

# Aberrant p53, mdm2, and Proliferation Differ in Glioblastomas from Long-Term Compared with Typical Survivors<sup>1</sup>

Eric C. Burton,<sup>2</sup> Kathleen R. Lamborn,  
Peter Forsyth, James Scott, Jason O'Campo,  
Jane Uyehara-Lock, Michael Prados,  
Mitchel Berger, Sandra Passe, Joon Uhm,  
Brian P. O'Neill, Robert B. Jenkins, and  
Ken D. Aldape<sup>3</sup>

Departments of Pathology [J. O., J. U. L., K. D. A.] and Neurological Surgery [E. C. B., K. R. L., M. P., M. B.], University of California, San Francisco, San Francisco, California 94143; Department of Medicine, Tom Baker Cancer Center University of Calgary, Calgary, Alberta, Canada 2N4N2 [P. F., J. S.]; and Departments of Laboratory Genetics [S. P., R. J.] and Neurology [J. U., B. P. O.], Mayo Clinic and Foundation, Rochester, Minnesota 55905

## ABSTRACT

**Purpose:** Glioblastoma multiforme (GBM) is a highly lethal neoplasm with a median survival of ~1 year. Only 2–5% of patients originally diagnosed with GBM will survive  $\geq 3$  years. Whether tumors from these long-term survivors (LTSs) exhibit molecular genetic differences compared with typical GBM survivors is not known.

**Experimental design:** Tumors from 41 patients initially diagnosed with GBM and having survival  $\geq 3$  years (LTS) was compared with 48 GBMs from short-term survivors (STSs, survival  $\leq 1.5$  years) for p53 aberrations (expression/mutation), epidermal growth factor receptor overexpression, mdm2 overexpression, and proliferation index.

**Results:** Nuclear p53 expression was significantly more frequent in the LTS group. However, no difference in the rate of p53 mutation was evident. Overexpression of epidermal growth factor receptor was slightly more frequent in the STS patients, but this is not statistically different. mdm2 overexpression was significantly more frequent in the STSs, and this group had a significantly higher median proliferation index.

**Conclusion:** Long-term GBM survivors were more likely to have p53-overexpressing tumors, although a differ-

ence in p53 mutation rate could not be detected. They were less likely to exhibit mdm2 overexpression and had a lower proliferation rate compared with typical GBM survivors.

## INTRODUCTION

GBM<sup>4</sup> represents an important clinical problem. Despite many years of intense investigation and novel therapies, the clinical outlook for patients with this tumor has not changed in two decades, and their median survival remains at ~1 year after initial surgical diagnosis (1). A greater understanding of the molecular genetic abnormalities involved in the pathogenesis, biological behavior, and therapeutic response of these tumors may identify new targets for therapy and thereby improve our ability to treat them successfully.

Over the past decade our knowledge of the mechanisms that result in the malignant transformation of gliomas has increased significantly. Aberrations in oncogenes and tumor suppressor genes have been characterized in glial tumors, and, based on this information, genetic subtypes of individual tumors have been identified (2). Specific aberrations have been found to be associated with the biological and clinical variability observed between patients with histologically similar tumors. To illustrate, anaplastic oligodendrogliomas that show combined loss of 1p/19q have been correlated with increased chemosensitivity and prolonged survival compared with individuals with tumors that have retained these chromosomal arms (3).

In regard to the molecular pathogenesis of GBM, among the most common aberrations are mutation in p53, amplification/rearrangement of the EGFR, and amplification/overexpression of mdm2. p53 regulates genes that induce G<sub>1</sub> cell-cycle arrest or apoptosis in response to genotoxic stress. p53 mutation was one of the first genetic aberrations identified in astrocytic tumors and is thought to be an early event in the pathogenesis of astrocytic tumors (4). Amplification/overexpression of the EGFR has been identified as one of the most common aberrations in GBM. Activation of the receptor by its ligand results in the inducement of downstream signaling pathways, including the ras-mitogen-activated protein kinase signaling pathway, which confers a growth advantage in astrocytes. An internally rearranged form, designated EGFR vIII, is frequently found in GBM (5). This mutant receptor is a truncated variant that is ligand independent and constitutively activated. mdm2 is also a frequently amplified gene in GBM. This protein forms a complex with p53 inhibiting its transcriptional ability. Thus, amplification and consequent overexpression of mdm2 are understood

Received 6/14/01; revised 10/22/01; accepted 10/30/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported in part by NIH Grants CA62399, CA13525, CA82103, and CA09291, a Robert Wood Johnson Foundation Grant (to K. D. A.) and the Robert Magnin Newman Fellowship (to E. C. B.).

<sup>2</sup> Present address: University of California Los Angeles, Neuro-Oncology Program, Los Angeles, CA 90095.

<sup>3</sup> To whom requests for reprints should be addressed, at Department of Pathology (Neuropathology), University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston TX 77030. Phone: (713) 792-7935; Fax: (713) 745-1105; E-mail: kaldape@mdanderson.org.

<sup>4</sup> The abbreviations used are: GBM, glioblastoma multiforme; STS, short-term survivor; LTS, long-term survivor; EGFR, epidermal growth factor receptor; KPS, Karnofsky performance score; UCSF, University of California, San Francisco; CI, confidence interval.

to be an alternative means by which p53 function is lost, in addition to p53 mutation (6).

