Analysis of proliferation markers and p53 expression in gliomas of astrocytic origin: relationships and prognostic value

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Consecutive paraffin sections of 105 astrocytomas and 15 oligoastrocytomas were examined for expression of p53, MIB-1 (Ki-67), and proliferating cell nuclear antigen (PCNA). The tumors had been examined previously for genetic abnormalities and by flow cytometry. Regardless of the tumor’s stage and grade and the patient’s age and gender, p53 expression was found in 40% of tumors. Although p53 expression was associated with a loss on chromosome 17p and was more frequent in aneuploid tumors, it had no association with survival time. The MIB-1 and PCNA labeling indices increased with increasing tumor grade but showed no association with other clinicopathological parameters. In individual tumors, there was poor concordance between any of the variables (MIB-1, PCNA, and p53). Results for p53 and MIB-1 were similar for both astrocytomas and oligoastrocytomas. The MIB-1 and PCNA values appeared to have prognostic utility in univariate analysis but not after adjusting for patient age and tumor grade. The poor concordance between MIB-1 and PCNA in individual tumors indicates that any one means of assessing proliferative potential in gliomas may not be reliable.

KEY WORDS • glioma • p53 • proliferating cell nuclear antigen • MIB-1

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As a group, gliomas are the most common primary intracerebral neoplasms and are classified according to their histological characteristics. Several grading systems are currently in use; their differences lie in their parameters, application, and reproducibility. As part of an ongoing project, we are attempting to identify biological markers that not only will aid in classification and grading of gliomas but will also be of utility in predicting the prognosis of patients with these tumors. Such markers will have a significant impact on therapeutic decision making.

It has been suggested that the proliferative potential of gliomas may be a strong predictor of prognosis. Bromodeoxyuridine (BudR) labeling studies and cytometric determinations of S-phase fractions have been shown to be of value, whereas immunostaining for proliferating cell nuclear antigen (PCNA) and Ki-67 molecules expressed in cycling cells are readily applicable tests of proliferative potential. Nonetheless, the interpretation of PCNA data has proved controversial and, until recently, the use of Ki-67 has been restricted to frozen material. Even so, both PCNA and Ki-67 labeling indices (LIs) have been shown to increase with increasing glioma grade and have been found to be predictors of poor survival in some studies. With the development of the new antibody MIB-1, which reliably detects a Ki-67 antigen in paraffin sections, it is now possible to compare directly PCNA and Ki-67 LIs in individual glioma specimens. The antibody MIB-1 has proved to be a reliable marker of proliferative potential. In their recent study of 48 astrocytomas, Sallinen, et al., found it to be prognostically significant and the most informative of the proliferation parameters they tested.

The p53 gene is altered in approximately one-half of all astrocytomas and is generally accompanied by overexpression of the protein product. In most of these cases, p53 overexpression can be assessed immunohistochemically. The p53 protein is intimately involved in regulation of the cell cycle. It is known to affect the transcription of p21^CIP1, a universal inhibitor of the cyclin-dependent kinases (CDKs), and Gadd45, the function of which remains unclear. Both p21 and PCNA form quarternary complexes with cyclins and CDKs in normal, but not transformed, cells and are important in regulating cell-cycle progression. The p53 protein apparently also modulates the expression of PCNA. It was of interest therefore to determine whether the presence of abnormal amounts of p53 protein were associated with increased rates of proliferation.

In this article we report on the prognostic value of p53 expression and the proliferation markers PCNA and MIB-1 in 120 patients with newly diagnosed cerebral astrocytomas, and we evaluate the relationships among these markers.

Materials and Methods

Patient Population
This work represents an ongoing prospective investigation, initiated in January 1987, of central nervous system tumors surgically removed at the Mayo Clinic. A variety of laboratory studies have been performed, all with the approval of the Mayo Clinic Institutional Review Board. This report focuses on 120 patients with infiltrative cerebral gliomas, all of which were morphologically classified using the World Health Organization (WHO) system. The tumors were graded according to the St. Anne–Mayo method. All patients were newly diagnosed tumors and had received no prior oncological or radiation therapy. Patients with low-grade astrocytomas (Grade 2) were treated with postoperative radiation (5000–6480 cGy). Patients with high-grade tumors (Grades 3 and 4) were treated with radiation (5000–6486 cGy) and adjuvant chemotherapy that included a nitrosourea compound. At present, there is no conclusive evidence that differences in chemotherapy affect the survival of our patients with high-grade gliomas.

