

# Analysis of Complex Relationships between Age, p53, Epidermal Growth Factor Receptor, and Survival in Glioblastoma Patients<sup>1</sup>

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## ABSTRACT

**Glioblastoma multiforme (GBM) carries a dismal prognosis. However, a range of survival times exists, and parameters that define prognostic groups may help to optimize treatment. To identify such prognostic groups, we analyzed tumor tissue from 110 cases of newly diagnosed GBM from two clinical protocols. Similar to other studies, we found no association of epidermal growth factor receptor (EGFR) overexpression (as assessed by immunohistochemistry), p53 immunopositivity, or p53 mutation with survival in the entire sample. However, EGFR overexpression showed trends toward worse prognosis in patients younger than the median age, but better prognosis in patients older than the median age. This interaction of EGFR with age group was statistically significant and led us to focus our further analyses on the younger patients. In this group, a statistically significant association of EGFR overexpression with worse survival was identified in the p53-negative but not p53-positive tumors. We found a similar result after screening these cases for mutations in p53: EGFR overexpression was negatively associated with survival only in the p53 wild-type cases. To confirm this unexpected result, this finding was reproduced in a validation sample of an additional 42 tumors from younger patients on the same two clinical protocols. This complex relationship between EGFR and p53 in younger patients remained in a multivariate analysis that incorporated additional prognostic variables. The results suggest that analysis of prognostic markers in GBM is complex, and maximal information may require analysis of subgroups based on age and the status of specific markers such as p53. In addition, they suggest a specific group of patients on which to focus promising therapies targeting EGFR.**

## INTRODUCTION

GBM<sup>3</sup> is the most common and lethal primary brain tumor in adults. It is nearly uniformly fatal, with a median survival of approximately 1 year, despite modern treatment modalities (1). However, a range of survival times exists around this median. Efforts to understand why some patients live longer or shorter than the average may provide insights into the biology of these neoplasms. To date, the most consistent predictor of survival in malignant gliomas is patient age at diagnosis. Multiple studies have shown that younger patients with these tumors live longer following initial diagnosis, even after adjusting for histological grade, size of tumor, KPS, extent of resection, and treatment following biopsy/resection (1–5). Despite falling within a

single histological grade, glioblastomas exhibit significant genetic heterogeneity. It is likely that these genetic differences, at least partially, account for the differences in survival time among patients with these neoplasms.

Efforts have been made by many groups to test whether the genetic alterations found in these tumors can, in addition to patient age, predict prognosis. The most common alterations in high-grade astrocytic gliomas include mutations in the *p53* gene, amplification and rearrangement of the *EGFR* gene, and amplification of a region of chromosome 12q that encodes murine double minute 2 (*MDM2*) and cyclin-dependent kinase 4 (*CDK4*) (6). Many of these alterations have been tested for potential as prognostic markers. However, there has been no consensus for the prognostic value of most of these commonly altered genes in GBM. For example, some investigators have found that gliomas with the *p53* gene mutation or immunopositivity (as a marker of abnormality in the p53 pathway) predict shorter survival (7–9), whereas others have found no statistically significant relationship (3, 10–14). In addition, one recent study showed that mutation in *p53* predicted longer survival in patients with GBM (15).

Similar to the situation with p53, no consensus has been established on the prognostic value of EGFR. The prognostic implications of *EGFR* amplification/overexpression in glioblastoma are controversial, with some groups claiming no association with survival (10, 11, 16) and others claiming that this aberration is a negative prognostic factor (7, 8, 17, 18).

Proliferation indices have not been consistently demonstrated to be associated with prognosis in high-grade astrocytomas. The MIB-1 antibody detects the Ki-67 antigen, which is expressed only in actively cycling cells (19). Although Ki-67 labeling index predicts prognosis in low-grade astrocytomas (20, 21), the studies in patients with GBM are less clear (22–24).

Taken together, these studies show no consistent relationship between these potentially prognostic molecular markers in GBM and patient outcome. The studies are frequently difficult to compare with each other because some were performed on a mix of tumor types and most did not study patients in clinical trials; thus, treatment modalities may have varied widely. There has also been a suggestion in the literature that there are subtypes of GBM based partially on EGFR and p53 status (25). To address these issues, we attempted to identify a group of patients for whom specific markers might be prognostic. The results of this study suggest that evaluation of specific markers in glioblastoma is complex, and analysis in specific subsets of patients may reveal prognostic information not identifiable when the entire sample is viewed as a whole.

