Brain Pathology 8: 655-667(1998)

RESEARCH ARTICLE

Survival of Patients with Glioblastoma Multiforme is not Influenced by Altered Expression of p16, p53, EGFR, MDM2 or Bcl-2 Genes

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Deregulated expression of one or more growth control genes including p16, p53, EGF receptor (EGFR), MDM2 or Bcl-2 may contribute to the treatment resistance phenotype of GBM and generally poor patient survival. Clinically, GBM have been divided into two major groups defined by (1) histologic progression from a low grade tumor ("progressive" or "secondary" GBM) contrasted with (2) those which show initial clinical presentation without a prior history ("*de novo*" or "primary" GBM). Using molecular genetic analysis for p53 gene mutations together with immunophenotyping for overexpression of EGFR, up to four GBM variants can be distinguished, including the $p53^{+}/EGFR^{-}$ progressive or the $p53^{-}/EGFR^{+}$ *de novo* variant. We examined the survival of 80 adult patients diagnosed with astrocytic GBM stratified by age category (>40, 41-60 or 61-80) to determine whether alterations in any one given growth control gene or whether different genetic variants of GBM (progressive *versus de novo*) were associated with different survival outcomes. Survival testing using Kaplan-Meier plots for GBM patients with or without altered expression of p16, p53, EGFR, MDM2 or Bcl-2 showed no significant differences by age group or by gene expression indicating a lack of prognostic value for GBM. Also the clinical outcome among patients with GBM showed no significant differences within each age category for any GBM variant including the progressive and *de novo* GBM variants indicating similar biologic behavior despite different genotypes. Using a pairwise comparison, one-third of the GBM with normal p16 expression showed accumulation of MDM2 protein and this association approached statistical significance ($0.01 < P < 0.05$) using the Bonferroni procedure. These GBM may represent a variant in which the $p19^{ARF}$/MDM2/p53 pathway may be deregulated rather than the p16/cyclin D-CDK4/Rb pathway.

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Introduction

Glioblastomas multiforme (GBM) are the most common malignant tumor of the central nervous system. They are largely refractory to the radiation and other adjuvant therapies in standard use today, accounting for the poor survivals of less than 24 months for most patients with glioblastoma (1, 2). Resistance to treatments may be associated with deregulation of some growth control genes including loss of normal p53 function, deregulated Bcl-2, or epidermal growth factor receptor (EGFR) expression, any one of which may interfere with the induction of drug- or radiation-induced apoptosis in GBM. Mutation of p53 genes in primary cultures of GBM has been associated with increased drug resistance to a large number of chemotherapeutic agents (3) and reintroduction of wild-type p53 by adenoviral transfer to p53-deficient glioma cells is associated with restoration of the apoptotic response (4). Similarly, deregulated expression of Bcl-2 or EGFR in glioma cells also has been correlated with drug resistance and decreased apoptotic response *in vitro* (5, 6). The p53 protein plays a central role in the signaling pathways required to mediate apoptosis. It interacts with the promoters of many different genes and can upregulate EGFR and MDM2 expression or repress Bcl-2 gene expression (7). Mutations inactivate the p53

Parameter	No. of patients (percent)	Median age (range in yrs)	Median survival (range in mos)	P value
Age group				
1) 22-40	12 (15%)	29.0 (22-39)	25.7 (4.3-119.8)	P <.05 (1 vs 3)
2) 41-60	34 (42.5%)	54.5 (41-60)	16.1 (4.5-104.2)	P >.05 (1 vs 2)
3) 61-80	34 (42.5%)	66.0 (61-80)	12.6 (2.3-24.9)	P <.05 (2 vs 3)
Sex				
Males	49 (61%)	57.0 (23-74)	15.8 (2.3-104.3)	P = 0.95
Females	31 (39%)	57.0 (22-80)	14.8 (2.7-119.8)	

Table 1. The Tukey-Kramer test was used to test differences between age groups; the Wilcoxon test for comparisons for all pairs was used for differences between sex.

Table 1. Summary of patient characteristics with glioblastoma.

