein (1, 2). Recent studies of other human cancers, however, have suggested that p53 gene alterations are not necessarily associated with immunohistochemically detectable p53 protein and that high levels of p53 protein may exist without mutations in exons 5–8 (3–5). We recently studied 34 astrocytomas using immunohistochemistry on fixed, embedded tissues with the monoclonal antibody Pab 1801 to demonstrate p53 protein accumulation, and SSCP and DNA sequence analysis of exons 5–8 to reveal mutations in the p53 gene (2). Ten tumors had p53 protein accumulation but no mutations by SSCP. We postulated that cases might have elevated levels of wild type p53 protein or p53 mutations outside of the conserved exons. To determine whether the accumulated protein was wild type or mutant, we used immunohistochemistry on frozen tumor tissues with antibodies Pab 240, which is mutant specific, and Pab 1801, which reacts with both wild type and mutant proteins. To confirm that these tumors did not harbor mutations in the less-conserved regions of the p53 gene, we performed SSCP on exons 4, 9, and 10, since rare mutations have been reported in these exons. Finally, because the product of the MDM2 oncogene binds to p53 protein, MDM2 gene amplification could lead to increased levels of MDM2 protein and, in turn, wild type p53 protein (6, 7). We therefore used slot-blot analysis of tumor and normal DNA to assay these tumors for MDM2 gene amplification.

Materials and Methods

Cases. All tumors were cerebral hemispheric gliomas from adults that were classified by a neuropathologist (D. N. L.) according to the WHO criteria. Cases 114 and 308 were astrocytoma, WHO grade II; cases 10, 12 and 82 were anaplastic astrocytoma, WHO grade III; cases 150 and 238 were anaplastic oligoastrocytoma, WHO grade III; and cases 18, 228, and 294 were glioblastoma multiforme, WHO grade IV. All cases were from primary resection specimens before the patients had been treated with either radiation or chemotherapy. These 10 tumors are a subset of those included in a previous study (2). Frozen tumor tissue was available for immunohistochemistry on seven of 10 cases (cases 10, 12, 18, 82, 228, 238, and 308). Tumor and blood DNA was available for SSCP and slot-blot analysis on nine of 10 cases (cases 10, 12, 18, 114, 150, 228, 238, 194, and 308).

Immunohistochemistry. Eight-μm sections of frozen tumors were cut on a cryostat and stored at −80°C. Sections were fixed in cold acetone, blocked with normal horse serum, and incubated with either Pab 1801 (diluted 1:4000) or Pab 240 (diluted 1:100) (Oncogene Science, Manhasset, NY) for 1 h at 4°C. Tissues were pretreated in 0.05% saponin before Pab 240 incubation. The sections were then exposed to biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA) and Streptavidin-HRP (Jackson Immuno Research, West Grove, PA), followed by a diaminobenzidine solution and methyl green counterstain. Controls were performed by omitting the primary antibody and by staining the human breast cancer cell line HTB26 (mutant p53) and human bladder cancer cell line HTB5 (wild type p53) (American Type Culture Collection, Rockville, MD) as positive and negative controls, respectively. In addition, normal human cerebellum was used as a negative tissue control. The slides were evaluated by two observers (M.-P. R., D. N. L.) for the presence and proportion of positively stained cells and the subcellular localization of the reaction product.

SSCP Analysis and DNA Sequencing. SSCP was performed as described (2, 8), with minor modifications. Exons 4, 9, and 10 of the p53 gene were amplified from tumor DNA using PCR conditions and oligonucleotides similar to those of Toguchida et al. (9), with the following exceptions. For exon 4, the primers used were 5'-ACTTCCTGAAAACAACGTTCT-3' and 5'-GAATC-CTAAAAGTGCTCAACA-3'. These primers amplify a 479-base pair amplicon which, after digestion withMsp I, results in 216-, 158-, and 105-base pair fragments. The amplification products were separated on 6% nondenaturing polyacrylamide gels with 10% glycerol overnight at 3 W. SSCP results were confirmed by a second identical assay. Cases with mobility shifts on SSCP were directly sequenced with Vent (exo- ) DNA polymerase and the CircumVent Thermal Cycle kit (New England BioLabs, Beverly, MA), using the SSCP primers.