It has not been firmly established which, if any, of the molecular genetic aberrations important for the pathogenesis of GBM represent prognostic factors. The identification of molecular genetic markers or combinations of markers that are associated with survival in patients with GBM would be beneficial for its diagnostic and prognostic potential and in identifying genes associated with aggressive biological behavior for targeted therapy. In this regard, the prognostic significance of these aberrations and of proliferation index in GBM remains unresolved despite multiple studies, some with conflicting results (7–11). We have found that the prognostic significance of these aberrations may be complex and best observed in subsets of patients with GBM (12). Known clinical parameters associated with better prognosis in GBM include younger age at diagnosis and high KPS (13). Nevertheless, most patients with this illness, including those that are young with a high KPS, succumb to it after a year despite intensive therapy. However, there is a range of survival time, which is not accounted for by current clinical or histological parameters. A small number of patients with histologically confirmed GBM survive 3 years or longer after initial surgical diagnosis (14, 15). Although they represent a minority (2–5%) of patients, a comparison of these patients with STSs would test the hypothesis that tumors from LTSs are genetically distinct from STSs. Previous studies of long-term GBM survivors have predictably identified, based on our understanding of clinical factors associated with outcome, that as a group these patients tend to be younger, and have a higher initial KPS and lower proliferation indices when compared with typical GBM survivors (14–18). But to date, specific molecular genetic differences have not been identified between long-term and short-term GBM survivors.

In this report, we compare two cohorts of GBM patients, one of which survived < 1.5 years after initial surgical diagnosis (STSs) with a group which survived  $\geq$  3 years (LTSs). The two groups were of similar median age to control for this important prognostic variable. Tumor samples from each group were tested for mdm2 and EGFR overexpression, proliferation index, and abnormalities in p53, including aberrant expression and mutation. The results of this comparison identify specific molecular differences between these two groups.

## MATERIALS AND METHODS

**Patient Population.** LTSs were obtained from three sources: the UCSF neuro-oncology service database (26 cases), the Tom Baker Cancer Center, University of Calgary (10 cases), and the Mayo clinic (5 cases). All of the LTSs were selected for the study on the basis of survival ( $\geq$ 36 months), an original histopathological diagnosis of glioblastoma, and the ability of the investigators to obtain paraffin-embedded tumor tissue or unstained slides. Cases with biopsy proven low-grade astrocytic precursors or radiographic evidence of a possible low-grade precursor were excluded. Slides from each case were reviewed, and a diagnosis of glioblastoma was confirmed using the WHO criteria for GBM (19).

The 48 STSs were all selected from the UCSF database. These were patients originally diagnosed with GBM who had a

survival of  $\leq$  18 months. Because most of the LTSs were in the younger age groups, STSs had an age constraint of  $\leq$  50 years to give the two groups similar median ages. Analysis on both patient groups was done only on pretreatment diagnostic tissue.

Although possible treatment differences between the two groups was not specifically considered in the comparison, standard treatment for GBM patients with these clinical characteristics was maximal resection followed by 60 Gy of external beam radiation. Many patients would also receive chemotherapy, either as an adjuvant to radiation or at recurrence. The UCSF patients, which comprise the majority of the LTSs and all of the STSs, were each registered in clinical trials at some point during the course of their illness. Therapy for the LTSs from the Calgary group has been discussed previously (14).

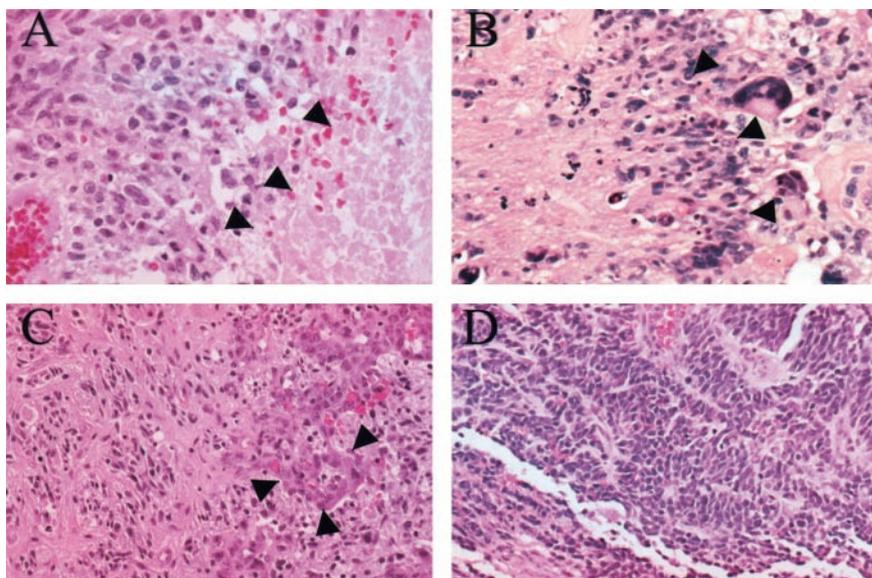
**Immunohistochemistry.** Once paraffin blocks were obtained, a block with representative tumor tissue was chosen for study and 5- $\mu$ m sections placed on positively charged slides. Multiple serial sections from each case were stored for later staining. The immunohistochemical procedures were routine and were performed as described previously (12). Primary antibodies used were all mouse monoclonals and were obtained and used as follows: anti-p53 (DO-7; Dako; 1:150 dilution), anti-EGFR (Clone 528; Calbiochem Oncogene Research; 1:50 dilution, which recognizes wild-type and the most common rearranged form), anti-mdm2 (Ab-1; Calbiochem; 1:150 dilution), and anti-Ki-67 (MIB-1; Dako; 1:500 dilution).

Each slide stained for p53, EGFR, mdm2, or MIB-1 was individually reviewed and scored by one neuropathologist (K. A.). Scoring for p53 and mdm2 was based on 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 positive. For purposes of statistical analysis, all of the 0 and 1 p53 and mdm2 scores 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.” Alternative, more stringent cutoff points for p53, mdm2, and EGFR were tested but did not significantly alter the results (not shown). 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.