## MATERIALS AND METHODS

**Patient Population.** All of the patients in this study were enrolled in one of two consecutive clinical trials (8822 and 6G901) administered by the Neuro-Oncology Service, University of California, between December 28, 1987, and December 26, 1995. In the first protocol, patients were treated with standard external beam radiotherapy, followed by 1,3-bis(2-chloroethyl)-1-nitrosourea and 6-thioguanine. A total of 134 GBM patients were enrolled in

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<sup>3</sup> The abbreviations used are: GBM, glioblastoma multiforme; EGFR, epidermal growth factor receptor; SSCP, single-strand conformation polymorphism; KPS, Karnofsky performance score; CI, confidence interval; HR, hazard ratio; IHC, immunohistochemistry; UCSF, University of California–San Francisco.

this study. In the second protocol, patients received either standard or hyperfractionated radiotherapy, plus or minus difluoromethylornithine. A total of 233 GBM patients were enrolled in this study. Protocols from both trials were approved by the UCSF Human Experimentation Committee, and informed consent was obtained from each patient. Selection for the current study was based on the diagnosis of GBM of the original resection and the ability of the investigators to obtain paraffin-embedded tumor tissue. One hundred ten cases were selected for the initial study, 57 of which were from protocol 6G901 and 53 from protocol 8822. In a comparison of the 6G901 patients who were included in this study *versus* those who were not, the median age (58.0 *versus* 57.1 years, respectively,  $P = 0.58$ , Mann-Whitney  $U$  test) and survival (42.1 *versus* 39.9 weeks,  $P = 0.27$ ) did not differ significantly. For the 8822 patients, there was also no difference in age (52.0 *versus* 53.6 years,  $P = 0.26$ ) or survival (62.0 *versus* 53.3 weeks,  $P = 0.21$ ) between those who were studied and those who were not. The median KPS was the same (90) for those patients who were studied *versus* those who were not among both protocols. The distribution of extent of resection (biopsy *versus* subtotal resection *versus* gross total resection) was 27%, 62%, and 11%, respectively, among the patients who were not studied, compared with 2%, 90%, and 8%, respectively, in the patients whose tissue was available for study in the initial sample. The difference in biopsy rates between included and excluded patients reflected the availability of tissue for study. There was a significant difference between the 6G901 and 8822 patients as a whole with respect to median age (57.1 *versus* 52.9 years,  $P = 0.02$ ) and survival (41.1 *versus* 57.2 weeks,  $P < 0.01$ ). Subsequent to the analysis of the original 110 patients, tumor tissue from an additional 42 cases was obtained from patients in these clinical protocols who were identified as younger than 55 at the time of initial surgery. Of the 152 total cases (110 patients in the original group and 42 patients in the validation group), 63 patients were enrolled on protocol 8822 and 89 patients were enrolled in protocol 6G901. All tissue analyzed represented tumor from the original resection before the administration of radio- or chemotherapy. Diagnosis of GBM was consistent with the WHO criteria in use today (26). Survival is expressed as weeks after initial surgery date.

**IHC.** Once paraffin blocks were obtained, a block with representative tumor tissue was chosen for study and 5- $\mu$ m sections were mounted on positively charged slides. Multiple serial sections from each case were stored for later staining. The immunohistochemical procedures were routine and are summarized as follows. Sections were deparaffinized in histological grade xylenes for 10 min and rehydrated through sequential 95%–70% ethanol and placed in PBS. For all antibodies, microwave antigen retrieval was performed by placing the slides in 50 mM citrate buffer (pH 6.0) and microwaving for 12 min at full power and 10 min at 20% power, followed by cooling for 15 min and two to three 5-min washes in PBS. For the MIB-1 antibody the sections were also treated with 0.025% trypsin for 15 min at 37°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS/0.05% Tween 20 or 3% hydrogen peroxide in methanol for 10–20 min. Sections were then washed two to three times in PBS and blocked for 20 min in the appropriate serum from the same species as the secondary antibody diluted to 10% in PBS. The primary antibody, diluted in PBS/10% serum, was applied to the sections in a humid chamber for 2 h at room temperature or overnight at 4°C. After washing two to three times in PBS, the secondary antibody was applied per directions in a kit from Vector Laboratories, Inc. Briefly, biotinylated antimouse was diluted in 10% normal horse serum/PBS 1:200, and sections were incubated at room temperature for 30 min. Detection of the antibody was performed with diaminobenzidine for 1–5 min. Sections were then counterstained with light hematoxylin and mounted. Primary antibodies were all mouse monoclonals and were obtained and used as follows: anti-p53 (DO-7, DAKO Corp.; 1:150 dilution), anti-EGFR [clone 528(4), Calbiochem Oncogene Research; 1:50 dilution, which recognizes both wild type and most common rearranged form (27)], and anti-Ki67 (MIB-1, DAKO Corp.; 1:500 dilution).

Each slide stained for p53, EGFR, or MIB-1 was individually reviewed and scored by at least one neuropathologist (M. L. S. or K. A.). Disagreements in scoring (<10% of cases) were resolved by review and discussion at a multi-headed microscope. Scoring for p53 was for nuclear staining on a four-point scale from 0–3. A score of 0 indicated no staining, 1 indicated <5% of nuclei with positive staining, 2 indicated 5–30% of nuclei stained, and 3 was >30% of nuclei with positive staining. For purposes of statistical analysis, all of the

p53 scores of 0 and 1 were later condensed to a score of “negative,” whereas the scores of 2 and 3 were condensed to a score of “positive.”

