

## EXPRESSION OF p53 AND PROGNOSIS IN CHILDREN WITH MALIGNANT GLIOMAS

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### ABSTRACT

**Background** The prognosis of children with high-grade gliomas is uncertain, even when clinical and histologic findings are considered. We investigated whether mutations in the *TP53* gene or the degree of expression of p53 protein in high-grade gliomas is associated with progression-free survival in children with these tumors.

**Methods** Paraffin-embedded specimens of malignant gliomas from children treated in the Children's Cancer Group study CCG-945 were assessed by mutational analysis of *TP53* (121 specimens) and immunohistochemical analysis of p53 (115 specimens). For mutational studies, areas of tissue that contained malignant glioma were isolated by microdissection, and the DNA was subjected to polymerase-chain-reaction-based amplification and sequencing of *TP53* exons 5, 6, 7, and 8. Immunohistochemical analysis was performed with the use of a microwave-enhanced antigen retrieval and an antibody that bound both wild-type and mutant p53.

**Results** We found a significant association between overexpression of p53 and outcome; this association was independent of histologic features, age, sex, the extent of resection, and tumor location. The rate ( $\pm$ SE) of progression-free survival at five years was  $44\pm 6$  percent in the group of 74 patients whose tumors had low levels of expression of p53 and  $17\pm 6$  percent in the group of 41 patients whose tumors had overexpression of p53 ( $P < 0.001$ ). A nonsignificant association was observed between mutations in *TP53* and outcome.

**Conclusions** Overexpression of p53 in malignant gliomas during childhood is strongly associated with an adverse outcome, independently of clinical prognostic factors and histologic findings. (N Engl J Med 2002;346:420-7.)

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**H**IGH-GRADE astrocytomas are the largest group of primary central nervous system tumors and generally have a poor prognosis.<sup>1-4</sup> However, many studies have found that a young age is a favorable prognostic factor,<sup>3,7</sup> suggesting that malignant gliomas in children might differ biologically from similar lesions in older patients.<sup>8</sup>

High-grade gliomas in adults arise through several distinct molecular pathways of tumorigenesis. So-

called primary malignant gliomas, which typically affect older patients and have a histologic grade of IV (i.e., glioblastoma multiforme)<sup>9</sup> at diagnosis, commonly show amplification of the epidermal growth factor receptor gene (*EGFR*),<sup>10-15</sup> which encodes a tyrosine kinase involved in cell replication. In contrast, secondary malignant gliomas that evolve from low-grade lesions generally affect young adults and often have mutations in *TP53*, which encodes the p53 protein, but rarely have amplification of *EGFR*.<sup>10-15</sup> To date, these features have not been linked to the prognosis of gliomas in adults, nor have other characteristic mutational events in high-grade astrocytomas.<sup>16-23</sup>

As compared with the extensive characterization of the molecular alterations in high-grade gliomas in adults,<sup>10,14,16,23</sup> there is limited information on the molecular abnormalities in pediatric gliomas.<sup>24-29</sup> In our previous studies of malignant gliomas from children, we found that overexpression of p53 and mutations in *TP53* were associated with adverse outcomes,<sup>25</sup> but the small size of the cohort did not allow us to assess whether the prognostic significance of these changes was independent of the histologic features of the tumor.

To investigate the clinical significance of these molecular changes more conclusively, we analyzed data on the multiinstitutional cohort of the Children's Cancer Group (CCG) study CCG-945, a large group of uniformly treated children with high-grade gliomas.<sup>30</sup> We examined mutations in *TP53*, overexpression of p53, or both and evaluated the association of these changes with outcome.

### METHODS

#### Population

CCG-945 included 231 children with non-brain-stem malignant gliomas who were treated with surgery, radiotherapy of the involved field, and one of two regimens of chemotherapy. A total

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of 172 patients who were between 18 months and 21 years of age at diagnosis and had intracranial malignant gliomas were randomly assigned to receive adjuvant therapy with either prednisone, lomustine, and vincristine or the "eight-drugs-in-one-day" regimen.<sup>30,31</sup> This regimen incorporated seven agents with some activity against pediatric brain tumors: lomustine, vincristine, hydroxyurea, procarbazine, cisplatin, cytosine arabinoside, and dacarbazine; methylprednisolone was added to reduce cerebral edema. The goal of this approach was to circumvent resistance to individual agents by exposing the tumor to multiple agents, albeit at a low dose intensity. Fifty-nine children younger than 18 months of age or with malignant gliomas of the spinal cord were nonrandomly assigned to the eight-drug regimen.