**p53 Genetic Analysis.** An effort was made to obtain p53 sequence on all of the cases. LTS and STS tumors for whom adequate DNA was available were analyzed for p53 mutations in exons 5–8. Established procedures were used to extract DNA from paraffin tissues. Sequencing on exons 5–8 was done after PCR amplification using established primers on an ABI sequencer as described previously (12).

**Statistical Analyses.** Survival time was established as the interval from initial surgery to patient death or last official contact. Fisher’s exact test was used to assess the significance of p53, EGFR, and mdm2 scores between the two groups. This method was also used to determine the significance of the p53



**Fig. 1** Histopathology of glioblastoma from LTSs. Representative photomicrographs of tumor specimens showing features of GBM. *A*, case 20 shows tumor necrosis (arrowheads). *B*, case 16 demonstrates marked nuclear atypia and pseudopallisading necrosis (arrows). *C*, case 14 shows microvascular proliferation characteristic of GBM (arrowheads). *D*, case 25 displays high cellularity and features of small cell GBM.

mutation rate, in addition to the presence of necrosis and microvascular proliferation between the two groups. Multivariate analysis (logistic regression) was done to identify associations of marker status with prognosis. A Mann-Whitney test was used to compare the values of Ki-67 index from each patient cohort. Cox proportional hazards regression was used to identify prognostic factors within the LTS group. Patients who were alive at last known contact were considered censored for the Cox analysis. Given the exploratory nature of this study, we report nominal *P*s without adjustment for multiple comparisons. This is done to reduce the chance of not identifying potentially important associations because of the reduction of power that accompanies standard methods of adjustment.

## RESULTS

**Patient Characteristics and Histological Features.** The LTS population consisted of 16 males and 25 females with a median age at diagnosis of 39 years (range = 21–75 years). Median survival for the group was 59 months (Kaplan-Meier). Fourteen patients were alive at last contact with median follow-up of 66.5 months (range = 38–179). Median KPS at initial diagnosis was 90 (range = 60–100).

For inclusion into this study, histopathology from all of the cases was reviewed to confirm that features of glioblastoma (WHO) were present. After this review, several cases initially diagnosed as GBM were deemed to have a significant oligodendroglial component and were excluded from this study. Prominent microvascular proliferation and necrosis are essential diagnostic features of GBM, and the WHO requires the presence of either for the diagnosis of GBM. As such, a comparison of these features was made between the two groups. For the LTSs, necrosis was present in 31 of 41 (76%) cases *versus* 40 of 48 (83%) STSs ( $P = 0.43$ ; Fisher's exact test). The frequency of microvascular proliferation also did not significantly differ between the two groups and was present in 36 of 41 (88%) LTSs *versus* 46 of 48 (96%) STSs ( $P = 0.24$ ; Fisher's exact test). Four

**Table 1** Clinical comparison of LTSs and STSs

A comparison of the median age (years), KPS, gender (% of cohort that is female), survival (months), and the *P*s of both patient groups. Statistical tests performed were Mann-Whitney (for age and KPS) and Fisher's exact test (for gender).

	LTS	STS	<i>P</i>
Age (yrs)	39	42	0.97
KPS	90	90	0.83
Gender (% F)	60%	38%	0.03
Survival (mo.)	59.0	11.5	

representative LTS cases demonstrating histological features diagnostic of GBM are shown in Fig. 1.

The STS cohort consisted of 48 patients (30 males and 18 females). The median age at diagnosis was 42 years (range = 22–50). Median survival for the STSs was 11.5 months (range = 3–18 months), and median KPS was 90 (range = 50–100). A comparison of the clinical features for the two patient groups is summarized in Table 1. There was no significant difference in median age (LTS 39 *versus* STS 42 years;  $P = 0.97$ ; Mann-Whitney) or median KPS (90 *versus* 90;  $P = 0.83$ ; Mann-Whitney) between the two groups. However, there were more women present in the LTS cohort than in the STS cohort (60% *versus* 38%;  $P = 0.03$ ; Fisher's exact test).

**Immunohistochemistry and p53 Mutations.** Table 2 shows the results of p53, EGFR, and mdm2 expression for LTSs along with the p53 mutation status for each case. An effort was made to obtain p53 sequence on all of the cases. Twenty of 41 LTSs had DNA sufficient for amplification and subsequent p53 gene sequencing for exons 5–8. Results for the 48 individual STSs are described in Table 3. Twenty-six of 48 STSs had p53 sequencing data.

A comparison of these results for the LTS and STS cohorts is summarized in Table 4. Significantly higher nuclear p53

Table 2 p53, EGFR, mdm2, and Ki-67, and status for LTS GBMs

Individual results are given by case number. Results based on immunohistochemistry are scored as “+” or “-”, except for Ki-67, which is expressed as percentage of positive cells. For the *p53* gene, the mutation is represented by showing the mutated codon and amino acid change.

Case	p53 protein	<i>p53</i> gene	EGFR	mdm2	Ki-67
1	+	codon 266 gly→arg	+	+	50.1
2	+	WT <sup>a</sup>	-	+	22.9
3	+	WT	+	+	31.3
4	+	codon 152 pro→leu	-	+	35.5
5	+	WT	+	+	16.4
6	-	ND	-	-	5.2
7	-	ND	-	-	24.0
8	+	ND	-	-	3.4
9	+	ND	-	-	11.4
10	+	ND	-	-	3.8
11	+	ND	-	-	16.8
12	+	ND	-	-	16.2
13	+	ND	-	+	12.5
14	-	ND	-	-	15.0
15	+	ND	-	-	6.6
16	-	WT	-	+	68.1
17	+	codon 273 arg→cys	-	-	6.9
18	+	ND	-	+	21.5
19	+	WT	-	-	9.5
20	+	ND	-	-	12.6
21	+	ND	-	-	50.1
22	+	WT	-	-	7.8
23	+	WT	-	+	85.2
24	+	ND	-	-	4.4
25	+	WT	-	+	56.0
26	+	ND	-	+	4.0
27	+	WT	-	-	20.3
28	+	WT	-	+	41.4
29	+	ND	+	+	38.4
30	+	codon 280 arg→gly	-	ND	5.9
31	-	ND	-	+	12.0
32	+	WT	-	-	34.1
33	+	WT	+	-	12.5
34	+	ND	-	+	10.8
35	+	ND	-	-	13.5
36	+	WT	-	+	23.0
37	+	codon 250 arg→cys	-	-	43.4
38	+	ND	-	-	19.0
39	+	WT	-	-	ND
40	+	ND	-	+	7.9
41	-	WT	-	+	15.2