The EGFR antibody typically stained the cell membrane, often with some accompanying cytoplasmic staining. Scoring was on a three-point scale with 0 indicating no staining, 1 indicating light or focal staining, and 2 indicating strong staining. For statistical analysis, a score of 0 was treated as “negative” and a score of 1 or 2 was considered “positive.”

MIB-1 scoring was accomplished by determining the percentage of positive nuclei after counting 1000 tumor cells, or as many as possible in the case of small specimens. For the purposes of statistical analysis, the MIB-1 score was later graded as 0, 1, 2, or 3 based on quartile determinations of the initial continuous scoring. These cutoffs were 16.75%, 27.50%, and 39.75%, defining four groups of 27–28 patients each. This categorization recognizes that the MIB index is not truly continuous, but rather semiquantitative (similar to EGFR and p53 scoring) and minimizes potential bias created by selection of the region of the tumor for determination of proliferation index. It also prevents a few extremely large values from dominating the analysis.

**p53 Genetic Analysis.** All cases from the original sample of 110 patients for which adequate DNA was available were analyzed for p53 mutations. SSCP analysis was performed for exons 5–8 on all cases using a novel fluorescent analysis recently developed at the UCSF Cancer Center (28). Briefly, forward primers were labeled with the fluorescent dye FAM, whereas the reverse primers were labeled with the fluorescent dye TET. The PCR products were run on an ABI 377, and data were analyzed using Genescan software (ABI). All SSCP shifts were sequenced after reamplification using established primers on an ABI sequencer. Five cases were also analyzed by SSCP using radioactively labeled nucleotides, as described previously (29), showing similar results, and two cases were identified by this method alone.

**Statistical Analyses.** Univariate analysis and Kaplan-Meier curves were generated with the statistical program PRISM by GraphPad. Log rank  $P$ s are reported for the dichotomous variables. Multivariate analysis (Cox proportional hazards regression) was performed with NCSS software and SAS. HRs and  $P$ s are noted for each variable in the multivariate analysis. The outcome variable for all analyses was death from all causes. No patients in the study were known to have died from treatment toxicity. Among patients in the four arms of protocol 6G901, no difference in survival was noted between any of the groups, so they were combined and considered a single protocol. Protocol 8822 was coded as “0,” and protocol 6G901 was coded as “1” in multivariate analysis. The extent of surgery, as estimated by the surgeon, was coded as biopsy = 0, subtotal resection = 1, and gross total resection = 2. KPS was coded so that a score of 100 was coded as “0,” 90 as “1,” 80 as “2,” and so forth. HRs provide information about the direction of an association (a numeral over 1 indicates an increased risk with the positive variable, and a numeral under 1 indicates a decreased risk) as well as the magnitude of the risk. To describe the EGFR-p53 interaction, new variables representing the four groups defined by these dichotomous variables were created to identify the EGFR HRs for the p53-negative and p53-positive groups. This analytic method was chosen to demonstrate increases in risk, if any, associated with EGFR based on p53 status (30). HRs with  $P < 0.05$  were considered statistically significant, and all significant HRs are designated as such in the tables or text. HRs were rounded to the first decimal.

To explore possible relationships between age group and the variables under study, age was dichotomized at the median value (55 years). Our goal was to divide the group into two groups of equal size and ask whether the predictive markers in each group were the same or different, not to identify the optimal age cutoff with respect to the prediction of outcome.

In Tables 2, 4, and 5, clinical variables (KPS, extent of resection, protocol status, and age) are not included in the models presented. However, additional analyses including these variables were run in each case, resulting in similar findings, which are stated below in the text.

## RESULTS

**Univariate Analyses.** In the initial sample of 110 patients, the median age was 55 and there were 42 women and 68 men. The median survival following the initial surgery was 51.3 weeks. Five patients were lost to follow-up and, therefore, censored at the last known

survival time, and the remainder died. Univariate analyses on this sample showed that age, KPS, MIB-1, and protocol status were significantly associated with survival, whereas neither EGFR nor p53 (either by expression or mutational analysis) showed an association with survival (Table 1).

**Analysis of EGFR.** Thirty-five percent (39 of 110) of the original 110 cases had positive EGFR staining. The mean age of EGFR-positive cases was greater than that of EGFR-negative cases (58 versus 52,  $P = 0.01$ , two-tailed  $t$  test). Given the strong influence of age on prognosis in these tumors, we divided the sample into younger and older age groups, based on the median age of 55. We tested for an association of EGFR with survival in each age group independently. As shown by Kaplan-Meier analysis in Fig. 1, EGFR positivity was significantly associated with an improved prognosis in patients of the older age group but showed a trend toward worse survival in the younger patients. These data suggested that the effect of EGFR might differ depending on age group. Corroborating this hypothesis, a significant statistical interaction between EGFR and age group was present in Cox proportional hazards testing ( $P < 0.01$  for the interaction term; EGFR HR, 1.7; 95% CI, 0.9–2.3 for younger patients and HR, 0.5; 95% CI, 0.3–0.9 for older patients).