Immunohistochemical Analysis
The antibodies used in this study included: DO-7, a p53-specific monoclonal antibody that reliably recognizes the p53 protein in paraffin sections; PAb 1801, a p53-specific polyclonal antibody that recognizes p53 protein in archival samples when antigen retrieval methods are used; MIB-1, a Ki-67–specific monoclonal antibody effective in paraffin-embedded sections;15,16 and PC10, an antibody that recognizes the PCNA antigen. These antibodies were used at concentrations of 1:100, 2 μg/ml, 1:60, and a 1:500 dilution, respectively. Negative controls included either mouse immunoglobulin (IgG) (for monoclonal antibodies) or rabbit IgG at 1 μg/ml. All dilutions were made in phosphate-buffered saline (pH 7.2) with 0.05% Tween-20 and 1% normal goat serum.

Sections were deparaffinized and endogenous peroxidase activity was quenched by a 20-minute exposure of sections to a 0.6% solution of hydrogen peroxide in methanol. One section only was assessed from each sample. Slides intended for PAb 1801 immunohistochemical analysis were pretreated with antigen retrieval solution and were subjected to microwave treatment (at boiling point) and incubated at 70°C for 30 minutes. Slides intended for MIB-1 immunohistochemical analysis were pretreated for 20 minutes with 10 mM citrate buffer that had been heated to boiling point. Nonspecific binding was blocked with 5% normal goat serum for 15 minutes. Diluted primary antibodies were applied, followed by incubation at either room temperature for 1 hour or overnight at 4°C. Sections were incubated with biotinylated goat anti–mouse IgG at 1:200 dilution for 30 minutes, followed by peroxidase-conjugated streptavidin at 1:500 for 30 minutes. Diaminobenzidine served as the chromogen and 0.2% methyl green as the counterstain.

Scoring of Tumor Cells
The proportion of neoplastic cells was recorded. Cytological atypia, defined as nuclear enlargement, irregularity, and hyperchromasia, defined neoplastic cells. These features are illustrated in previous publications establishing the utility of the St. Anne–Mayo grading system.15,16 Expression of p53 protein was scored as follows: none or a rare occurrence (< 1%) of nuclei stained, 1 to 10%, between 10 and 50%, or more than 50% of neoplastic nuclei stained. Tumors having more than 10% of cell nuclei stained were deemed p53 positive. The PCNA and MIB-1 LIIs were determined with a digital analyzer, using previously described methods.60 For PCNA, only nuclei with dense granular or diffuse staining were considered positive. Lightly stained nuclei were not included. The MIB-1 exhibited strong diffuse staining that was readily identified. A total of 10 separate fields were read for each sample and the mean labeled/total percentage nuclear area was recorded. The LI calculated in our study reflects the proportion of malignant nuclei stained. Select samples were run in multiple experiments for both p53 and the proliferation markers. The data showed good reproducibility, as did control tissues run in each experiment. Occasional sections were lost during the staining procedure and some samples had excessive backgrounds that precluded reliable reading. Thus, the sample numbers for each of the markers varied slightly.

DNA Ploidy and Chromosome 17 Loss of Heterozygosity
The methods of analysis for DNA ploidy and chromosome 17 loss of heterozygosity (LOH) and the results of the analyses of the tumor specimens used in this report have been previously described.25

Statistical Analysis
Wilcoxon and chi-square tests were used to compare distributions of clinicopathological characteristics among subsets of patients. Nonparametric Spearman rank correlation coefficients were used to assess the degree of linear association between pairs of markers. As tumor response and progression can be difficult to ascertain in patients with gliomas, survival was used as the endpoint. Survival distributions from the date of tissue acquisition were estimated with Kaplan–Meier curves and compared among patient subsets with log-rank tests.45

To assess the association of survival time with multiple clinicopathological characteristics, multivariate analyses were performed using both Cox proportional hazards models and classification and regression tree (CART) models for censored survival data, as developed by LeBlanc and Crowley.46 In preliminary investigations such as this, a CART analysis can be particularly helpful because, unlike the Cox analysis, it allows large numbers of variables to be investigated without specifying expected interactions, and it can use variables with missing values for some subjects.