<sup>a</sup> WT, wild type; ND, not determined because of unamplifiable DNA or unavailable slides.

protein accumulation was present in the LTS (85%) versus the STS group (56%;  $P < 0.01$ ). However, there was no difference in the mutation rate between the two cohorts (25% versus 31%;  $P = 1.0$ ). The rate of p53 overexpression for the LTSs with p53 mutations was 100% (5 of 5), whereas the STS rate of p53 expression for mutated cases was 88% (7 of 8). LTSs in which no mutation could be detected had a p53 positivity rate of 87% (13 of 15) versus 61% (11 of 18) for STSs. Whereas EGFR overexpression was more common in the STSs (25%) than the LTSs (12%), this result was not statistically significant. mdm2 overexpression was more frequent in the STS tumors (75%) as compared with the LTSs (45%;  $P < 0.01$ ). The median MIB-1 labeling index was higher (28.1%) for typical survivors compared with 15.7% for the LTS group ( $P < 0.01$ ).

Table 3 p53, EGFR, mdm2, and Ki-67, and status for STS GBMs

Individual results are given by case number. Results based on immunohistochemistry are scored as “+” or “-”, except for Ki-67, which is expressed as percentage of positive cells. For the *p53* gene, the mutation is represented by showing the mutated codon and amino acid change.

Case	p53 protein	<i>p53</i> gene	EGFR	mdm2	Ki-67
1	-	WT <sup>c</sup>	+	+	40.0
2	+	ND	-	-	19.6
3	+	WT	-	+	7.9
4	+	WT	-	+	19.0
5	+	ND	-	-	18.6
6	+	WT	-	+	62.1
7	-	ND	-	+	53.7
8	-	WT	+	+	15.4
9	-	WT	-	+	69.9
10	-	WT	-	+	28.3
11	-	ND	-	-	16.1
12	+	WT	-	+	35.5
13	+	WT	-	+	38.2
14	-	ND	-	+	23.1
15	-	ND	-	-	35.8
16	-	ND	-	+	26.6
17	+	ND	-	+	50.1
18	-	ND	+	-	27.8
19	+	codon 273 arg→his	-	+	12.7
20	+	codon 132 lys→thr	+	+	17.8
21	+	ND	-	-	16.1
22	-	WT	+	+	39.1
23	+	ND	-	+	59.4
24	+	ND	-	-	29.1
25	-	ND	-	+	39.0
26	+	ND	-	+	14.7
27	-	WT	-	+	33.6
28	+	WT	+	+	21.4
29	+	WT	-	+	20.6
30	+	WT	+	+	58.0
31	-	ND	+	+	41.0
32	+	WT	-	+	15.4
33	+	codon 273 arg→his	-	-	26.6
34	-	ND	-	+	55.3
35	+	ND	-	+	23.9
36	+	ND	-	+	50.2
37	+	codon 152 pro→leu	-	+	46.2
38	+	codon 175 arg→his	-	-	30.2
39	-	codon 237 met→ile	-	-	37.9
40	+	WT	-	+	58.1
41	+	WT	+	+	26.9
42	-	ND	-	+	22.5
43	-	ND	-	+	6.2
44	+	codon 273 arg→cys	+	-	7.3
45	-	WT	+	+	17.9
46	-	ND	+	+	51.6
47	-	ND	-	-	10.8
48	+	G-C splice site intron 4	-	+	44.1

<sup>a</sup> WT, wild type; ND, not determined because of unamplifiable DNA and/or unavailable slides.

Because STSs were more likely to be immunonegative for p53, immunopositive for mdm2, and have a high proliferation index, we asked whether combinations of these variables could additionally separate the two groups. To facilitate this, we used a cutoff of 20% to dichotomize the Ki-67 index. Table 5 shows that 13 of 48 (27%) of the STS patients showed this molecular profile, whereas only 1 of 41 (2%) of the LTS group showed this. p53/Ki-67 and p53/mdm2 profiles also appeared to distin-

**Table 4** Comparison of marker status of LTS to STS GBMs

Results for p53 protein, EGFR, and mdm2 are expressed as the percentage of positive cases within each group. p53 gene is expressed as percentage of mutations in exons 5–8. Except for Ki-67, *P*s are from Fisher's exact test. Ki-67 indices are expressed as medians and are compared using a Mann-Whitney test. The SDs/SEs for the Ki-67 are LTS: 19.0/3.0 and STS 16.5/2.4.

Marker	LTS	STS	<i>P</i>
p53 protein	85% (35/41)	56% (27/48)	<0.01
p53 gene	25% (5/20)	31% (8/26)	1.0
EGFR	12% (5/41)	25% (12/48)	0.12
mdm2	45% (18/40)	75% (36/48)	<0.01
Ki-67	15.7 (range 3.4–85.2)	28.1 (range 6.2–69.9)	<0.01

guish these two groups of patients, although these profiles were not as sensitive (Table 5).