**p53 Immunohistochemical Analysis.** The fact that EGFR seemed to be differentially associated with survival led to an approach in which patients in the two age subgroups were treated as different groups for analytic purposes. We analyzed relationships of p53 expression in these two groups. IHC for p53 was positive in 30 of 53 younger patients and 24 of 57 older patients. p53 was not associated with survival in either group, and an age interaction was not identified (data not shown). We tested for relationships between p53, EGFR, and survival in either group. A statistically significant interaction between p53 and EGFR was identified in the younger patients ( $P < 0.01$ ). To illustrate this, Kaplan-Meier curves relating EGFR to survival were generated treating the p53-negative and p53-positive younger patients as two different groups (Fig. 2). EGFR positivity was a negative prognostic factor only in the p53-negative group (Fig. 2A). Table 2 shows the results of Cox regression, which indicated that EGFR was a significant predictor of outcome (EGFR overexpression was associated with shorter survival) only in the p53-negative subgroup. When adjustments for additional prognostic variables, including age, KPS, extent of resection, and protocol status were included in this model, a statistically significant EGFR-p53 interaction ( $P = 0.01$ ) remained. In the older patients, no interaction between EGFR and p53 was identified ( $P = 0.23$ ). Specifically, the EGFR HR in the p53-negative group (HR, 0.6; 95% CI, 0.2–0.9) was not significantly different from that of the p53-positive group (HR, 0.4; 95% CI, 0.2–1.4).

The difference in direction of association of EGFR and survival based on age group led to an examination of the two groups as independent samples. An additional difference between the younger

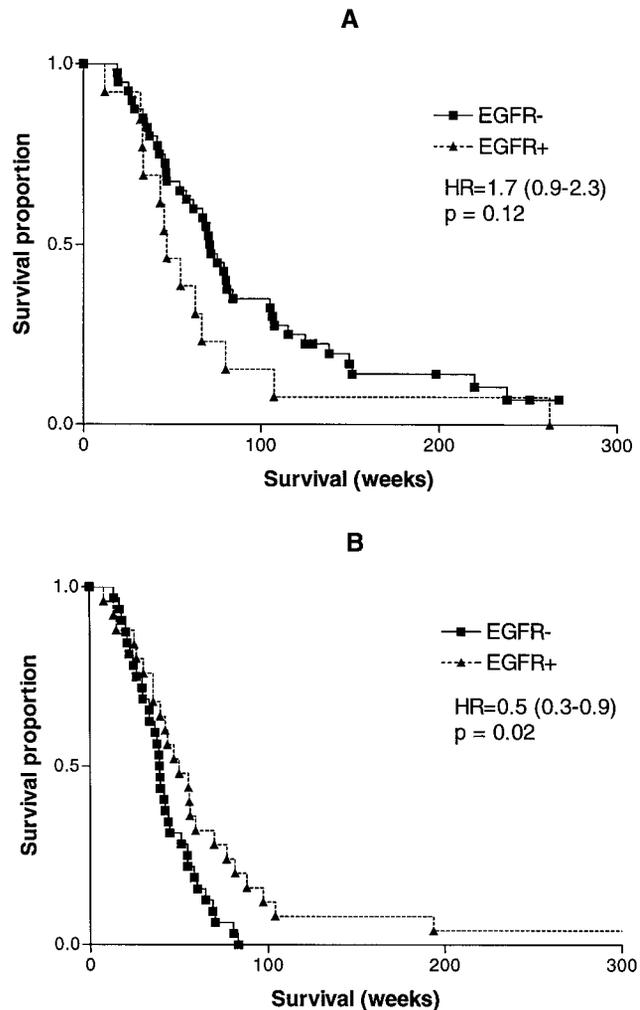


Fig. 1. Kaplan-Meier analysis comparing EGFR status with survival in patients  $<55$  ( $n = 53$ ; A) and patients  $\geq 55$  ( $n = 57$ ; B) years of age in the original sample of 110 patients. HRs, 95% CIs, and  $P$ s shown are based on Cox analysis. ■, EGFR-negative tumors; ▲, EGFR-positive tumors.

and older patients was that the range of survival times was greater in the younger patients (25th and 75th percentiles, 41.2 and 107.5 weeks, respectively) than in the older patients (25th and 75th percentiles, 29.4 and 59.3 weeks, respectively). For this reason, subsequent analyses to characterize molecular profiles relating to prognosis were focused on the younger group of patients.

