The Relationship between TP53 Mutations and Overexpression of p53 and Prognosis in Malignant Gliomas of Childhood


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ABSTRACT

The prognosis for children with high-grade gliomas remains somewhat unpredictable. Although prolonged disease control is sometimes achieved after surgery, radiotherapy, and chemotherapy, most patients exhibit rapid disease progression. Because p53-dependent apoptosis mechanisms are involved in the cytotoxic effects of irradiation and chemotherapy, we questioned whether p53 status might be associated with outcome in childhood malignant gliomas. Therefore, we examined p53 status, both immunohistochemically and by direct sequencing of exons 5-8, in a series of 29 archival pediatric malignant non-brainstem gliomas treated consecutively at our institution between 1975 and 1992. Eighteen tumors had dense p53 staining in the majority of cells, although only 11 had mutations of the p53 gene (TP53). On univariate analysis, there was a significant association between p53 overexpression and a shorter progression-free survival (PFS) and overall survival (OS; P = 0.019 and 0.013, respectively; rank sum test). In addition, there was a significant association between TP53 mutations and a poorer PFS (P = 0.04), and a strong trend toward a shorter OS among patients with TP53 mutations (P = 0.06). Median PFS and OS for patients with TP53-mutated tumors were 6 months and 16 months, respectively, and for those with p53 overexpression 5.5 months and 14 months, respectively, versus 16 months and 25 months, respectively, for those without TP53 mutations and 25 months and >4 years, respectively, for those without p53 overexpression. The percentage of patients in this series with TP53 mutations (37.9%) was substantially higher than in previous studies of childhood gliomas and comparable to the frequency of mutations noted in adult gliomas. However, both TP53 mutation and p53 overexpression were significantly less frequent in tumors from children younger than 4 than from older children (P = 0.02 and 0.01, respectively). These results indicate that p53 mutation and expression status may be associated with prognosis in childhood malignant gliomas, and thus may provide a basis for stratifying patients biologically in future malignant glioma studies.

INTRODUCTION

Astrocytomas comprise 40% of childhood intracranial neoplasms (1, 2). Of these, 20% are high-grade lesions, such as AA or GBM, that arise within the cerebral or cerebellar hemispheres (1, 3). Although numerous studies have noted that young age is a favorable prognostic factor in patients with malignant gliomas (4, 5) and that children account for a disproportionate percentage of long-term survivors in most studies (6-8), it remains uncertain whether this relationship is simply due to the fact that children have a preponderance of lower-grade (grade III versus IV) tumors or other favorable prognostic features (3-6), or that children may receive more aggressive treatment than adults (3, 9). Alternatively, it has been suggested that gliomas in children may differ from those that arise in adults in terms of their molecular pathogenesis. Mutations of the p53 gene (TP53) in particular have been reported to be uncommon in childhood gliomas (10-12), which contrasts with their frequent detection in adult gliomas (11, 13-15). Because p53-dependent apoptosis has been implicated in mediating the toxicity of radiotherapy and chemotherapy (16-19), it is conceivable that the low incidence of TP53 mutations among childhood gliomas may account for their more favorable response to adjuvant therapy and their generally better overall prognosis than adult tumors. This hypothesis has been validated in other tumor types, in which TP53 mutations or nuclear accumulation of p53 (which has been used as an indirect correlate of TP53 mutations) has been associated with poor in vivo chemosensitivity (20) and an increased rate of disease progression (21-23). Conversely, transfer of a wild-type p53 gene to a tumor cell line lacking normal p53 has been associated with facilitation of apoptosis (24) and enhancement of chemosensitivity in vitro (25).

In the present study, we examined whether nuclear accumulation of p53, as assessed immunohistochemically, and TP53 mutations, as assessed by direct gene sequencing, were associated with prognosis among a cohort of children with malignant gliomas treated consecutively at our institution between 1975 and 1992. A previous outcome study of such patients indicated that approximately 30% of children had long-term disease control and the remainder had a prognosis that was comparable to the generally poor outcomes of adults (9). The current study demonstrates that the incidence of TP53 mutations in childhood malignant gliomas is comparable to that of adult tumors and that p53 overexpression and mutations in childhood gliomas are associated with a less favorable prognosis.