The CART modeling procedures generate a regression tree consisting of nodes (subsets of patients) by successively splitting each node into two nodes. At each splitting stage, the variable that splits the node into two subsets with the most homogeneous intragroup hazard rates but the greatest difference in mean hazard rates between groups (that is, the greatest difference in survival) is chosen as the optimum split for that node. Only complete data for a particular variable are used to define a particular split. If data for a particular variable that produces the optimum split were missing for any patients in a given node, then the CART software calculated the correlation of the remaining variables for patients in the node and used the most highly correlated variable (called the surrogate variable) to classify the patients with missing data into appropriate subsets.

Sources of Supplies
Biogenex Laboratories (San Ramona, CA) provided the DO-7 and the antigen retrieval solution used in the PAb 1801 immunohistochemical analysis; Dako Corporation, Inc. (Carpinteria, CA) provided the PC10, biotinylated goat anti–mouse IgG, and peroxidase-conjugated streptavidin. The MIB-1 was obtained from Amac, Inc. (Westbrook, ME), and the PAb 1801 from Cambridge Research Biochemical (Valley Stream, NY).

Results

Patient Characteristics
As shown in Table 1, the 120 patients included in this study consisted of 77 males (64%) and 43 females with a median age of 56 years (range 6–80 years). The study included 15 patients (12%) with mixed oligoastrocytomas and 105 with astrocytomas of the diffuse or fibrillary type. Cytologically, 49 (46.7%) of the latter tumors were of the fibrillary cell type, 20 (19%) of the gemistocytic type, and eight (7.6%) of the giant-cell type. Two tumors were gliosarcomas. Of the 120 tumors, 86 (72%) were classified WHO Grade 4, 19 (16%) Grade 3, and 15 (13%) Grade 2. Fifty-two (43%) of the patients had undergone gross-total tumor resection. At the time of data analysis, 92 patients (77%) had died, and the median duration of follow-up review was 3.24 years for the remaining 28. The median survival time for the entire group was 17.8 months.
Proliferation Markers and p53

Table 1 provides a summary of the values of the proliferation markers and p53 expression for several patient subsets defined by various patient and tumor characteristics.

**Proliferating Cell Nuclear Antigen.** The LI of 107 gliomas with a PCNA determination ranged from 0 to 91.2%, with a mean value of 22.96% (± 19.5%) and a median value of 17.16%. Proliferating cell nuclear antigen LI values of 5% or less were found in 22 patients (20.6%), values of 6 to 20% in 40 patients (37.4%), values of 21 to 40% in 27 patients (25.2%), and values of more than 40% in the remaining 18 patients (16.8%). The PCNA LI increased significantly with increasing tumor grade (p = 0.0057), as shown in Fig. 1A. There was no evidence of any linear association of PCNA LI with any other parameter (Table 1 and Figs. 1B and C). Patients with mixed oligoastrocytomas had lower PCNA LI values; these patients also had lower tumor grade and younger age. The MIB-1 Antibody. For the 104 patients (87%) whose tumors showed staining with MIB-1, the LI ranged from 0 to 75.5%, with mean and median values of 20.72% (± 16.13%) and 18.5%, respectively. Patients with oligoastrocytomas did not have a significantly different MIB-1 LI from the other gliomas analyzed. As observed for PCNA, Fig. 1A shows that the MIB-1 LI increased significantly with grade. There was no evidence of any linear association between MIB-1 scores and any other parameter (Table 1 and Figs. 1B and D).

**The p53 Protein.** For the 102 patients (85%) whose tumors exhibited p53 immunostaining, nuclear staining in more than 10% of neoplastic cells was observed in the tumors of 41 patients (40%) with DO-7 and 45 patients (44%) with PAb 1801. Tumors showing only rarely stained cells were not included in the positive group. No cytoplasmic staining was observed. The percentage of cells stained in any given tumor was highly concordant between the two p53 antibodies (data not shown). Table 1 shows that the percentage of p53 positive tumors was not significantly associated with tumor grade, patient age, or gender but was significantly different among tumor subsets defined by ploidy and chromosome 17 LOH.