Multivariate analysis, using logistic regression with stepwise backward elimination, was performed. The inclusion of p53, EGFR, mdm2, MIB-1, KPS, age, necrosis, and microvascular proliferation as covariates identified p53 expression and mdm2 as independently associated interactions with survival. When only these two variables were included, p53 positivity was a favorable prognostic factor (odds ratio = 0.24; 95% CI 0.08–0.71; *P* < 0.01), whereas mdm2 positivity was associated with poor survival (odds ratio = 3.26; 95% CI 1.31–8.15; *P* = 0.01).

Because the LTS cohort showed a wide range of survival time, we tested whether any of the clinical characteristics or individual marker analyses were associated with survival time within this group. On univariate analyses using Cox regression, only EGFR overexpression was significantly associated with survival. The association of positive EGFR expression with worse survival remained after adjustment for relevant clinical variables (age and KPS) showing a hazard ratio of 7.1 (*P* = 0.03).

## DISCUSSION

Relevant clinical and biological markers can be found by comparing molecular genetic aberrations in tumors from patients that comprise opposite ends of a survival spectrum (20–22). To date, little data exist comparing GBM patients with LTS with typical GBM survivors. If it can be determined that genetic differences exist between these two groups, additional investigation could lead to molecular markers that better predict patient survival or more importantly lead to therapies targeted at the most critical genetic aberrations that prescribe biological aggressiveness in a tumor. By using immunohistochemical analysis for p53, mdm2, EGFR, and proliferation index, and additionally determining p53 mutation status in a subset of these tumors, this study suggests the molecular patterns from the glioblastomas in patients that survive long-term have molecular aberrations distinct from those of typical survivors.

Previous studies of long-term GBM survivors have found that this group of patients tend to be younger than the median age of all GBM patients (55–60 years; Refs. 14, 15, 17, 23), a finding reproduced in this study. Given the importance of age and prognosis, we selected a group of typical survivors of similar median age. Histological review of the LTSs revealed

**Table 5** Multimarker profile comparison of LTS and STS patients

Since STS patients were more likely to be p53-negative, mdm2-positive, and have a higher Ki-67 labeling index, the number of cases from each group showing each pairwise profile and the combination of all three. Ki-67 scores were dichotomized, where “Ki-67+” indicates a labeling index of >20%.

Profile	LTS	STS	<i>P</i>
mdm2+/Ki-67+	11/41 (27%)	27/48 (56%)	<0.01
p53–/Ki-67+	2/41 (5%)	16/48 (33%)	<0.01
p53–/mdm2+	3/41 (7%)	16/48 (33%)	<0.01
p53–/mdm2+/Ki-67+	1/41 (2%)	13/48 (27%)	<0.01

that no significant differences in microvascular proliferation and necrosis, hallmarks of GBM, could be identified. As part of that review, several cases were noted to show features of oligodendroglioma, a glioma with an improved prognosis. This finding has been reported previously (24); therefore, these cases were excluded from this analysis to include only those cases with diagnostic features of GBM.

We found that LTSs with GBM had a higher rate of abnormal p53 protein expression, as 85% of these cases were scored positive, compared with 56% of typical survivors. The fact that previous studies in unselected populations of GBMs show a p53-immunopositive rate of ~60% suggests that the tumors from the LTS group are different from most GBMs in regard to the rate of p53 expression (25, 26). The high rate of p53 immunopositivity led us to hypothesize that the LTS tumors would exhibit a correspondingly higher rate of p53 mutations. However, an unanticipated finding was the similarity in p53 mutation rates between the two cohorts (LTS 25%, STS 31%). The underlying reason for the high rate of p53 protein accumulation in the absence of detectable mutations in the LTS is not clear. Because our sequencing was limited to exons 5–8, it remains formally possible that the LTS may exhibit mutations outside this highly conserved region, although such mutations are rare (27). Because numerous LTS cases were obtained from procedures performed many years ago, in most cases we were unable to obtain DNA of sufficient amount and quality to test whether mutations were present in additional exons. Whereas cytoplasmic localization of wild-type p53 has been proposed as a mechanism by which p53 function is abrogated, we found no cytoplasmic p53 staining in our cohort (28). The p53 network is complex, and it is possible that an increased frequency of nuclear wild-type p53 overexpression is a sign of abnormalities in other components of the p53 pathway (29). Our data suggest that there are mechanisms operating in LTS GBMs that result in the stable expression of wild-type p53. Supporting this hypothesis is a report published in 1996 by Morita *et al.* (23). They found p53 immunopositivity in the absence of any detectable p53 gene mutations in a study of LTSs. Discordant p53 expression has also been described as operative in various studies of astrocytic tumors along with other tumor types (25, 30, 31). One consideration from these results is that the molecular aberration(s) that account(s) for stable expression of wild-type p53 in GBM may be used in part to distinguish, either quantitatively or qualitatively, LTS GBM from STS GBM.

p53 expression was identified as a positive prognostic factor by logistic regression analysis including both groups, and

LTSs in this study were predominantly p53 positive/EGFR negative. This finding is in contrast with a previous study that found p53 positivity/EGFR negativity to be a poor outcome predictor in young patients (12). Moreover, that study showed the prognostic significance of EGFR varied with the p53 status of the tumor. This interaction between p53 and EGFR was not found in the LTS cohort. One possible explanation for these discrepancies is, in choosing the LTS GBMs, we have selected tumors that are truly biologically different from the majority of GBMs. As such, the relevance of molecular markers and patient outcome can only be assessed in the context of similarly identified clinical cohorts, which is not the case between these two studies.