**Analysis of the p53 Gene.** Because immunopositivity of p53 may not be a reliable indicator of p53 gene mutation, we determined the p53 gene status to determine whether a similar interaction existed between EGFR expression and p53 gene status in the younger patients. We tested for p53 mutations, using SSCP of exons 5–8, followed by direct sequencing of abnormally migrating exons. Sufficient material was available to ascertain p53 gene status in 88 of the 110 patients from the original sample, and 46 of 53 patients from the younger group. Overall, mutations were detected in 17 of 88 cases (19%) and are outlined in Table 3. Most (14 of 17) of the cases in which p53 was found to be mutated occurred in younger patients, similar to what has been described previously (25). All cases showing p53 mutation demonstrated immunopositivity of p53, except case 497, in which a G-A mutation resulted in a premature stop codon. Overall, p53 gene status was highly correlated with p53 expression status ( $P < 0.01$ , Fisher's exact test). However, although 16 of 17 of the p53 mutant cases were p53 positive by IHC, 25 of 71 (35%) cases in which

Table 1. Univariate analyses of EGFR, p53, age, KPS, protocol, and MIB-1 proliferation index in the original group of 110 patients

Univariate Cox analyses were performed using EGFR IHC, p53 IHC, p53 mutation status (p53 gene), and age group as dichotomous variables. MIB-1 values were separated on a quartile basis. HRs,  $P$ s, and 95% CIs are as indicated. The HR for age as a continuous variable indicates the increase in hazard on a per year basis.

Variable	HR	95% CI	$P$
EGFR (+ vs. -)	1.1	0.7–1.6	0.61
p53 IHC (+ vs. -)	1.1	0.7–1.5	0.82
p53 gene (mutant vs. wild type)	0.8	0.5–1.2	0.49
Age (continuous)	1.05	1.03–1.07	$<0.01$
Age ( $\geq 55$ vs. $<55$ )	2.0	1.4–3.0	$<0.01$
KPS	1.3	1.1–1.6	$<0.01$
Extent of resection	0.9	0.5–1.5	0.60
Protocol (6G901 vs. 8822)	2.1	1.3–3.4	$<0.01$
MIB (quartiles)	1.3	1.1–1.5	$<0.01$

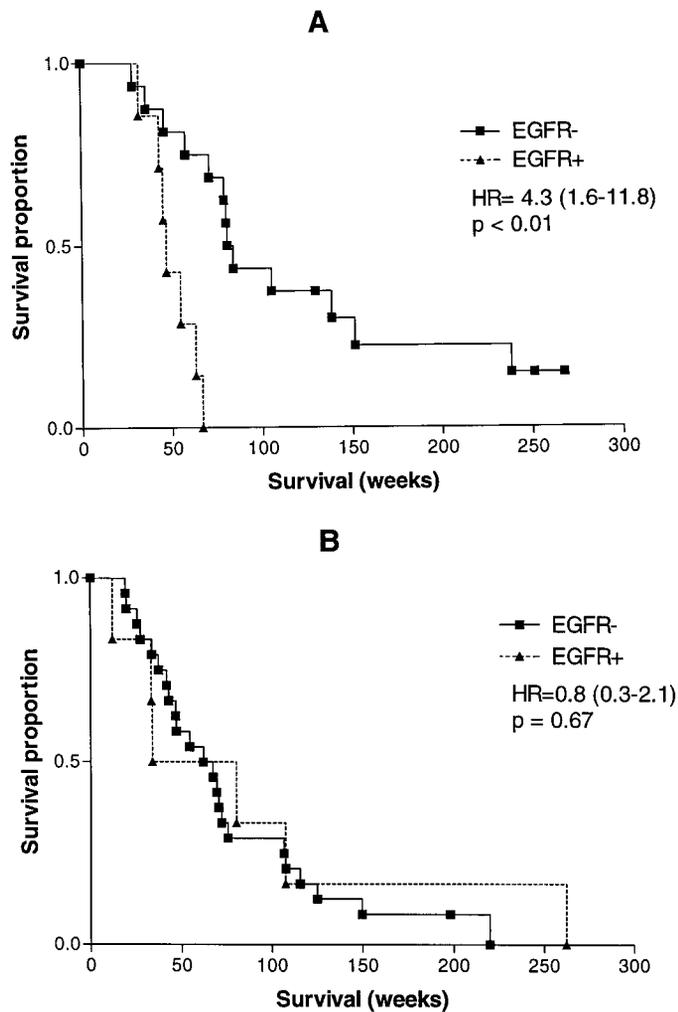


Fig. 2. Kaplan-Meier analysis comparing EGFR status with survival in younger patients from the original sample ( $n = 53$ ), with stratification by p53 immunohistochemical analysis. HRs, 95% CIs, and  $P$ s shown are based on Cox analysis. A, p53-negative cases ( $n = 23$ ). B, p53-positive cases ( $n = 30$ ). ■, EGFR-negative tumors; ▲, EGFR-positive tumors.

Table 2 Association of EGFR with survival in patients <55 years ( $n = 53$ ) based on p53 immunohistochemical status

Cases were scored for p53 and EGFR expression by IHC, and median survival (weeks after initial surgery) was determined for each of the four groups of cases based on marker status (left). A Cox multivariate analysis was performed to test for associations of EGFR status with survival in p53- and p53+ subgroups. Adjustments for age, KPS, extent of resection, and protocol status resulted in similar findings (see text). HRs, 95% CIs, and  $P$ s are as indicated on the right.