The study enrolled patients from 60 institutions between April 1985 and May 1990. The protocol required that each institution provide histopathological confirmation of the presence of a high-grade astrocytoma (i.e., a glioblastoma multiforme, an anaplastic astrocytoma, or another eligible grade III glioma, such as an anaplastic mixed glioma). The outcomes were among the best reported for patients with these tumors, with an overall rate ( $\pm$ SE) of survival at five years of  $36 \pm 6$  percent, with no significant difference between the two treatment groups.<sup>30</sup>

For the present study, tissue collection was initiated by the Pediatric Branch of the Cooperative Human Tissue Network in the context of a CCG biology study, CCG-B975, which was approved by the institutional review board of Children's Hospital of Pittsburgh. Specimens were coded by the Cooperative Human Tissue Network to mask clinical, histologic, and outcome data from the investigators. The material provided for analysis was reexamined by two neuropathologists to confirm that adequate amounts of tissue were available for the planned studies.

### Analysis for Mutations in TP53

Paraffin-embedded tumor specimens were used for all analyses. Slides were reviewed, and blocks that contained malignant glioma were sectioned at a thickness of 4  $\mu$ m. Sections were stained with hematoxylin and eosin to confirm that characteristic tissue had been obtained; adjacent sections were subjected to immunohistochemical staining for p53 or microdissection-based analysis for TP53 mutations. Exons 5, 6, 7, and 8 were examined specifically because they encompass most TP53 mutations detected in astrocytic<sup>32</sup> and nonastrocytic<sup>33</sup> tumors.

For mutational analysis, tissue specimens from regions with the highest degree of anaplasia were removed directly from tumor sections with the use of microdissection techniques as described.<sup>25,34-36</sup> Individual exons were amplified by the polymerase chain reaction (PCR) with the use of the following primer pairs: exon 5, GCACTACTCCCCTGCCTCAA (sense) and GCCCAGCTGCTCACCATCGC (antisense); exon 6, GGGTCCCCAGGCCTCTGATT (sense) and CCTCCCAGAGACCCAGTT (antisense); exon 7, CTTGCCACAGGTCTCCCCAAG (sense) and GCAGGCCAGTGTGCAGGGTGG (antisense); and exon 8, TTTCTATCCTGAGTAGTGG (sense) and GGCTCTCTCCACCGCTTCTTG (antisense), as reported.<sup>25,36</sup> The PCR products were isolated and directly sequenced by dideoxy chain termination with the use of <sup>35</sup>S-labeled deoxyadenosine triphosphate (dATP) as previously described.<sup>25,36</sup> Sequences were read from autoradiograms of 6 percent polyacrylamide gels.

### Expression of p53

Tumor-containing sections were baked at 60°C for 30 minutes, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3 percent hydrogen peroxide and methanol. Slides were rehydrated in phosphate-buffered saline. Microwave-enhanced antigen retrieval<sup>37</sup> was performed by boiling the slides in 10 mM citrate buffer (pH 6.0). Nonspecific antibody binding was blocked

by incubation with a protein-blocking reagent (Immunon, Pittsburgh) for 20 minutes. Sections were then incubated overnight at 4°C with anti-p53 antibody (DO-7, Dako, Carpinteria, Calif.) at a 1:300 dilution in common antibody diluent (BioGenex, San Ramon, Calif.). This antibody recognizes a determinant of wild-type and mutant p53 in formalin-fixed sections.<sup>38</sup>

Slides were rinsed in phosphate-buffered saline and incubated with a biotin-labeled secondary antibody, followed by a streptavidin-horseradish peroxidase conjugate. Bound antibody was revealed with the use of the substrate 3,3'-diaminobenzidine.<sup>39</sup> Sections were counterstained with Mayer's hematoxylin, washed, dehydrated with graded concentrations of ethanol, cleared in xylene, mounted, and examined microscopically.

Positive and negative controls were included with each batch of sections to confirm the consistency of the analysis. Specimens were examined independently for immunoreactivity with the use of an antibody against a histologically verifiable internal positive control antigen (i.e., staining of the Ki-67 antigen by the MIB1 antibody [Immunotech, Westbrook, Me.] in mitotic figures in tumor), to identify cases in which a lack of immunoreactivity for p53 might indicate problems of tissue preservation, rather than a lack of protein expression. Such cases were excluded from the outcome analysis.