**Correlation Between Variables.** There was little correlation between pairs of immunohistochemical markers. Figure 2 shows the scatterplot of corresponding values in each tumor for PCNA versus MIB-1. The correlation coefficient was nearly zero. This was also the case when we examined PCNA versus p53 (DO-7) and MIB-1 versus p53 (DO-7) (data not shown).
Correlation of Variables With Survival

Figure 3 displays the Kaplan–Meier survival curves for the patient subsets defined by grouped values of each of the immunohistochemical markers, that is, low, medium, and high values of PCNA (Fig. 3A); low versus high MIB-1 LI (Fig. 3B); and low versus high p53 staining percentages (Fig. 3C). These figures suggest that survival time is significantly reduced as either the PCNA LI or the MIB-1 LI is increased (log rank p = 0.0131 and p = 0.0069, respectively) but is approximately the same for both high and low p53 staining values. We found no differences in survival times between patients with p53 immunoreactivity, compared to those without, within any particular grade (p = 0.41, p = 0.71, and p = 0.85 for Grades 2, 3, and 4 tumors, respectively, log-rank test), or when tumors with more than 50% of cells staining were compared with those with less than 50% or no p53 cell staining (p = 0.51, log-rank test).

To determine whether PCNA and MIB-1 remained significantly associated with survival time after adjustment for the effects of other clinicopathological variables, multivariate analyses were performed using Cox and CART models. Table 2 provides a summary of the results of the Cox modeling process.

The probability values presented for the one-variable models show that survival time is significantly associated with each of the following variables when considered individually: patient age, tumor grade, three cell types (astrocytomas of the fibrillary, gemistocytic, and oligoastrocytoma histology types), and PCNA and MIB-1 LIs. Moreover, the risk ratios show that the risk of dying increases for higher values of age, grade, PCNA, and MIB-1 as well as the presence of the fibrillary cell type but is reduced in the presence of the relatively favorable gemistocytic and oligoastrocytoma cell types.

The probability values obtained for each of the vari-
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Variables in the multivariate “full” Cox models containing all the clinicopathological variables together with one or all of the immunohistochemical variables show that length of survival is significantly associated only with age and grade. None of the cell types or other variables retained any significant association with survival duration after adjustment was made for the effects of age and grade.

Figure 4 displays the classification tree generated by the CART modeling process. It identified six groups of patients with distinctly different survival distributions (Fig. 5), which are defined as follows: 1) 15 patients with Grade 2 tumors; 2) 19 patients with Grade 3 tumors; 3) 26 patients with Grade 4 tumors, age less than 56.5 years, and PCNA LI greater than 8.7; 4) 24 patients with Grade 4 tumors, age equivalent to 56.5 to 66.5 years, and PCNA LI greater than 8.7; 5) 14 patients with Grade 4 tumors, age less than 66.5 years, and PCNA LI less than 8.7; and 6) 22 patients with Grade 4 tumors and age greater than 66.5 years.

Discussion

The overall goal of this series of investigations was to identify biological markers that might aid in the morphological classification of gliomas and in the prediction of prognosis for patients affected by these tumors. Currently, few clinical and pathological factors have been identified as being prognostically useful. Patient age and tumor grade have been most strongly associated with prognosis. Several reports have suggested that p53 overexpression and proliferative potential, as measured by PCNA or Ki-67, are correlated with prognosis. In general, however, these studies have yielded widely varying results, have used small numbers of patients, and often have not used multivariate analysis.

We examined the expression of p53 and the proliferation markers PCNA and MIB-1 in paraffin sections of 120
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newly diagnosed cerebral gliomas, all but 15 of which were astrocytic. The advantage of using immunohistochemical analysis on paraffin-embedded material is the preservation of tissue architecture, which allows the staining properties of tumor cells to be directly assessed.