	Median survival (no. of cases)		Cox analysis (EGFR+ vs. EGFR-)		
	EGFR-	EGFR+	HR	95% CI	$P$
p53-	82 (16)	47 (7)	4.3	1.6-11.8	<0.01
p53+	57 (24)	64 (6)	0.8	0.3-2.1	0.67

a mutation could not be detected were positive by IHC, suggesting that a significant number of cases showed p53 dysregulation without a mutation in the exons tested.

Similar to the analysis for p53 expression, in the younger patients a significant EGFR-p53 interaction ( $P = 0.02$ ) was observed when p53 gene status was used as the p53 variable. Therefore, cases from this younger age group were analyzed separately for an association of EGFR with survival based on p53 gene status. Cox regression (Table 4) showed that, analogous to the results using p53 IHC data, EGFR was significantly associated with worse survival only in the p53

wild-type cases. This interaction remained ( $P = 0.03$ ) after adjustments for age, KPS, extent of resection, and protocol status were made.

**Validation Sample.** To validate the relationships of EGFR, p53, and survival in the younger patients, we screened an independent sample of cases to test the reproducibility of the p53-EGFR interaction and the association of each marker with prognosis. An additional set of 42 cases from younger patients (<55 years of age) on the same two clinical protocols used in the original sample were tested for p53 and EGFR by IHC. Similar to the results obtained in the original sample, a significant EGFR-p53 interaction was identified ( $P < 0.01$ ; Table 5), which remained identifiable as a statistical trend ( $P = 0.08$ ) after adjustment for age, KPS, extent of resection, and protocol. In addition, EGFR was significantly associated with survival only in the p53-negative group, which provided validation of the hypothesis generated by the initial sample.

**Analysis of MIB-1 Index.** We asked whether the proliferation index could add additional information to the molecular analyses

Table 3 p53 mutational analysis of tumors

Exons 5-8 were subject to SSCP analysis. Abnormally migrating exons were re-amplified and subjected to direct sequencing. The table indicates all of the mutations identified in the 88 cases analyzed. Patient age and p53 expression score are included, as indicated.

Case	Age	p53 score	Exon	Mutation
152	44	Positive	5	TGC cys-TAC tyr 176
262	39	Positive	8	CGT arg-CAT his 273
263	47	Positive	5	AAG lys-ACG thr 132
317	52	Positive	8	TGT cys-TTT phe 275
362	50	Positive	5	CCG pro-CTG leu 152
381	32	Positive	8	CGT arg-TGT cys 273
386	48	Positive	5	CGC arg-CAC his 175
403	35	Positive	7	ATG met-ATA ile 237
407	71	Positive	8	CGT arg-CAT his 273
440	40	Positive	5	CCG pro-CTG leu 152
445	53	Positive	8	CGT arg-TGT cys 273
446	56	Positive	5	TAC tyr-TGC cys 163
452	38	Positive	8	CGT arg-TGT cys 273
464	58	Positive	5	CCC pro-ACC thr 151
492	34	Positive	N/A	G-C splice site intron 4
495	38	Positive	N/A	A-T splice site intron 6
497	44	Negative	5	TGG trp-TGA-stop 146

Table 4 Association of EGFR with survival in patients <55 years ( $n = 46$ ) based on p53 gene status

Cases were screened for p53 gene status, and median survival (weeks after initial surgery) was determined for each of the four groups of cases based on gene status (left). A Cox multivariate analysis was performed to assess correlations of EGFR status with survival in p53 wild-type (p53wt) and p53 mutant (p53mut) subgroups. Adjustments for age, KPS, extent of resection, and protocol status resulted in similar findings (see text). HRs, 95% CIs, and  $P$ s are as indicated on the right.

	Median survival (no. of cases)		Cox analysis (EGFR+ vs. EGFR-)		
	EGFR-	EGFR+	HR	95% CI	$P$
p53wt	80 (22)	46 (10)	3.4	1.5-8.0	<0.01
p53mut	61 (12)	148 (2)	0.4	0.1-1.9	0.27

Table 5 Association of EGFR with survival in a validation sample of patients <55 years ( $n = 42$ ) based on p53 immunohistochemical status

Cases were scored for p53 and EGFR expression by IHC, and median survival (weeks after initial surgery) was determined for each of the four groups of cases based on marker status (left). A Cox multivariate analysis was performed to test for correlations of EGFR with survival in p53- and p53+ subgroups. Adjustments for age, KPS, extent of resection, and protocol status resulted in similar findings (see text). HRs, 95% CIs, and  $P$ s are as indicated on the right.