Sections were examined for p53 immunoreactivity by an observer who was unaware of the histologic diagnoses, outcomes, or clinical features. Only cells with dense nuclear staining were interpreted as positive. Tumors were categorized as expressing little or no p53 (a grade of 0 or 1), similar to normal brain, or as overexpressing p53, with staining observed in a sizable subgroup of cells (25 to 50 percent; grade 2), most cells (50 to 75 percent; grade 3), or nearly all cells (>75 percent; grade 4) in the high-power field in areas with maximal staining. Overexpression was therefore defined semiquantitatively, based on the percentage of p53-expressing cells in the tumor.

### Central Pathological Review

Histopathological inclusion criteria for the CCG-945 clinical study were based solely on the pathological diagnosis made at each institution, but it has since been recognized that the classification of gliomas in children is extremely challenging and subject to differences of opinion even among skilled reviewers.<sup>40</sup> To eliminate the possibility that results in the present study may have been influenced by diagnostic variations among the pathologists at each institution, all specimens in the current study were independently reviewed in a blinded fashion by a senior neuropathologist to ensure consistent classification based on contemporary guidelines from the World Health Organization.<sup>9</sup> We then examined the association of overexpression of p53 and mutations in TP53 with outcome, using the diagnosis made by the reviewer in place of the diagnosis made at the institution and excluding cases believed to be discordant (e.g., not high-grade gliomas) or equivocal.

### Statistical Analysis

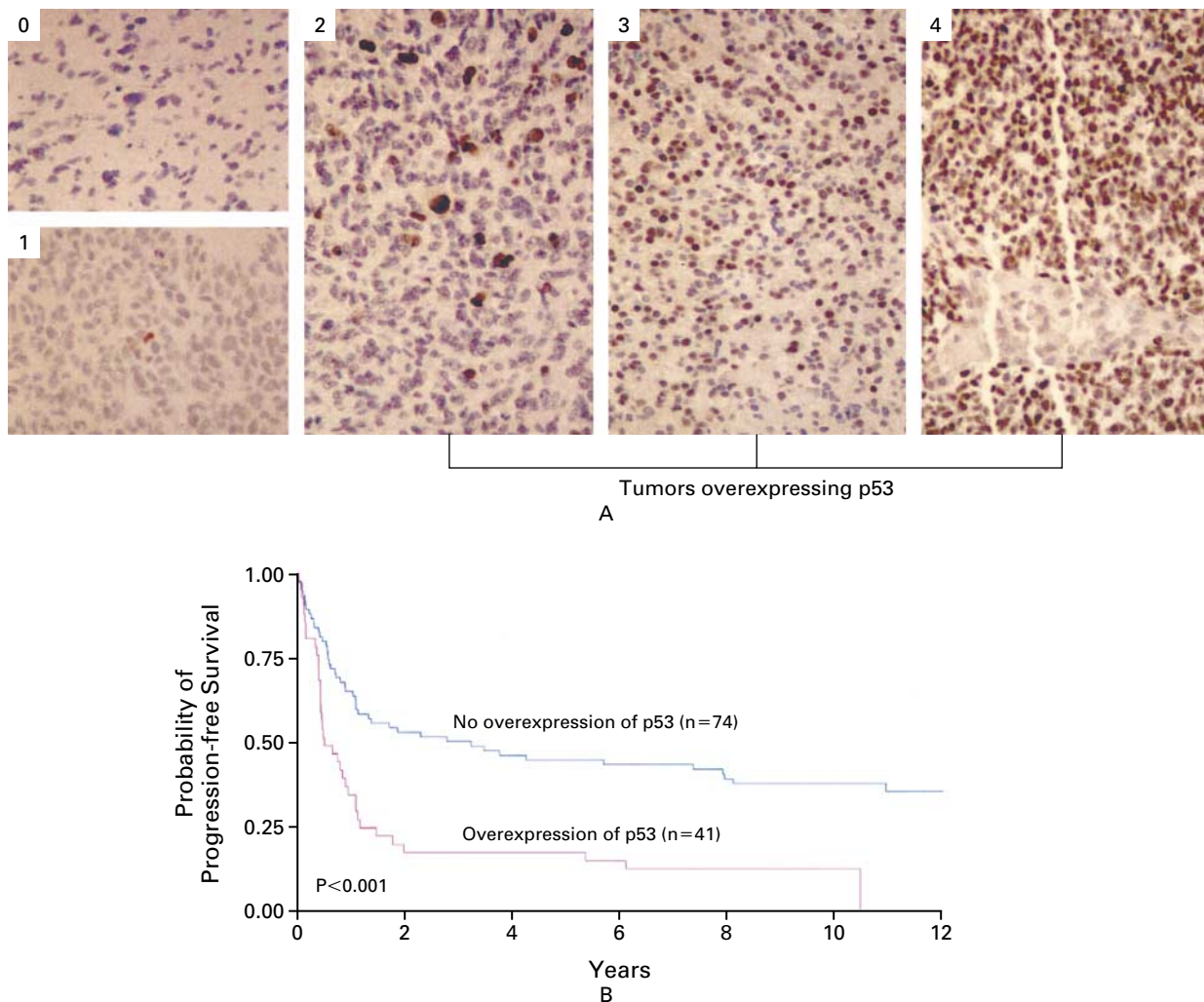
For the outcome analysis, tumors were classified according to the presence or absence of overexpression of p53 or mutations in TP53. The primary end point was progression-free survival, defined as the time from study entry to disease progression. Comparisons of outcome were based on the log-rank test; estimates of survival, with standard errors, were calculated from the product-limit estimate.<sup>41</sup> Because it was considered that expression of p53 and the status of TP53 might correlate with the histopathological diagnosis, an analysis of the association between those findings and outcome was performed with a stratified log-rank test,<sup>41</sup> which adjusted for a variety of potential prognostic factors. Comparisons of the distribution of p53 expression and mutation status between different subgroups of patients were based on a two-sided Fisher's exact test and a  $\chi^2$  test, as appropriate.<sup>42</sup>