EGFR has been studied as a prognostic factor in gliomas singly and in combination with other molecular markers (9, 32, 33). However, a consensus has not been reached in regard to the significance of this marker as a prognostic factor. In this study, overexpression of EGFR was more common in the STS group, but this difference was not statistically significant ( $P = 0.18$ ). However, on Cox analysis of the LTS group in isolation, EGFR expression was a negative prognostic factor even after adjustment for age and KPS. Yet, the significance of this finding is tempered somewhat because of the small number of EGFR-positive cases in this cohort. Chromosome 7p amplification (where EGFR resides) is common in glioblastomas, and it is possible that this may represent a better marker than EGFR expression to differentiate the two groups. However, in a study comparing genetic aberrations identified by comparative genomic hybridization, we have found that 7p amplification rates were slightly increased but not significantly different in short-term (6/24, 25%) versus long-term GBM survivors (6 of 39; 15%),<sup>5</sup> a finding which corroborates our EGFR expression data.

Several studies have looked at clinical outcome and mdm2 expression with mixed results. One report found conflicting results on the prognostic significance of mdm2 depending on clinical end point being measured (overall survival versus disease-free survival at recurrence; Ref. 34). Another study found mdm2 positivity to be a negative predictor of survival (35). Consistent with this, the proportion of mdm2-positive cases was significantly higher in our STS group. Similarly, proliferation rate has not been a consistent predictor of outcome in GBM (36–38). The median proliferative index in our LTS cohort was lower than that in the typical survivors. This finding is consistent with other studies that have looked at this question in LTSs and found the longer survivors to have lower proliferative indices than the short-term group (14). It is possible that the use of patients at the extreme ends of survival allows one to identify differences not present when cases with a narrow range of survival time are examined.

No single marker predicted long-term survival in this study, and we asked whether multimarker profiles might improve the molecular distinction between the two groups. Because STS cases were more likely to be p53-negative, mdm2 positive, and have a high Ki-67 labeling index, cases fitting this

profile were compared between the two groups. Table 5 shows that only 1 LTS case showed this profile, whereas 13 of 48 STS cases showed this. Differences were more modest when only two markers were used. While clearly not specific, this raises the possibility that multimarker profiles may improve our ability to predict or rule out long-term survival in GBMs. We have found by comparative genomic hybridization that some of the LTS cases show combined loss of 1p and 19q,<sup>5</sup> a marker of improved outcome in oligodendroglioma (3, 39). Whereas only 6 of 39 LTS cases showed 1p/19q loss, 0 of 24 STSs has these combined losses, suggesting that this may be a relatively specific but by no means universal marker profile in long-term GBM survivors.

These findings support the hypothesis that disparate survival seen in patients with GBM may be rooted in genetic differences between the tumors. In contrast, a similar study to this done by Kraus *et al.* (40) compared two groups of patients (42 patients total) with GBM, segregated by time to tumor progression ( $\leq 6$  versus  $\geq 24$  months) after surgery, as a surrogate for overall survival. No association of p53, EGFR, and mdm2 with time to tumor progression was identified. Several relevant differences between this and the current study can be noted. The sample size in our study was larger, increasing the statistical power to detect differences. In addition, they found only 1 of 40 cases to be mdm2-positive, whereas more than half of our cases were mdm2-positive, perhaps a reflection of the different antibodies used. The average patient age in their study (50 years of age) was older than in our cohort (42 years of age). Finally, it is possible that the use of a surrogate marker for survival in their study may explain in part the differences between this and the current study.

Glioblastomas have been stratified into subgroups based on p53 mutation and EGFR expression being mutually exclusive events (41). EGFR-amplified glioblastomas are more commonly seen in the group of patients with tumors that develop *de novo* (primary GBM). p53-mutated glioblastomas are more commonly seen in patients with GBMs that have developed from less malignant precursors (secondary GBM). Clinically, patients with secondary GBMs must have previous biopsy evidence of a lower grade tumor or perhaps radiographic evidence of the same. Lengthy symptomatology has also been used as a criterion. Whereas a detailed history of previous radiology and clinical symptoms is not known for our cohorts, a minimal requirement for inclusion was that the initial histology was diagnostic of GBM. One consideration that must be addressed is that the molecular differences between our patient groups may in part be attributable to differing ratios of primary or secondary GBMs included in each group. However, we see in this study that the rate of p53 mutation and EGFR overexpression did not differ between the STS and LTS groups. Therefore, it is likely that the two cohorts did not differ in their proportions of these GBM subtypes.

An unexpected finding was a relatively high proportion of women among the LTS group (60%). Gliomas are more common in men, as evidenced by the gender proportion in the STS group (30 men and 18 women). Analysis of data from our previous study (12) revealed no association of gender with marker status (not shown). Whereas one large study has reported that women with malignant glioma may have improved survival

<sup>5</sup> E. Burton and K. Aldape, unpublished observations.

compared with men (42), gender is not a well-established prognostic factor in GBM, and the reproducibility of this gender difference awaits confirmation from additional studies.

To our knowledge, this is the largest sample size of long-term GBM survivors that has been compiled and examined for molecular markers. We have found that there are differences in the tumors of GBM patients that survive longer than 3 years when compared with tumors from a cohort of patients with similar age and KPS but with poor survival. We noted differences in the rate of abnormal p53 expression (without differences in the mutation rate), mdm2 overexpression, and proliferation rate. Studies examining additional genetic differences in tumors from patients exhibiting disparate survival may shed more light on the biologically important aberrations in these aggressive tumors.

## ACKNOWLEDGMENTS

We thank the University of California, San Francisco Brain Tumor Research Center tissue bank for providing tissue samples.