	Median survival (no. of cases)		Cox analysis (EGFR+ vs. EGFR-)		
	EGFR-	EGFR+	HR	95% CI	$P$
p53-	56 (17)	32 (8)	4.4	1.7-11.7	<0.01
p53+	55 (12)	83 (5)	0.5	0.2-1.5	0.23

Table 6 Final multivariate model for younger patients with GBM, with HRs for EGFR based on p53 immunohistochemical status

Younger patients from the initial ( $n = 53$ ) and validation sample ( $n = 42$ ) were grouped and entered into a multivariate model, which included adjustments for age, KPS, protocol status, and extent of resection. In this model, significant variables included KPS (HR, 1.4; 95% CI, 1.2–1.7;  $P < 0.01$ ) and protocol status (HR, 2.2; 95% CI, 1.4–3.6;  $P < 0.01$ ). Age as a continuous variable was not significant ( $P = 0.36$ ), nor was extent of resection ( $P = 0.69$ ). When included in this model MIB-1 was not significant ( $P = 0.13$ ) and was, therefore, excluded from the final model. Initial KPS and/or extent of resection were not available on 10 of the 95 patients; therefore, the total number of patients analyzed was 85.

	Median survival (no. of cases)		Cox analysis (EGFR+ vs. EGFR-)		
	EGFR-	EGFR+	HR	95% CI	$P$
p53-	66 (28)	44 (14)	3.6	1.8–7.4	<0.01
p53+	56 (33)	82 (10)	0.7	0.3–1.4	0.28

performed on these tumors. In univariate analysis of the original sample of 110 patients, high MIB-1 was associated with worse survival (Table 1). A significant MIB-1-age interaction was not observed. Specifically, a high MIB-1 index showed a trend toward worse survival in younger patients (HR, 1.2;  $P = 0.09$ ; 95% CI, 0.97–1.55) and was significantly associated with worse survival in the older patients (HR, 1.3;  $P = 0.03$ ; 95% CI, 1.0–1.7). Subsequent analyses focused on the younger patients for reasons stated above. In this group, the MIB-1 proliferation index was analyzed for its relationship to survival in the p53-negative and p53-positive subgroups. In the initial sample of 53 younger patients, the MIB-p53 interaction was statistically significant ( $P < 0.01$ ) and MIB-1 seemed to be associated with worse survival only in the p53-negative cases (data not shown). However, the importance of MIB-p53 interaction was not confirmed in the validation sample of 42 younger cases. In this group, the MIB-p53 interaction was not significant ( $P = 0.94$ ) and MIB-1 was associated with worse survival in both the p53-negative group (HR, 4.6; 95% CI, 1.4–14.7) and in the p53-positive group (HR, 4.9; 95% CI, 1.2–19.2). This suggested that, in contrast to EGFR, the association of the proliferation index with survival was not reproducibly dependent on p53 status.

**Final Multivariate Models.** For the entire group of 95 younger patients, multivariate models were tested, which initially included EGFR, p53, and MIB-1, a three-way interaction term (EGFR-p53-MIB), all possible two-way interaction terms, and adjustments for age, KPS, and protocol status. After elimination of nonsignificant interactions, the only interaction term that remained significant was that for EGFR-p53. The MIB-1 proliferation index was not significant in this multivariate model. This interaction is displayed in Table 6 by identifying the EGFR HRs separately in the p53-negative versus p53-positive cases. As shown, EGFR was a predictor of negative survival in the p53-negative cases, but not in p53-positive cases (Table 6).

## DISCUSSION

GBM is a biologically aggressive neoplasm, with a median survival of  $\sim 1$  year following initial diagnosis (1). Although a range of survival times exists around this median, it remains controversial as to which tumor markers in these neoplasms are associated with prognosis (3, 7–18, 31). However, it is possible that these markers are important in defining genetic subgroups with regard to tumor biology and possibly treatment selection. We began with a sample of 110 cases of newly diagnosed GBM from patients on two clinical protocols and, similar to previous studies, we found limited prognostic information in the sample as a whole. We, therefore, attempted to identify a subset of cases in which the specific markers were associated with survival. EGFR was found to be differentially prognostic in the older versus younger patients. We focused on the younger patients

and found that the negative association of EGFR overexpression with survival existed only in cases that did not show aberrant p53 expression. To confirm this unexpected result, we validated it by: (a) using p53 mutational status as the p53 measure in these cases; and (b) confirming the result in an independent sample of younger patients.

Age is one of the most consistent variables associated with survival in malignant gliomas. Older patients fare much worse than younger patients among tumors of similar grade. Although the molecular basis of this relationship, if any, is not understood, we found that the association of EGFR overexpression with survival may differ depending on the age group. EGFR overexpression showed trends toward worse survival in the younger patients, but a better survival in the older patients (Fig. 1). These trends had the effect of canceling each other out when the sample is viewed as a whole and may explain why the literature has not shown a consistent association of EGFR with prognosis in astrocytic neoplasms. In several studies of astrocytic neoplasms of multiple grades, EGFR overexpression or amplification has been shown to be associated with a worse prognosis (7, 8, 17, 18). Other studies have found no such prognostic relationship (10, 11, 16). It is possible that differences in distributions of age may, in part, explain the lack of consistent findings in these studies. With respect to additional clinical-prognostic variables, we found that initial KPS was associated with survival, but extent of resection was not (Table 1). Multiple factors, including reliability of reporting and location of the lesion complicate the analysis of extent of resection as a prognostic variable (32).