Gene	Age group	No. of IHC+ (% patients)	No. of IHC- (% patients)	P value
p16	22-40	8 (73%)	3 (27%)	0.34
	41-60	19 (61%)	12 (39%)	
	61-80	24 (77%)	7 (23%)	
p53	22-40	7 (58%)	5 (42%)	0.59
	41-60	16 (47%)	18 (53%)	
	61-80	20 (59%)	14 (41%)	
EGFR	22-40	4 (33%)	8 (67%)	0.59
	41-60	10 (29%)	24 (71%)	
	61-80	14 (42%)	19 (58%)	
MDM2	22-40	7 (64%)	4 (36%)	0.52
	41-60	16 (48%)	17 (52%)	
	61-80	14 (44%)	18 (56%)	
Bcl-2	22-40	5 (45%)	6 (55%)	0.77
	41-60	15 (47%)	17 (53%)	
	61-80	16 (55%)	13 (45%)	

Table 2. Abbreviations: IHC, immunohistochemistry positive (+) or negative (-); P value assesses the hypothesis within the gene expression and between age groups as determined by Chi-square Test.

Table 2. Gene expression by age group in glioblastoma.

gene in over 50% of GBM, amplification and overexpression of EGFR occurs in greater than one-third of GBM, and Bcl-2 is up-regulated in approximately 50% of the cases (8-13). Therefore, deregulated expression of one or more growth control genes may contribute to the treatment resistance phenotype of GBM.

GBM have been divided into two major groups defined by (1) histologic progression from a low grade tumor ("progressive" or "secondary" GBM) contrasted with (2) those which show initial clinical presentation without a prior history ("*de novo*" or "primary" GBM) (14-16). Recently, molecular genetic analysis together with immunophenotyping have shown that tumors that progress generally harbor mutations of the p53 gene ($p53^+$) but rarely show overexpression of the EGF receptor ($EGFR^-$), whereas *de novo* tumors most often contain the wildtype p53 gene ($p53^-$) and frequently show amplification and aberrant overexpression of the EGFR ($EGFR^+$) or MDM2 proteins (8, 11, 12, 17-23). In addition, loss of major portions of chromosome 10 occurs with high frequency in GBM and is virtually invariably associated with amplification of EGFR (24, 25). Putative prognostic factors such as growth fraction index, apoptotic index or inactivation of growth regulatory proteins such as p53 have been unsuccessful to date in predicting the biological behavior of GBM (26-34).

The aims of this study were (1) to determine the expression of critical growth control genes such as p16, p53, EGFR, MDM2 and Bcl-2 in a large series of GBM and (2) to relate the genetic findings stratified with respect to age with patient survival; the second aim includes a specific question as to whether genetically defined variants of GBM representing the progressive tumor variant ($p53^+/EGFR^-$) compared with the *de novo* tumor variant ($p53^-/EGFR^+$) differed in their biological behaviors within age groups using patient survival as the clinical outcome.

Materials and Methods

Patient Population. Archival paraffin blocks were obtained for 95 adult patients with the diagnosis of GBM who had surgery at New York University Medical Center (NYUMC) between 1979 and 1991. Histopathologic grading of the tumors was reevaluated by the neuropathologist (D.C.M.) at the time of the present study using the revised WHO classification scheme except that necrosis was required for the diagnosis of GBM (35). In some cases, additional stains were used to confirm classification as an astrocytic tumor. Fifteen cases were rejected because they were not GBM *i.e.* there were non-astrocytic neoplastic components, or there was insufficient tissue for the study. A total of 80 tumors were obtained from a patient population consisting of 49 males and 31 females with WHO grade IV astrocytomas. All tumors were selected to be supratentorial; 45% were located in the left hemisphere and 55% in the right. All preoperation Karnofsky performance scores were 70 or greater. The standard of care at NYUMC during the time interval 1977-1991 of this study was aggressive neurosurgical resection followed by radiation and adjuvant therapy. In our series, aggressive clinical approaches have not altered the median survival of patients with GBM from 12 months (13).