Slot-Blot Analysis. To generate a probe for MDM2, a 96-base pair amplicon from the 5' end of the published sequence (7) was amplified using PCR with the primers 5'-GTCTGTACCTACTAGTGTTG-3' and 5'-AATACT-TCAATAAGCAATTG-3' and an annealing temperature of 52°C. The PCR product was extracted with phenol:chloroform, precipitated, and run on a 4% NuSieve agarose gel (American Bioanalytical, Natick, MA). The product was isolated and cleaned with the Spin-Bind DNA extraction unit (FMC Bioproducts, Rockland, ME) and labeled with [α-32P]dATP and [α-32P]dCTP using the random priming method. Tumor DNA (300, 30, and 10 ng) and blood DNA (300 ng) were blotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using the Minifold II Slot blot System (Schleicher and Schuell). The membranes were prehybridized with salmon sperm DNA for 2 h, followed by hybridization with the labeled probe for 18 h at 59°C. Membranes were washed briefly at room temperature and X-OMAT film was exposed for 2 h at −80°C. Amplification was assessed by comparing signal intensity between tumor and blood DNA.

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3 The abbreviations used are: SSCP, single strand conformation polymorphism; WHO, World Health Organization; PCR, polymerase chain reaction.

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Results and Discussion

To determine whether the 10 astrocytomata with p53 protein accumulation and no conserved region mutations had wild type or mutant protein, we studied these tumors with immunohistochemistry using two antibodies, PAb 1801 and PAb 240. PAb 1801 is a human-specific monoclonal antibody that recognizes an epitope near the NH₂ terminus of both wild type and mutant p53 protein (10). PAb 1801, however, does not recognize most nonsense mutations (2). In this study, immunohistochemistry on frozen sections showed that all seven available cases were positive with the PAb 1801 antibody (Fig. 1, left), confirming our previous results with this antibody on formalin-fixed, paraffin-embedded tissues (2). In most cases, approximately one-third of cells stained with PAb 1801. Positive reaction product was observed only in nuclei, not in cytoplasm, and varied in intensity, with some nuclei staining more strongly than others. PAb 240 is a monoclonal antibody that reacts with an epitope in the middle of only mutant forms of mouse and human p53 protein (11). Immunohistochemistry with PAb 240 showed positive staining in only one case (case 18), with moderately strong nuclear staining in scattered cells (Fig. 1, right). No cytoplasmic staining was noted with PAb 240. The positive control cell line HTB26 showed moderate to strong nuclear staining with both PAb 1801 and PAb 240 antibodies. The normal cerebellum, the negative control cell line HTB5, and omission of the primary antibody all resulted in no immunohistochemical reaction. The results suggest that the accumulated p53 protein may be wild type in six of the seven studied astrocytomata.

To confirm that these cases did not have p53 mutations outside of exons 5–8, we performed SSCP analysis on exons 4, 9, and 10. Previous studies of astrocytomata that have included exons 2–11 have shown that p53 mutations are exceedingly rare outside of exons 5–8, with only one exon 4 mutation, one exon 9 mutation, and no mutations in other nonconserved exons in 128 astrocytomata (1, 12–14). In other human cancers, mutations outside of exons 5–8 are uncommon but have also been noted in exons 4 and 9 (9, 15). Four of the nine available astrocytomata (cases 12, 18, 150, and 228) had SSCP migration shifts in exon 4, but sequencing showed that these four shifts were caused by the common codon 72 polymorphism (CCC/CGC). No other migration alterations were noted (Fig. 2), making it unlikely that these astrocytomata have mutations in p53 coding regions. Intrinsic mutations cannot be excluded in these cases but have been noted only rarely in astrocytomata (1, 16) and other human tumors (17). It is possible that our one case (case 18) with positive PAb 240 staining has an intrinsic mutation. For the remaining cases, however, these data provide further support that the accumulated p53 protein may be wild type.