MATERIALS AND METHODS

Patient Population. Children with high-grade non-brainstem gliomas first diagnosed between 1975 and 1992 were identified from a detailed review of the Tumor Registry of the Children’s Hospital of Pittsburgh. All patients were under age 18 at diagnosis and had undergone computed tomography or magnetic resonance imaging both preoperatively and postoperatively. None had received previous therapy. Detailed information on tumor location and extent of resection based on imaging and operative criteria had been ascertained on each of these children in a previous study (9).

Virtually all patients had been included in prospective multicenter trials of the Children’s Cancer Group that evaluated the efficacy of various chemotherapy regimens (26, 27) in conjunction with radiotherapy. Patients older than 3 years of age generally received at least 5000 cGy to the tumor and a generous margin of the surrounding brain. In patients younger than 3, radiation was deferred or the total dose was reduced to minimize neurotoxicity. Patient outcome was assessed by review of hospital and outpatient charts. If a patient had not been evaluated within the previous 6 months, a telephone interview was conducted with the family. Neuroimaging was obtained regularly on these patients, and their films were reviewed for evidence of recurrent disease. Each...
patient alive at the time of review had neuroimaging available within the previous year.

Assessment of Tumor Histology. The original slides and paraffin blocks were reviewed again independently by two neuropathologists (A. J. M. and R. L. H.) who had no knowledge of the patients' outcome. Where appropriate, additional staining or immunohistochemistry was performed from the original blocks. The tumors were graded by WHO criteria (28). Children with pure AA or GBM were included in the study, as were patients with mixed gliomas that displayed a predominance of AA. Patients with anaplastic oligodendroglioma or ganglioglioma and those with pleomorphic xanthoastrocytoma were excluded, because these tumors differ prognostically from other malignant gliomas. In all cases, there was complete agreement between the two neuropathologists with regard to the final histopathological diagnosis.

p53 Immunohistochemistry. Formalin-fixed paraffin-embedded specimens were used for all aspects of this study. Slides from the original tumor specimen were reviewed, and blocks containing malignant glial tissue were sectioned at a thickness of 4 mm. Adjacent sections were: (a) stained with H&E to confirm that characteristic tissue had been obtained; (b) subjected to immunohistochemistry for p53; or (c) processed for TP53 mutation analysis. Sections to be used for p53 immunohistochemical analysis were processed within several days of cutting to avoid loss of immunoreactivity, which we and others have observed to occur if antibody staining is significantly delayed.

Slides for immunohistochemical analysis were baked at 60°C for 15 min, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol and water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol for 20 min. Slides were then rehydrated in PBS and rinsed in 10 mm citrate buffer (pH 6.0). Microwave antigen enhancement (29) was performed by boiling the slides in 10 mm citrate buffer for 5 min at 80% power (Kenmore, 700 W). Additional buffer was added, and the slides were boiled for another 5 min and then allowed to cool to room temperature for 1 h. Slides were rinsed in PBS, and nonspecific antibody binding was blocked by incubation with 10% normal horse serum (Vector Laboratories, Burlingame, CA) in PBS for 20 min.

Sections were then incubated for 2 h at room temperature with anti-p53 antibody (DO1, Santa Cruz Laboratories, Santa Cruz, CA) at a concentration of 2 μg/ml in blocking buffer. This antibody recognizes a denaturation-stable determinant of both wild-type and mutant p53 (30). Control sections were treated with blocking buffer alone. Slides were then rinsed 3 times with PBS, and antibody binding was localized using horse anti-mouse IgG (Vector Laboratories, 1:200). After additional rinsing in PBS, antibody binding was visualized using a Vector ABC Elite kit using the substrate 3,3′-diaminobenzidine with nickel enhancement (31) according to the manufacturer’s protocol. The slides were then counterstained with Mayer’s hematoxylin for 2 min, incubated in 1% ammonium hydroxide, and washed in distilled water. The sections were dehydrated through graded concentrations of ethanol, incubated in xylene for 5 min, mounted, and examined using a light microscope. p53 staining was quantitated by examining 5–10 high-power fields (approximately 2000 cells). Positive and negative controls (colon adenocarcinoma with a known TP53 mutation and normal brain tissue, respectively) were included with each batch of sections to confirm the consistency of the analysis. p53 staining was graded as follows: (1) absent or rare; (2) present in a minority of cells; (3) present in approximately 50–75% of cells; or (4) present in virtually all cells. To ensure consistency of grading, all immunohistochemical assessments were done by a single neuropathologist (R. L. H.), and only tumor cells with dense nuclear staining that obscured nuclear detail were graded as positive. Tumors with p53 expression in the majority of cells (grades 3 and 4) were classified as exhibiting p53 overexpression.