Our initial results suggested that our GBM cases might best be studied as two separate groups based on age. Because the patients younger than the median age showed a greater range of survival times, we focused our subsequent analyses on this group. Because aberrations of p53 are common in this group of patients (25), we asked whether any relationship existed between EGFR, p53, and survival in these patients. Unexpectedly, EGFR overexpression was strongly associated with worse survival only in those cases that were immunohistochemically negative for p53, and not in p53-positive cases. We validated this result in two ways. First, we screened these tumors for p53 mutations. We found a mutation rate of 19%, comparable with that found in other studies (25). In addition, the majority of the tumors in which mutations were identified (14 of 17) occurred in patients younger than the median age, also consistent with the previous literature (25). Mutations in p53 are usually missense, resulting in prolongation of the half-life of the protein, rendering it immunodetectable. We found that all but one of the cases in which a mutation was identified showed p53 immunopositivity, indicating that lack of abundant protein was a fairly reliable indicator of a wild-type gene. The converse was not true, as many cases showing p53 immunopositivity in the absence of a detectable mutation were observed. Immunopositivity of p53 in the absence of a detectable mutation in exons 5–8 may represent a marker of abnormality in the p53 pathway, either a mutation outside the “hotspots” in p53 itself or a downstream lesion, resulting in abrogation of the growth-suppressive effects of wild-type p53. This suggests that p53 expression may be a more inclusive marker of p53 pathway aberrations than mutational analysis of exons 5–8. The fact that EGFR was associated with survival only in the p53 wild-type cases corroborated the results from the IHC data.

As a second method of validation, we reproduced this result in an independent sample, using 42 additional cases of younger patients from the same two clinical protocols as in the original study. In this case, we had a specific prior hypothesis based on the findings of the analysis of the first patient group, and we were able to confirm this hypothesis: EGFR positivity is a negative prognostic factor for survival in younger patients with tumors that are p53 negative, and the same is not true when tumors are p53 positive. The EGFR HRs in the

initial group of young p53-negative patients and the comparable patients in the validation set were very consistent (4.3. *versus* 4.4, respectively). This suggests that EGFR may be a more important prognostic marker in a specific subset of cases and that efforts to identify the role of EGFR in a whole sample of cases may mask the ability to identify a prognostic association.

We next explored the proliferation index as a prognostic marker in the younger patients. High MIB-1 was associated with worse survival in the younger patients and showed a trend toward worse survival in the older patients in univariate analyses. Whereas a significant interaction was identified between MIB-1 and p53 expression in the original group of younger patients, this was not confirmed when p53 genetic analysis was used as the p53 measure, nor was a MIB-1-p53 interaction observed in the validation group.

Previous studies have suggested that GBM can be defined as primary *versus* secondary based on clinical characteristics (initial histological diagnosis and length of symptoms before initial surgery). This distinction is supported by mutually exclusive EGFR amplification and p53 mutation, respectively. It should be noted that although all of the patients in our study had an initial histological diagnosis of GBM, information was not available as to length of symptoms before biopsy. Hence, we do not know if these patients meet the strict criteria for primary GBM as described previously (25).

If confirmed in subsequent studies, the results of our study may be of clinical use by identifying a subgroup of patients for whom a specific marker is related to prognosis. These findings may also be of use in the selection of patients for specific clinical trials. For example, efforts are under way to specifically target the EGFR protein in glioblastoma to neutralize its oncogenic effects (33, 34). If EGFR plays a more important role in biological aggressiveness in a particular subset of patients, this subgroup may be of interest to preferentially include in such a clinical trial. Examination of the Kaplan-Meier curves in Fig. 2 suggests that EGFR+/p53- cases had a particularly poor prognosis, suggesting that these patients may represent good candidates for anti-EGFR therapies.

In summary, we find that EGFR expression showed significant association with prognosis in a subset of patients with glioblastoma, as defined by age and p53 status. This relationship was identified through the unexpected finding that EGFR positivity: (a) was related to improved survival in older patients but worse survival in younger patients; and (b) in the younger patients, EGFR predicted a worse prognosis only in those tumors that were immunohistochemically negative for p53. This finding was validated both by an examination of p53 mutational status and by reproducing this result in an independent sample of cases from patients on the same clinical protocols as in the original sample. Glioblastoma is a morphologically defined entity that likely "lumps" together tumors of a variety of genetic profiles. The results suggest that the identification of prognostic markers in these genetically heterogeneous tumors may be complex and may require stratification by factors such as patient age and the specific genetic profile of the tumor. It is perhaps not surprising that a specific marker is variably associated with prognosis in GBM, given that genetic subsets of these tumors have been recently defined on the basis of p53 and EGFR status, and these profiles are, in turn, related to age at diagnosis. Additional studies are likely to further highlight the genetic complexity of specific prognostic markers of this morphologically defined entity.

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## Analysis of Complex Relationships between Age, p53, Epidermal Growth Factor Receptor, and Survival in Glioblastoma Patients

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