**RESULTS**

**Characteristics of the Patients**

Specimens from 148 of the 231 patients were available for analysis; 115 could be assessed for p53 expression, and 121 contained sufficient tissue for analysis of *TP53* mutations. Twenty-four specimens that lacked immunoreactivity for internal positive control antigens (e.g., staining of cells with mitotic figures with use of the MIB1 antibody for staining of Ki-67 antigen) and 9 specimens that lacked sufficient tissue to permit reliable assessment were pro-

spectively excluded from analysis of p53 expression, whereas 27 specimens that lacked sufficient tumor tissue to permit microdissection of foci with over 80 percent malignant glioma cells were excluded from mutational analysis. The rate of progression-free survival at five years in the group of patients whose specimens could be assessed for p53 immunoreactivity ( $35 \pm 4$  percent) did not differ from that of the group of 92 children for whom specimens were not available or had insufficient tissue ( $35 \pm 5$  percent) or the 24 with specimens that could not



**Figure 1.** Immunohistochemical Analysis of p53 and the Rate of Progression-free Survival According to the Levels of p53 Expression. In Panel A, immunohistochemical analysis shows various levels of p53 expression. Tumors that had p53 expression in no cells (0) or very few cells (1) were defined as having normal expression. Tumors with dense expression that exceeded the normal levels in brain and that was either focal (2 or 3) or diffuse (4) were defined as having overexpression. The mean rate of progression-free survival at five years was  $44 \pm 6$  percent among the 74 children with tumors that had normal levels of expression, as compared with  $17 \pm 6$  percent among the 41 children whose tumors had overexpression of p53 ( $P < 0.001$ ) (Panel B).

**TABLE 1.** RATES OF PROGRESSION-FREE AND OVERALL SURVIVAL AS A FUNCTION OF p53 STATUS.\*

VARIABLE†	NO. OF PATIENTS	PROGRESSION-FREE SURVIVAL AT 5 Yr		SURVIVAL AT 5 Yr	
		PROBABILITY	P VALUE	PROBABILITY	P VALUE
		%		%	
Grade of p53 expression			<0.001		<0.001
0-1	74	44±6		51±6	
2-4	41	17±6		20±6	
Mutation status			0.13		0.08
<i>TP53</i> -	81	38±5		42±6	
<i>TP53</i> +	40	24±7		28±7	
Grade and mutation status			0.004; <0.001 for trend		0.003; <0.001 for trend
0-1 and <i>TP53</i> -	41	46±8		51±8	
0-1 and <i>TP53</i> +	14	41±14		50±13	
2-4 and <i>TP53</i> -	20	25±10		30±10	
2-4 and <i>TP53</i> +	13	8±7		8±7	

\*Plus-minus values are means ±SE.