## REFERENCES

1. Fine, H. A. The basis for current treatment recommendations for malignant gliomas. *J. Neuro-Oncol.*, *20*: 111–120, 1994.
2. Louis, D. N., and Gusella, J. F. A tiger behind many doors: multiple genetic pathways to malignant glioma. *Trends Genet.*, *11*: 412–415, 1995.
3. Cairncross, J. G., Ueki, K., Zlatescu, M. C., Lisle, D. K., Finkelstein, D. M., Hammond, R. R., Silver, J. S., Stark, P. C., Macdonald, D. R., Ino, Y., Ramsay, D. A., and Louis, D. N. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J. Natl. Cancer Inst.*, *90*: 1473–1479, 1998.
4. Sidransky, D., Mikkelsen, T., Schwchheimer, K., Rosenblum, M. L., Cavane, W., and Vogelstein, B. Clonal expansion of p53 mutant cells is associated with brain tumour progression. *Nature (Lond.)*, *355*: 846–847, 1992.
5. Feldkamp, M. M., Lala, P., Lau, N., Roncari, L., and Guha, A. Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens. *Neurosurgery*, *45*: 1442–1453, 1999.
6. Biernat, W., Kleihues, P., Yonekawa, Y., and Ohgaki, H. Amplification and overexpression of MDM2 in primary (*de novo*) glioblastomas. *J. Neuropathol. Exp. Neurol.*, *56*: 180–185, 1997.
7. Baxendine-Jones, J., Campbell, I., and Ellison, D. p53 status has no prognostic significance in glioblastomas treated with radiotherapy. *Clin. Neuropathol.*, *16*: 332–336, 1997.
8. Chozick, B. S., Pezzullo, J. C., Epstein, M. H., and Finch, P. W. Prognostic implications of p53 overexpression in supratentorial astrocytic tumors. *Neurosurgery*, *35*: 831–838, 1994.
9. Etienne, M. C., Formento, J. L., Lebrun-Frenay, C., Gioanni, J., Chatel, M., Paquis, P., Bernard, C., Courdi, A., Bensadoun, R. J., Pignol, J. P., Francoual, M., Grellier, P., Frenay, M., and Milano, G. Epidermal growth factor receptor and labeling index are independent prognostic factors in glial tumor outcome. *Clin. Cancer Res.*, *4*: 2383–2390, 1998.
10. Jaros, E., Perry, R. H., Adam, L., Kelly, P. J., Crawford, P. J., Kalbag, R. M., Mendelow, A. D., Sengupta, R. P., and Pearson, A. D. Prognostic implications of p53 protein, epidermal growth factor receptor, and Ki-67 labelling in brain tumours. *Br. J. Cancer*, *66*: 373–385, 1992.
11. Newcomb, E. W., Cohen, H., Lee, S. R., Bhalla, S. K., Bloom, J., Hayes, R. L., and Miller, D. C. Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. *Brain Pathol.*, *8*: 655–667, 1998.
12. Simmons, M. L., Lamborn, K. R., Takahashi, M., Chen, P., Israel, M. A., Berger, M. S., Godfrey, T., Nigro, J., Prados, M., Chang, S., Barker, F. G., II, and Aldape, K. Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. *Cancer Res.*, *61*: 1122–1128, 2001.
13. Salmon, I., Dewitte, O., Pasteels, J. L., Flament-Durand, J., Brotschi, J., Vereerstraeten, P., and Kiss, R. Prognostic scoring in adult astrocytic tumors using patient age, histopathological grade, and DNA histogram type. *J. Neurosurg.*, *80*: 877–883, 1994.
14. Scott, J. N., Rewcastle, N. B., Brasher, P. M., Fulton, D., MacKinnon, J. A., Hamilton, M., Cairncross, J. G., and Forsyth, P. Which glioblastoma multiforme patient will become a long-term survivor? A population-based study. *Ann. Neurol.*, *46*: 183–188, 1999.
15. Chandler, K. L., Prados, M. D., Malec, M., and Wilson, C. B. Long-term survival in patients with glioblastoma multiforme. *Neurosurgery*, *32*: 716–720, 1993.
16. Vertosick, F. T., Jr., and Selker, R. G. Long-term survival after the diagnosis of malignant glioma: a series of 22 patients surviving more than 4 years after diagnosis. *Surg. Neurol.*, *38*: 359–363, 1992.
17. Salvati, M., Cervoni, L., Artico, M., Caruso, R., and Gagliardi, F. M. Long-term survival in patients with supratentorial glioblastoma. *J. Neuro-Oncol.*, *36*: 61–64, 1998.
18. Scott, J. N., Rewcastle, N. B., Brasher, P. M., Fulton, D., Hagen, N. A., MacKinnon, J. A., Sutherland, G., Cairncross, J. G., and Forsyth, P. Long-term glioblastoma multiforme survivors: a population-based study. *Can. J. Neurol. Sci.*, *25*: 197–201, 1998.
19. Kleihues, P. C., and Webster K. Pathology, and Genetics of Tumours of the Nervous System. IARC Scientific Publ. Lyon, France: IARC, 2000.
20. Goff, B. A., Muntz, H. G., Greer, B. E., Tamimi, H. K., and Gown, A. M. Oncogene expression: long-term compared with short-term survival in patients with advanced epithelial ovarian cancer. *Obstet. Gynecol.*, *92*: 88–93, 1998.
21. Kakeji, Y., Maehara, Y., Tomoda, M., Kabashima, A., Ohmori, M., Oda, S., Ohno, S., and Sugimachi, K. Long-term survival of patients with stage IV gastric carcinoma. *Cancer (Phila.)*, *82*: 2307–2311, 1998.
22. Shimada, Y., Imamura, M., Shibagaki, I., Tanaka, H., Miyahara, T., Kato, M., and Ishizaki, K. Genetic alterations in patients with esophageal cancer with short- and long-term survival rates after curative esophagectomy. *Ann. Surg.*, *226*: 162–168, 1997.
23. Morita, M., Rosenblum, M. K., Bilsky, M. H., Fraser, R. A., and Rosenfeld, M. R. Long-term survivors of glioblastoma multiforme: clinical and molecular characteristics. *J. Neuro-Oncol.*, *27*: 259–266, 1996.
24. Kraus, J. A., Wenghoefer, M., Schmidt, M. C., von Deimling, A., Berweiler, U., Roggendorf, W., Diets, S., Dietzmann, K., Muller, B., Heuser, K., Reifemberger, G., and Schlegel, U. Long-term survival of glioblastoma multiforme: importance of histopathological reevaluation. *J. Neurol.*, *247*: 455–460, 2000.
25. Louis, D. N., von Deimling, A., Chung, R. Y., Rubio, M. P., Whaley, J. M., Eibl, R. H., Ohgaki, H., Wiestler, O. D., Thor, A. D., and Seizinger, B. R. Comparative study of p53 gene and protein alterations in human astrocytic tumors. *J. Neuropathol. Exp. Neurol.*, *52*: 31–38, 1993.
26. Bruner, J. M., Saya, H., and Moser, R. P. Immunocytochemical detection of p53 in human gliomas. *Mod. Pathol.*, *4*: 671–674, 1991.
27. Rubio, M. P., von Deimling, A., Yandell, D. W., Wiestler, O. D., Gusella, J. F., and Louis, D. N. Accumulation of wild type p53 protein in human astrocytomas. *Cancer Res.*, *53*: 3465–3467, 1993.
28. Gu, J., Kawai, H., Wiederschain, D., and Yuan, Z. M. Mechanism of functional inactivation of a Li-Fraumeni syndrome p53 that has a mutation outside of the DNA-binding domain. *Cancer Res.*, *61*: 1741–1746, 2001.
29. Vogelstein, B., Lane, D., and Levine, A. J. Surfing the p53 network. *Nature (Lond.)*, *408*: 307–310, 2000.
30. Lang, F. F., Miller, D. C., Pisharody, S., Koslow, M., and Newcomb, E. W. High frequency of p53 protein accumulation without p53

- gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene*, 9: 949–954, 1994.
31. Koga, H., Zhang, S., Kumanishi, T., Washiyama, K., Ichikawa, T., Tanaka, R., and Mukawa, J. Analysis of p53 gene mutations in low- and high-grade astrocytomas by polymerase chain reaction-assisted single-strand conformation polymorphism and immunohistochemistry. *Acta Neuropathol.*, 87: 225–232, 1994.
  32. Waha, A., Baumann, A., Wolf, H. K., Fimmers, R., Neumann, J., Kindermann, D., Astrahantseff, K., Blumcke, I., von Deimling, A., and Schlegel, U. Lack of prognostic relevance of alterations in the epidermal growth factor receptor-transforming growth factor- $\alpha$  pathway in human astrocytic gliomas. *J. Neurosurg.*, 85: 634–641, 1996.
  33. Zhu, A., Shaeffer, J., Leslie, S., Kolm, P., and El-Mahdi, A. M. Epidermal growth factor receptor: an independent predictor of survival in astrocytic tumors given definitive irradiation. *Int. J. Radiat. Oncol. Biol. Phys.*, 34: 809–815, 1996.
  34. Korkolopoulou, P., Christodoulou, P., Kouzelis, K., Hadjiyannakis, M., Priftis, A., Stamoulis, G., Seretis, A., and Thomas-Tsagli, E. MDM2 and p53 expression in gliomas: a multivariate survival analysis including proliferation markers and epidermal growth factor receptor. *Br. J. Cancer*, 75: 1269–1278, 1997.
  35. Rainov, N. G., Dobberstein, K. U., Bahn, H., Holzhausen, H. J., Lautenschlager, C., Heidecke, V., and Burkert, W. Prognostic factors in malignant glioma: influence of the overexpression of oncogene and tumor-suppressor gene products on survival. *J. Neuro-Oncol.*, 35: 13–28, 1997.
  36. Torp, S. H., Helseth, E., Dalen, A., and Unsgaard, G. Relationships between Ki-67 labelling index, amplification of the epidermal growth factor receptor gene, and prognosis in human glioblastomas. *Acta Neurochir.*, 117: 182–186, 1992.
  37. Wakimoto, H., Aoyagi, M., Nakayama, T., Nagashima, G., Yamamoto, S., Tamaki, M., and Hirakawa, K. Prognostic significance of Ki-67 labeling indices obtained using MIB-1 monoclonal antibody in patients with supratentorial astrocytomas. *Cancer (Phila.)*, 77: 373–380, 1996.
  38. Pigott, T. J., Lowe, J. S., and Palmer, J. Statistical modelling in analysis of prognosis in glioblastoma multiforme: a study of clinical variables and Ki-67 index. *Br J. Neurosurg.*, 5: 61–66, 1991.
  39. Smith, J. S., Perry, A., Borell, T. J., Lee, H. K., O'Fallon, J., Hosek, S. M., Kimmel, D., Yates, A., Burger, P. C., Scheithauer, B. W., and Jenkins, R. B. Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. *J. Clin. Oncol.*, 18: 636–645, 2000.
  40. Kraus, J. A., Glesmann, N., Beck, M., Krex, D., Klockgether, T., Schackert, G., and Schlegel, U. Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. *J. Neuro-Oncol.*, 48: 89–94, 2000.
  41. Kleihues, P. O. Hiroko Primary, and Secondary glioblastomas. From concept to clinical diagnosis. *Neuro-oncology*, 1: 44–49, 1999.
  42. Sant, M., van der Sanden, G., and Capocaccia, R. Survival rates for primary malignant brain tumours in Europe. EURO CARE Working Group. *Eur. J. Cancer*, 34: 2241–2247, 1998.

# Clinical Cancer Research

## Aberrant p53, mdm2, and Proliferation Differ in Glioblastomas from Long-Term Compared with Typical Survivors<sup>1</sup>

Eric C. Burton, Kathleen R. Lamborn, Peter Forsyth, et al.

*Clin Cancer Res* 2002;8:180-187.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/8/1/180>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link <http://clincancerres.aacrjournals.org/content/8/1/180>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.