Immunohistochemistry. 6-μm sections were cut from formalin-fixed paraffin embedded tissue blocks and stained using standard streptavidin-biotin-complex immunoperoxidase methods as described (36-38). The primary antibodies used were as follows: for p53 (BP53-12, BioGenex) a mouse monoclonal antibody used at

Gene	Age group	Median survival IHC+ patients (range in mos)	Median survival IHC- patients (range in mos)	P value
p16	22-40	20.3 (4.3-119.8)	27.1 (25.8-37.7)	0.65
	41-60	15.3 (4.7-104.3)	24.6 (4.5-67.3)	0.54
	61-80	13.2 (2.3-24.9)	9.7 (3.8-15.8)	0.06
p53	22-40	27.1 (19.2-97.8)	20.1 (4.3-119.8)	0.28
	41-60	13.9 (4.5-60.9)	29.7 (6.0-104.3)	0.08
	61-80	12.2 (2.3-24.9)	13.9 (2.7-21.7)	0.77
EGFR	22-40	19.9 (14.2-37.7)	26.4 (4.3-119.8)	0.17
	41-60	12.2 (4.5-67.3)	19.5 (4.7-104.3)	0.31
	61-80	14.5 (3.8-21.7)	12.1 (2.3-24.9)	0.52
MDM2	22-40	25.6 (19.2-119.8)	20.0 (4.3-37.7)	0.36
	41-60	16.1 (4.7-104.3)	17.2 (4.5-60.9)	0.29
	61-80	13.8 (3.8-24.9)	12.3 (2.3-20.9)	0.64
Bcl-2	22-40	25.8 (19.2-85.1)	20.3 (4.3-119.8)	0.80
	41-60	21.8 (4.7-104.3)	11.7 (4.5-67.3)	0.19
	61-80	12.6 (2.3-24.9)	9.7 (2.7-18.2)	0.53

Table 3. Abbreviations: IHC, immunohistochemistry positive (+) or negative (-); P value determined by Kaplan-Meier and Log-Rank procedures.

Table 3. Patient survival by gene expression in glioblastoma stratified for age.

1:100; for EGFR (clone E30, BioGenex) a mouse monoclonal antibody used at 1:20; for p16 (clone C-20, Santa Cruz Biotechnology, Inc.) a rabbit polyclonal used at 1:100; for mdm-2 (clone IF2, Oncogene Science) a mouse monoclonal antibody used at 1:20; for Bcl-2 (clone 124, DAKO) a mouse monoclonal antibody used at 1:40. Negative controls consisted of adjacent tissue sections incubated with 1% normal rabbit serum instead of primary antibody. Sections from tonsil tissue were used as a positive control for p16 or Bcl-2 immunostaining in their respective assays to evaluate consistency of immunostaining between experiments. Brain tumor specimens previously characterized for showing accumulation for p53, or overexpression of EGFR, or MDM2 protein in >50% of the cells were used as positive controls, respectively. Samples were coded and the immunostaining assessed jointly at a multi-head microscope. For p53, p16 or MDM2 immunostaining only nuclear staining was regarded as positive; for EGFR immunostaining positive samples showed cytoplasmic and cell surface membrane staining; for Bcl-2 immunostaining positive samples showed cytoplasmic staining. Specimens with less than 5% immunopositive tumor cells were scored as negative. Positive samples were grouped according to the frequency of immunopositive tumor cells as: +, 5-25%; ++, 26-50%; and +++, >50% cells immunopositive. The p53, EGFR, p16 and Bcl-2 immunostaining has been previously reported on a cohort of 26 glioblastoma patients (8, 13, 36, 38).

SSCP Analysis for p53 Mutations. DNA was extracted from paraffin sections as described (39). Mutations in exons 4-8 of the p53 gene were screened using the PCR-SSCP assay as described previously (37). Samples showing variant SSCP bands were reamplified in two or more independent PCR-SSCP assays to confirm detection of the p53 mutation. Sequence analysis of PCR amplified products was performed on a semiautomated sequencer (Applied Biosystems model 373).

Statistical Analysis. Patient follow-up was defined as the interval from initial diagnosis through patient death or last official contact (scheduled follow-up or personal contact) as of October 1, 1997. Product-Limit survival testing was performed using Kaplan-Meier plots with hypothesis testing by Log-Rank procedures to assess the significance of an association of protein staining (positive vs negative) for each of the genetic variables with length of survival (40). Differences between groups of interval scaled variables was performed by the Wilcoxon non-parametric test (41). Testing for differences between more than two groups used Kruskal-Wallis evaluation (41) followed by the Tukey-Kramer procedure for planned all pairs (means) comparisons (42). Proportions in the pairwise comparison of gene expression were evaluated using Chi-square analysis (42). The Bonferroni procedure was used when multiple pairwise comparisons were made of proportions (43). Since four pairwise comparisons were made, this adjusted the threshold for significance from $P = 0.05$ to $P = 0.01$.