†Grade 0 or 1 indicates normal expression of p53, and grades 2, 3, and 4 overexpression. *TP53*- indicates the absence of a *TP53* mutation, and *TP53*+ the presence of such a mutation.

be assessed for p53 expression (42±10 percent, P=0.7).

**Expression of p53 and Outcome**

Of 115 assessable tumors, 74 showed little or no expression of p53, whereas 41 showed overexpression, with dense p53 immunoreactivity in more than 25 percent of cells (Fig. 1A). A striking difference in outcome between these two groups was apparent. The rate of progression-free survival at five years was 44±6 percent in the group of 74 patients whose tumors expressed low levels of p53 and 17±6 percent in the 41 patients whose tumors overexpressed p53 (P<0.001) (Fig. 1B). A strong inverse association between the level of expression of p53 and overall survival was also observed (Table 1).

***TP53* Mutations and Outcome**

Of 121 assessable tumors, 40 (33.1 percent) had a mutation in exons 5 through 8 of *TP53*. The presence of such a mutation was associated with an adverse prognosis, although this association did not reach statistical significance (Table 1). Table 1 also shows that among the 88 patients with tumors that could be assessed for both expression of p53 and mutations in *TP53*, 41 with tumors that had neither overexpression of p53 nor a mutation in *TP53* had a rate of progression-free survival at five years of 46±8 percent, whereas 13 with tumors that had both a mutation in *TP53* and overexpression of p53 had a rate of progression-free survival at five years of only 8±7 percent (P=0.004) (Fig. 2). The outcome

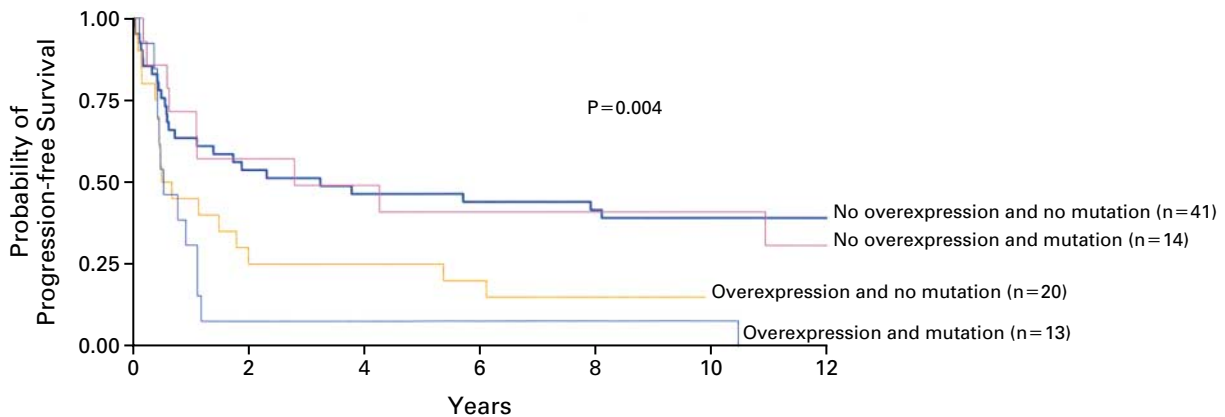
was intermediate in the 20 patients whose tumors overexpressed p53 but had no detectable mutations (Table 1 and Fig. 1) (P for trend, <0.001).

Although overexpression of p53 was more common in tumors classified as glioblastomas (58 percent) than in anaplastic astrocytomas (26 percent) or other eligible grade III gliomas (21 percent, P<0.002 according to the  $\chi^2$  test), the association between overexpression of p53 and adverse outcome remained after stratification according to histologic subtypes (P=0.005) (Table 2). Stratification for age, the extent of resection, the location of the tumor, and other variables did not diminish the association between overexpression of p53 and outcome (P<0.001, according to the stratified log-rank test) (Table 3).