Overall, 40% of the gliomas overexpressed p53. This proportion was similar in all grades of tumor and did not show any association with patient age or gender. Gemistocytic gliomas were most likely to be p53 positive, whereas there were no significant differences in the frequency of p53 expression in the other histological types, including oligoastrocytomas. Overexpression of p53 was of no prognostic significance in this series of patients.

A wide range of values was obtained for the LIs of PCNA and MIB-1 (Ki-67). These values tended to increase with increasing grade of malignancy, and poor survival time was significantly associated with elevated values of both indices in univariate analyses. Multivariate analysis, however, failed to reveal any independent prognostic value for either index except, possibly, for PCNA in the subset of patients with Grade 4 tumors and age less than 66.5 years, as determined by CART modeling. There was poor concordance between these two markers of proliferation, and no association was observed between p53 expression and PCNA or MIB-1 LI. Oligoastrocytomas had lower PCNA scores but were otherwise similar to the other cell types (MIB-1 and p53).

Although overexpression of the p53 protein and mutations of the p53 gene can occur in a significant proportion of astrocytomas, controversy exists regarding the relationship between p53 abnormalities and clinicopathological variables, particularly survival time. The p53 gene mutations and/or overexpression are found in both high- and low-grade astrocytomas, and many studies, including our own, fail to show an association of p53 expression and tumor grade. Similarly, p53 generally has had limited, if any, prognostic value. Expression of p53 was associated with both LOH on chromosome 17p and aneuploidy. As a corollary, LOH on chromosome 17p, on which the p53 gene resides, was found not to be a significant prognostic factor in a previous study of gliomas. Aneuploidy was not associated with survival in univariate analysis, although it may be significant in younger patients with high-grade astrocytic tumors. In a recent study, Kyritsis, et al. reported prognostic significance of p53 immunoreactivity in anaplastic gliomas but not glioblastomas. We were unable to show prognostic value for p53 staining within any grade of glioma in this study. Whether p53 overexpression is more common in young or female patients is not clear. There was no evidence of such associations in the present series of tumors.

Discordance between reported studies of p53 abnormalities in astrocytomas may reflect the use of differing antibodies or immunostaining methodologies, variation in the criteria of p53 positivity, and differences in the number and histological nature of the tumors studied. The DO series of antibodies are the most sensitive for use on paraffin-embedded material, but they have been infrequently applied. The DO-7 antibody recognizes an epitope within the first 45 amino acids of p53 and recognizes both mutant and wild-type p53. Several reports included Grade 1 tumors; one of these also included recurrent astrocytomas, with staining as low as 0.8% being deemed p53 positive. Some studies, including the present one, report staining in more subjective terms, usually citing a cutoff of 10 to 20% as positive. The significance of rarely stained nuclei remains unclear. The p53 gene is located on chromosome 17p and mutations in this gene are often associated with LOH at 17p. It is important to note that although p53 overexpression is often associated with the presence of p53 mutations and 17p LOH, mutations that result in truncated products (insertions and deletions resulting in frameshifts and mutations leading to premature stop codons) are generally negative. Furthermore, p53 overexpression...
protein expression may be stabilized by some other means.19,20 Studies of proliferation markers in astrocytomas generally indicate that, although high values are associated with tumor grade,2,3,30,36,37,56,64 mean values for Grade 2 astrocytomas ranged from 2 to 14%, values for Grade 3 tumors from 9 to 49%, and values for Grade 4 tumors from 18 to 59%.3,37 Given such overlap, it is of no surprise that skepticism has been expressed regarding use of PCNA in assessment of proliferation in astrocytomas.35,43

Increased Ki-67 immunoreactivity (on frozen tissue), although associated with increasing tumor grade, has rarely been shown to be significantly associated with poorer patient survival.28,43,72 There are two reports on MIB-1 staining in gliomas.54,59 The values obtained in this series closely reflect those reported by Sallinen, et al., but are higher in the low-grade tumors than those of Onda and associates.54 We and Sallinen, et al., found MIB-1 LIs to be inversely associated with survival in univariate analysis. Whereas, using multivariate analysis, Sallinen, et al., found that patient age and tumor grade remained the most significant independent predictors of survival, with MIB-1 immunoreactivity failing to reach significance.