TP53 Mutation Analysis. Exons 5–8 were specifically examined because these regions encompass the vast majority of TP53 mutations that have been detected in astrocytic (13) and nonastrocytic (32) tumors. For these studies, at least two separate tumor-containing microscopic targets were chosen for topographic genotyping, based on examination of adjacent H&E-stained and p53-stained sections. In this technique, minute tissue targets from the regions of highest anaplasia and/or densest p53 staining are removed directly from the unstained serial sections and placed in tubes for PCR amplification. Where available, paired control samples were taken from nonneoplastic brain adjacent to the tumor. TP53 exons 5–8 were amplified individually using oligonucleotide primers as reported previously (33, 34). The PCR products were then isolated with glass beads (BIO 101, La Jolla, CA) and directly sequenced by dideoxy chain termination using [32P]dATP (Sequenase, United States Biochemical Corp., Cleveland, OH). Oligonucleotide sequencing primers for each exon have been described previously (33, 34). Sequences were read from overnight-exposed autoradiograms of 6% polyacrylamide gels.

Statistical Analysis. To assess the association of p53 expression and mutations with outcome in the current study, actuarial survival curves were first generated using the Kaplan-Meier method, with tumor progression and death as the two end points (35). Patients who died perioperatively (n = 3) were excluded from outcome analyses. The relationship between p53 expression and mutations and both PFS and OS was then examined in a series of univariate analyses performed using a rank sum test to assess the strength of association between individual parameters and outcome (36). Because a previous analysis of this (9) and other (26, 37–43) groups of children with malignant gliomas indicated that resection extent, tumor location, age, and tumor histology were significantly associated with outcome, we also examined the relationship between these factors and p53 expression or mutation status using Fisher’s exact test. Finally, multivariate regression analysis (44) was performed to identify those factors that were independently associated with outcome after adjusting for the other covariates.

RESULTS

Patient Characteristics. Thirty-one patients were identified as having malignant non-brainstem gliomas that met the criteria for inclusion in the current study. The clinical, diagnostic, and therapeutic details of these patients were described previously (9). In 29 of these patients, sufficient histopathological material was available for performance of the p53 immunohistochemical and mutation analyses. Relevant features of these patients are summarized in Table 1. The median OS for the group was 18 months and for the 26 surviving the perioperative period, 19 months. The median PFSs were 7 months and 10.5 months, respectively. Nine patients were alive (all without evidence of disease) at last follow-up, with a median survival of 84 months (range, 55–142 months); eight of these patients were progression-free throughout the follow-up period.

p53 Immunohistochemistry and Mutation Analysis. Table 1 shows the results of p53 immunohistochemistry, TP53 mutation status, and other prognostic features in this series. Eighteen of the tumors exhibited dense p53 staining in the majority of cells, whereas 11 had much lower levels of p53 expression (Fig. 1). In contrast to the frequency of p53 immunostaining, TP53 mutations were detected in only 11 of the tumors. Fig. 2 illustrates a representative result, demonstrating the findings of topographic genotyping from a sample in which both tumor and peritumoral brain were available. TP53 mutations were seen in a number of sites within the coding regions examined. Two tumors exhibited deletions of the p53 gene within exons 6 and 8, respectively.

Relationship between p53 Overexpression, Mutations, and Outcome. On univariate analysis, nuclear accumulation of p53 was associated significantly with both PFS and OS. Median PFS for patients with p53 overexpression was only 5.5 months compared with 25 months for those with low levels of p53 expression (P = 0.019, rank sum test; Fig. 3). Median OS for patients with p53 overexpression was only 14 months compared with >4 years for those without this feature (P = 0.013; Fig. 4). In addition, the presence of TP53 mutations was strongly indicative of an adverse prognosis. Median PFS for patients with TP53-mutated tumors was only 6 months compared with 16 months for those without TP53 mutations (P = 0.04, rank sum test; Fig. 5). Median OS for patients with TP53-mutated tumors was only 16 months compared with 25 months for those without TP53 mutations (P = 0.06; Fig. 6). In comparing the two techniques for their accuracy in predicting outcome, TP53 mutation analysis proved to be less sensitive, but in some ways more specific, in predicting unfavorable outcomes. For example, 9 of 10 patients with TP53 mutations who survived the perioperative period had a short (<4 year) survival
The natural text is as follows:

p53 IN CHILDHOOD MALIGNANT GLIOMAS

Characteristics and outcome of patients are arranged in order of increasing PFS. The first three patients died perioperatively and are censored from outcome analysis. Age and survival are listed in months (mo). Patients with a value under F/U (follow-up) are alive and disease-free at the time of this report.