**Analysis Based on Central Review of the Histopathological Findings**

Inclusion in the original clinical study was based on the histopathological diagnosis made at each institution, but the criteria for classifying pediatric malignant gliomas have since evolved and include additional guidelines for distinguishing aggressive-appearing low-grade gliomas from high-grade gliomas.<sup>9</sup> Samples from all tumors we analyzed were independently reviewed by a senior pediatric neuropathologist, who was unaware of the clinical outcome or the results of p53 analysis, with the use of current guidelines from the World Health Organization<sup>9</sup> to ensure both consistency and stringency in the classification process.

Notwithstanding the elimination of 34 patients



**Figure 2.** Relation of Mutations in *TP53* and Overexpression of p53 to Outcome in Children with High-Grade Gliomas. The 41 children who had tumors that exhibited neither overexpression of p53 nor mutations in *TP53* had a mean rate of progression-free survival at five years of 46±8 percent, as compared with 41±14 percent among the 14 with *TP53* mutations but no p53 overexpression, 25±10 percent among the 20 children who had tumors with overexpression of p53 and no mutations, and 8±7 percent among the 13 children who had tumors with both overexpression and mutations (P=0.004; P for trend, <0.001).

**TABLE 2.** RATES OF PROGRESSION-FREE AND OVERALL SURVIVAL AS A FUNCTION OF THE LEVEL OF EXPRESSION OF p53, WITH STRATIFICATION ACCORDING TO THE HISTOPATHOLOGICAL DIAGNOSIS MADE AT EACH INSTITUTION.\*

HISTOPATHOLOGICAL DIAGNOSIS AND GRADE OF p53 EXPRESSION	NO. OF PATIENTS	PROGRESSION-FREE SURVIVAL AT 5 YR		OVERALL SURVIVAL AT 5 YR	
		PROBABILITY	P VALUE†	PROBABILITY	P VALUE‡
		%		%	
Anaplastic astrocytoma					
0-1	39	36±8	0.27	49±8	0.09
2-4	14	29±12		29±12	
Glioblastoma multiforme					0.06
0-1	16	44±12	0.04	38±12	
2-4	22	9±6		14±7	
Other grade III gliomas					0.03
0-1	19	63±11	0.03	68±11	
2-4	5	20±8		20±8	

\*Plus-minus values are means ±SE. Grade 0 or 1 indicates normal expression of p53, and grades 2, 3, and 4 overexpression.

†P=0.005 for the comparison across strata.

‡P=0.002 for the comparison across strata.

for whom the diagnosis could not be confirmed by central review, there was still a strong association between p53 status and outcome in the remaining patients. The rate of progression-free survival at five years was 32±7 percent in the group of 44 patients whose tumors did not overexpress p53 and 14±6 percent in the 37 whose tumors did overexpress p53 (P=0.01). This association was maintained in an analysis stratified according to the histologic features

of the tumor (P=0.03). The 13 patients whose tumors showed both overexpression of p53 and mutations in *TP53* had a rate of progression-free survival at five years of 8±7 percent, as compared with 32±9 percent in the 25 patients whose tumors lacked either feature, 33±19 percent in the 6 that had mutations but no overexpression, and 24±10 percent in the 17 that had p53 overexpression without *TP53* mutations (P for trend=0.06).

**TABLE 3.** PROGRESSION-FREE SURVIVAL AS A FUNCTION OF THE LEVEL OF EXPRESSION OF p53, WITH STRATIFICATION ACCORDING TO CHARACTERISTICS OF THE PATIENTS.\*

CHARACTERISTIC	NO OVEREXPRESSION (0-1)		OVEREXPRESSION (2-4)		P VALUE†	P VALUE STRATIFIED FOR CHARACTERISTIC
	NO. OF PATIENTS	PROGRESSION-FREE SURVIVAL AT 5 YR	NO. OF PATIENTS	PROGRESSION-FREE SURVIVAL AT 5 YR		
		%		%		
Age						
<3 yr	20	29±10	7	29±17	0.63	<0.001
≥3 yr	54	50±7	34	15±6	<0.001	
Sex						<0.001
Male	40	37±8	23	17±8	0.08	
Female	34	53±9	18	17±9	<0.001	
Histologic diagnosis (institutional)						0.005
Anaplastic astrocytoma	39	36±8	14	29±12	0.27	
Glioblastoma multiforme	16	44±12	22	9±6	0.04	
Other	19	63±11	5	20±18	0.03	
Histologic diagnosis (review)						0.03
Anaplastic astrocytoma	20	20±9	13	23±12	0.50	
Glioblastoma multiforme	17	28±11	21	10±6	0.08	
Other high-grade glioma	7	71±17	3	0±0	0.05	
Tumor site‡						<0.001
Supratentorial	59	47±7	37	19±6	<0.001	
Infratentorial	8	25±15	4	0±0	0.11	
Other or mixed	6	33±19	0	0±0		
Residual tumor§						<0.001
≤1.5 cm <sup>2</sup>	27	63±9	12	25±13	0.003	
>1.5 cm <sup>2</sup>	39	33±8	26	12±6	0.008	
Extent of resection						<0.001
≤90%	42	26±7	28	11±6	0.007	
>90%	32	69±8	13	31±13	0.009	