Discordance among the various published Ki-67 and PCNA LI studies could be due to a number of factors. Various PCNA antibodies define different epitopes and may have different cell cycle distributions. Furthermore, some controversy exists on the optimum way to assess PCNA values.11,68 Results of PCNA LI studies do not always correlate with those of other markers of proliferation23,27,42,58,70 and, in some systems, cells that stain with PCNA define a different population of cells from those that incorporate BUdR or stain with Ki-67.10 Such factors may complicate a direct comparison of published studies. Using the original Ki-67 antibody raised against a crude nuclear fraction of L428 cells, labeling closely correlated with BUdR indices in gliomas, thus suggesting value in assessment of proliferative potential.49 In gastric mucosa, MIB-1 accurately reflects the S phase fraction as determined by BUdR labeling, whereas PCNA does not.27 Recently, Onda and associates54 have shown that, in gliomas, MIB-1 labeling correlated with other parameters of proliferation, although correlation between different methods was often less than predicted.

In our present study, there was clearly a lack of concordance between the markers of proliferation, particularly when adjacent sections were assessed. The proliferation markers similarly showed little, if any, association with either the percentage of S- or G2+M fractions (data not shown). The poor concordance between MIB-1 and PCNA LIs in individual tumors suggests that any one means of assessing proliferative potential in gliomas may not be reliable. This conclusion is supported by a growing number of astrocytoma studies.25,43,58 It has been noted, for instance, that quantification of cells in a single part of the cell cycle does not fully explain the growth that contributes to tumor cell kinetics,57 in that such methods do not take into consideration rates of cell loss (apoptosis and...
necrotic processes), length of the cell cycle, or rates of cell turnover.

As has been reported by others, we did not observe an association between p53 expression and tumor cell proliferation, as measured by PCNA or MIB-1 staining. Although the mean Ki-67 LI has been reported to be higher in p53-positive gliomas, these variables showed no such correlation in the individual tumors. Recent evidence indicates that p53 mediates the transcription of a range of target genes that affect cell cycle arrest and apoptosis. Among these is p21<sup>WAF1/Cip1</sup>, which acts as a CDK inhibitor. The p21<sup>WAF1/Cip1</sup> gene has been shown to bind PCNA and to mediate the inhibition of the replication, but not the repair function, of PCNA. The p53 gene also affects transcription of Gadd45, which also may act in the cell cycle. However, both p21 and Gadd45 may also be induced in a p53-independent manner. It has also been suggested that PCNA expression may become deregulated within some tumor types, but whether p53 is involved has not been formally addressed.

Overall, the role that alterations in the p53 gene play in the development and progression of gliomas remains unclear. Because p53 protein overexpression and p53 gene mutations are found in all grades of astrocytomas, it has been hypothesized that alterations in p53 occur early in the development of gliomas. In addition to altering transcription and affecting the ability of cells to respond to DNA damage, an absence of p53 function may also confer resistance to apoptosis. It was recently observed that gliomas with wild-type p53 tended to express bcl-2, which blocks apoptosis. Expression of bcl-2 does not correlate with the presence or degree of malignancy in gliomas. These data indicate that many gliomas may develop an early resistance to apoptosis. It should be noted, however, that some glioblastomas with wild-type p53 and a normal chromosome 17 have abnormalities of chromosome 10. In this series of tumors, chromosome 10 abnormalities were associated with poor survival in univariate, but not in multivariate, analysis.

In conclusion, in this group of 120 patients with infiltrative gliomas the strongest predictors of survival were patient age and tumor grade. The overexpression of p53 protein was not correlated with survival in this analysis. In addition, the proliferation markers PCNA and MIB-1 (Ki-67) were associated with survival in univariate, but not in multivariate, analysis. The poor concordance of these two markers further indicates that they should be used with caution, if at all, in the clinical setting. It may be that in situations in which the histological typing or grading is uncertain, the use of MIB-1 may help to determine when tumors are likely to be associated with a poor prognosis. Thus the MIB-1 LI deserves further evaluation to determine whether it is of independent prognostic value. Such studies should focus on patients enrolled in treatment trials, thus eliminating the confounding variable of treatment effect on survival.

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