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*Patient characteristics, treatment, and outcome are updated from the previous report of our clinical series by Campbell et al. (9).*

**Locations:** H, supratentorial hemispheric; C, cerebellum; T, thalamic/basal ganglia.

**Resection:** GT, gross total; ST, subtotal; B, biopsy.

**RTX:** Radiotherapy; expressed in Gy, delivered in 180—200 Gy/day fractions.

**CTX:** Chemotherapy; VCP, vincristine/CCNU/prednisone; 8:1, "8 drugs in one day" regimen.

**Numbers under p53 histo refer to staining distribution:** (1) few or no cells stained; (2) minority of cells stained; (3) 50—75% of cells stained; and (4) virtually all cells stained.

**p53 mutations indicate exon, codon number, and amino acid substitution, where applicable.**

**F/U, months to last contact (patient alive).**

**Fluorouracil.**

**Etoposide/5-fluorouracil.**

**Etoposide/Ifosfamide/Mesna.**

The one exception had a deletion rather than a true mutation. versus 8 of 16 without mutations, whereas 13 of 16 children with p53 overexpression had a short survival versus 4 of 10 without overexpression.

**Relationship between p53 Expression and Other Prognostic Factors.** In previous studies, we (9) and others (26, 37—43) have identified a number of other prognostic factors that are associated with outcome in children with malignant gliomas. These include: (a) extent of resection; (b) tumor location; (c) age; and (d) tumor histology. Therefore, we examined whether TP53 mutation or p53 overexpression were associated with any of these factors. There was no significant association between TP53 mutations or p53 overexpression and either resection extent (gross total versus subtotal) or tumor location (cerebral hemispheric versus midline; P > 0.1). However, the subgroup of children with cerebellar lesions had a particularly high frequency of mutations (4 of 5; P = 0.054).

Similarly, there was no significant association between histology and TP53 mutations, which were detected in 2 of 8 AAs, 9 of 19 glioblastomas, and 0 of 2 anaplastic mixed gliomas (P > 0.2). However, there was a strong association between histology and p53 overexpression, which was present in 15 of 19 glioblastomas versus 3 of 10 AAs and mixed gliomas (P = 0.02).

In addition, a striking association between p53 status and age was noted. Mutations were detected in 0 of 7 tumors from children under age 4 compared with 11 of 22 from older children (P = 0.02). The youngest child with a TP53-mutated tumor was 56 months old at diagnosis. The frequency of such mutations in children 4—8 years old (3 of 6) was comparable to that of children older than 8 (8 of 16). p53 overexpression was also less common in younger children. Only 1 of 7 tumors from young children exhibited p53 overexpression versus 17 of 22 tumors from older children (P = 0.01).

Finally, multivariate analysis incorporating each of the above prognostic factors demonstrated that in the present series, only resection extent and p53 expression status were independently associated with either PFS or OS (P < 0.05) after adjusting for the other covariates.

**DISCUSSION**

Outcome for the 29 patients in the present series was well within the range reported previously for childhood malignant gliomas (9, 26, 37—43) with a median OS of 18 months, a median PFS of 7 months, and a 5-year PFS of approximately 30%. Thus, although a substantial percentage of patients are "cured" of their disease, the majority die of tumor progression within several years of diagnosis. The basis for the widely differing outcomes of children with histologically similar tumors has remained largely an enigma. Although recent studies have demonstrated that children with superficially situated cerebral hemispheric tumors who undergo aggressive tumor removal followed by radiotherapy and chemotherapy enjoy a relatively favorable prognosis (9, 42, 43), these factors do not entirely account for the diversity of outcomes in affected patients. Because adjuvant radiotherapy and chemotherapy comprise an essential element in the postoperative management of malignant gliomas, and because recent studies have indicated that loss of normal p53 function may have an adverse effect on radiosensitivity and chemosensitivity, we questioned whether this
it has been shown that even in the absence of TP53 mutations, p53 expression can be increased by cell cycle-specific variations, amplification of MDM2, DNA damage, and anoxia, which can lead to stress-induced increases in the expression of wild-type p53 (49), making p53 histological analysis a potentially more sensitive, but less specific tool than topographic mutation analysis for identifying TP53-mutated tumors. In addition, tumors with TP53 deletions may exhibit

factor was associated with outcome in children with malignant gliomas.