\*Plus-minus values are means ±SE.

†P values are for the comparison between the patients whose tumors had normal expression of p53 and those whose tumors had overexpression.

‡Data were missing for one patient.

§Data were missing for eight patients.

## DISCUSSION

Because p53-dependent pathways influence the cytotoxic effects of conventional chemotherapy and radiotherapy,<sup>43-46</sup> we investigated whether there is an association between the expression of p53 and outcome in children with malignant gliomas, as has been observed in several cancers that affect adults.<sup>47-51</sup>

We found increased expression of p53 in about 35 percent of high-grade gliomas in children and determined that this feature was an adverse prognostic factor in a cohort of children who were treated uniformly with surgery, radiotherapy, and chemotherapy. The frequency of *TP53* mutations was greater than in previous series of gliomas in children<sup>52-54</sup> and more in line with the frequency of such mutations in the secondary malignant gliomas that affect young adults.<sup>15,16,53</sup> The true frequency of *TP53* mutations in malignant gliomas in children may be even greater, because we did not examine all regions of the gene. Overexpression of p53, which is often used as

a surrogate indicator of alterations in p53 functional status, may occur for reasons other than a mutation in *TP53*.<sup>51,55</sup> Whatever the mechanism, however, this phenomenon was associated with an extremely adverse outcome. The frequency of overexpression of p53 increased significantly with increasing tumor grade (i.e., grade IV vs. grade III), suggesting that dysregulation of p53-mediated pathways might be an important step in the malignant progression of these tumors.

The strong association between overexpression of p53 and poor outcome contrasts with the lack of such an association in previous studies of malignant gliomas in adults.<sup>56,57</sup> This discrepancy suggests that primary malignant gliomas in children and adults have different distributions of molecular lesions. One example is that pediatric glioblastomas infrequently show amplification of *EGFR*,<sup>24,26,27</sup> which is a common feature in primary glioblastomas in adults.<sup>10,12-15,17,18,21-23</sup> Although the high frequency of overexpression of p53 in pediatric malignant gliomas

resembles the pattern seen in secondary malignant gliomas in adults, which develop from previously low-grade gliomas, it is important to emphasize that low-grade gliomas in children rarely undergo spontaneous progression to malignant gliomas.<sup>1</sup> Accordingly, malignant gliomas in children and secondary malignant gliomas in adults probably have different molecular defects. In addition, pediatric malignant gliomas may well consist of distinct molecular subgroups. As previously noted by our group, *TP53* mutations are significantly less common in malignant gliomas in children younger than three years of age than in tumors in older children.<sup>25,58</sup>

Our study was distinguished by its large size and consistent management.<sup>30</sup> This combination increased the chance of identifying molecular features that might have an overriding influence on prognosis. The fact that both chemotherapy and radiotherapy were used may have influenced the magnitude of the prognostic associations, because the efficacy of both types of treatment may have been affected by p53 status. The observation that the independent prognostic association between overexpression of p53 and outcome was apparent regardless of whether the histologic classification was performed by central review or by the institutional pathologist supports the view that p53 is a useful marker in both settings.

Although our observations indicate that p53 status is an important prognostic marker in pediatric malignant gliomas, it did not distinguish high-grade gliomas with a good prognosis from those with a poor prognosis. Instead, it distinguished children with high-grade gliomas who had an extremely unfavorable prognosis after treatment from those with a significantly more favorable, but still suboptimal, prognosis. For this reason, our observation does not support reduction of therapy in patients with a favorable p53 status. It shows, however, that the prognosis in children with malignant gliomas can be influenced by molecular changes in the tumor. We believe that molecular analyses should be included in prospective evaluations of novel treatments, particularly those that incorporate intensification of chemotherapy and radiotherapy.

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