Cases of lung (4), breast (18, 19), and ovarian carcinoma (20) have been reported that have p53 protein accumulation, as detected by PAb 1801 immunohistochemistry, but no mutations in exons 2–11. PAb 240 immunohistochemistry, however, was not performed on these cases to distinguish wild type from mutant protein. Davidoff et al. (21) have reported overexpression of a stable wild type p53 protein in four of five human neuroblastoma cell lines. The p53 protein in these cell lines was detected by immunoprecipitation with both PAb 1801 and PAb 421, which react with both the wild type and mutant forms of p53, but could not be detected with the mutant-specific PAb 240. The cell lines had no mutations in the conserved regions of the p53 gene. Furthermore, Barnes et al. (22) have documented accumulation of wild type p53 protein in the normal cells of two members of a cancer-prone family. Although high levels of wild type p53 would be assumed to have a tumor suppressor function, abnormally bound p53 may be inactive, as is hypothesized for normal p53 bound to viral oncoproteins. Furthermore, accumulated wild type p53 protein may be sequestered in the cytoplasm, away from its nuclear targets (23), although we did not observe distinct cytoplasmic staining in our cases. Tuck and Crawford (24) have shown that overexpression of wild type p53 in normal fibroblasts may enhance the tumorigenic potential of these cells. High levels of wild type p53 protein may also accumulate in rapidly proliferating nonneoplastic cells, and it is possible that similar high levels may arise in dividing tumor cells independent of p53 mutation (3, 25). Finally, nuclear levels of wild type p53 protein may increase after various types of DNA damage, and such accumulation may represent a common response to DNA injury (26). In summary, increasing evidence argues that wild type p53 protein levels may be elevated in the absence of gene mutations in some cases. At the same time, it remains possible that elevated wild type p53 protein may be associated with mutations in intrinsic sequences that are not examined by current molecular genetic techniques.

Wild type p53 protein has a short half-life and is generally not detectable by immunohistochemistry (27), whereas mutant p53 proteins often have extended half-lives (28) and can be detected immunohistochemically. In our cases, therefore, the wild type protein could have an extended half-life. One possible explanation is that wild type...
p53 is bound by another protein which elongates its half-life. A candidate for such a protein is the p90 product of the MDM2 oncogene. p90 binds to both wild type and mutant p53 protein and has transforming properties (6). MDM2 amplification has been noted in approximately one-third of human sarcomas (7). For these reasons, we evaluated our cases for MDM2 gene amplification. Slot-blot analysis demonstrated equal amounts of MDM2 gene in tumor and blood DNA in the nine astrocytomas studied (Fig. 3). Another study has recently reported a similar lack of MDM2 amplification in human astrocytomas (29). Other mechanisms must therefore exist to produce accumulation of wild type p53 protein in a subset of human astrocytomas. Recent data have suggested that increased protein stability is a more likely mechanism for wild type p53 protein accumulation than p53 overexpression (26). Further elucidation, however, awaits the identification of other human proteins that bind p53 and clarification of factors that control p53 expression (30).

The present findings provide further evidence that immunohistochemistry using the PAb 1801 antibody does not correlate well with p53 gene analysis in human astrocytic tumors. We have previously shown that PAb 1801 does not stain astrocytoma cells with nonsense mutations (2). In turn, the present findings indicate that PAb 1801 may not reliably distinguish wild type from mutant protein in human astrocytomas. Jaros et al. (31) recently demonstrated that PAb 1801 immunopositivity correlates with poor clinical survival in astrocytoma patients and attributed the immunopositivity to presumed mutations in p53. An alternative explanation may be that PAb 1801 immunopositivity reflects accumulated wild type p53 protein and that tumors with high levels of normal p53 protein are less susceptible to the DNA-damaging effects of radiation and chemotherapy than those tumors with p53 gene mutations (30). The biological basis of clinical correlations with PAb 1801 immunohistochemistry may therefore need to be reevaluated in light of the present data.

References
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