The present study yielded several novel observations:

(a) The frequency of TP53 mutations in pediatric malignant gliomas (11 of 29; 37.9%) was greater than previously estimated (10—12) and more in line with frequencies that have been observed in adult gliomas (11, 13—15). Moreover, the sites of mutations in our patients were generally similar to those noted in previous studies of adult gliomas. However, several unusual mutations (e.g., Pro278His and Leu145Val) were noted that were presumably associated with functional inactivation of p53 based on the high levels of immunoreactivity observed in these specimens. The greater incidence of TP53 mutations in the current study versus earlier reports may largely reflect that previous studies of childhood gliomas included a majority of pilocytic tumors, which are grade 1 lesions that have an excellent prognosis; only a small number of specimens were truly malignant (10—12). In contrast, one study that examined a small series of intrinsic brainstem gliomas of childhood, which are almost uniformly malignant lesions, noted a high frequency of TP53 mutations (45).

(b) TP53 mutations and p53 overexpression were associated with a less favorable PFS and OS, which is in keeping with recent findings in other tumor types (20—23). In agreement with previous reports (12, 46—48), it was apparent that overexpression of p53 did not always indicate the presence of detectable TP53 mutations in malignant gliomas. This may reflect, in part, that in some of these tumors, populations of TP53-mutated cells eluded detection in our mutation analysis or that mutations may have occurred outside of exons 5—8, which would not have been detected by our techniques. Alternatively,
variable staining, which ranges from absent (as would be expected in normal cells with wild-type p53) to moderate, as noted in cases 3 and 22 in the current study, presumably depending on the degree to which a shortened p53 transcript may be formed.

Furthermore, the immunohistochemical studies clearly are more technique-dependent and observer-dependent and, as such, are inherently vulnerable to false positive and false negative observations. Factors such as the use of microwave antigen enhancement, the time elapsed between sectioning and staining, and the choice of the secondary antibody labeling technique have a significant impact on the density of staining seen in individual specimens. In view of the vagaries of these techniques, it is perhaps not surprising that previous studies that have sought to determine whether a correlation exists between p53 expression and outcome among adult gliomas have obtained conflicting results, with some groups reporting such an association (50–53) and others failing to identify such a relationship (49, 54, 55). In this context, the results of the present study using direct assessment of TP53 mutations from carefully selected regions of anaplasia within the tumor specimen, in addition to immunohistochemical analysis, provide more convincing evidence that mutation of this gene constitutes an adverse prognostic factor in childhood gliomas.

(c) The presence of TP53 mutations occurred independently of histological diagnosis among children with malignant gliomas. The lack of an association between TP53 mutations and histology was not surprising, because in studies of adult malignant gliomas, TP53 mutations have been identified at a relatively consistent frequency in grade III and grade IV tumors (11, 13, 14).

(d) The finding that tumors from children under age 4 had a significantly lower frequency of TP53 mutations and p53 overexpression than those from older children raises the issue of whether such tumors may have arisen by distinct molecular pathways. In this regard, it is interesting that four of the nine children who were disease-free, long-term survivors in this series were among the seven patients who were younger than 4. Although this association between age and prognosis did not reach statistical significance (P = 0.09), the trend toward more favorable outcomes among young children than older children has also been noted by other groups (56).

In summary, the results of the present study indicate that the presence or absence of TP53 mutations or p53 overexpression in malignant gliomas of childhood constitutes a prognostic feature that may provide a basis for stratifying patients biologically in future malignant glioma studies. In conjunction with other strong prognostic factors, such as resection extent, this would facilitate efforts to identify prospectively those patients who are unlikely to have a favorable response to conventional treatment modalities, and who thus might be appropriate candidates for more intensive or novel therapeutic strategies.

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