Results

Clinical data. The demographic and survival parameters of the patient population with GBM are summarized in Table 1. The study population consisted of 12 patients with a median age of 29 years (range 22-39), 34

Age group	Variant of GBM	No. of patients with variant	Median Survival (range in mos)	P value
22-40	p53-/EGFR-	4	22.9 (4.3-119.8)	ND
	p53+/EGFR-	4	56.1 (25.8-97.8)	
	p53-/EGFR+	1	14.2	
	p53+/EGFR+	3	20.5 (19.2-37.7)	
41-60	p53-/EGFR-	13	30.8 (6.0-104.3)	0.16
	p53+/EGFR-	11	15.2 (4.7-60.9)	
	p53-/EGFR+	5	11.7 (6.1-67.3)	
	p53+/EGFR+	6	13.6 (4.5-20.5)	
61-80	p53-/EGFR-	6	9.1 (2.7-17.7)	0.19
	p53+/EGFR-	14	12.7 (2.3-24.9)	
	p53-/EGFR+	8	14.7 (3.8-21.7)	
	p53+/EGFR+	6	9.4 (5.7-18.2)	

Table 4. Abbreviations: p53 status was determined by single-strand conformation polymorphism assay to detect p53 gene mutations (p53+); immunohistochemistry was used to detect EGFR expression (EGFR+); P value was determined by Kaplan-Meier and Log-Rank procedures; ND=not determined due to inadequate sample size.

Table 4. Patient survival by glioblastoma variant stratified for age.

Gene expression			P value
	p53 negative	**p53 positive**	
p16 negative	10	12	0.84
p16 positive	25	27	
EGFR negative	23	29	0.62
EGFR positive	14	14	
MDM2 negative	19	20	0.81
MDM2 positive	17	20	
Bcl-2 negative	20	16	0.10
Bcl-2 positive	13	23	
	p53 SSCP negative	**p53 SSCP positive**	
EGFR negative	26	25	0.70
EGFR positive	15	12	
	EGFR negative	**EGFR positive**	
p16 negative	11	11	0.12
p16 positive	36	16	
MDM2 negative	23	16	0.30
MDM2 positive	26	11	
Bcl-2 negative	22	14	0.46
Bcl-2 positive	25	11	
	MDM2 negative	**MDM2 positive**	
p16 negative	15	7	0.04
p16 positive	21	29	
Bcl-2 negative	17	18	0.91
Bcl-2 positive	17	17	
	p16 negative	**p16 positive**	
Bcl-2 negative	11	23	0.18
Bcl-2 positive	6	27	

Table 5. P value determined by Chi-square Test.

Table 5. Associations of altered gene expression in glioblastoma.

patients with a median age of 54.5 years (range 41-60) and 34 patients with a median age of 66.0 years (range 61-80). An association was found between younger age and survival. For patients aged 22-40, the median survival was 25.7 months which was significantly ($P < 0.05$) longer compared with patients aged 61-80 whose median survival was 12.6 months. In addition, patients aged 41-60 had a median survival of 16.1 months which was significantly ($P < 0.05$) longer compared with patients aged 61-80 whose median survival was 12.6 months. There were 49 males and 31 females. The median age of 57.0 years was the same for each sex. The median survivals of 15.8 months for males and 14.8 months for females was not significantly different ($P=0.95$). In our sample, we found a comparative ratio of brain tumors of 1.58:1, men *versus* women similar to the ratio of 1.5:1 reported by others (16).

Gene expression by patient age. Tissue sections from 80 GBM were analyzed for the presence or absence of expression of the protein encoded products of the p16, p53, EGFR, MDM2 and Bcl-2 genes; the results are summarized in Table 2. Since patient age is a known prognostic factor for patients with GBM (1, 2, 14, 15) and was demonstrated to be so for this patient population (Table 1), the results for gene expression are grouped with respect to age categories as described in clinical data. A comparison of patient age and frequency of gene expression showed no significant differences (Table 2). For any given genetic variable (p16, p53, EGFR, MDM2 or Bcl-2), age was not associated with any significant shifts of genetic profiles.

Patient survival by gene expression in glioblastoma stratified for age. To determine whether expression of specific growth control genes would be of prognostic value in predicting survival among patients with GBM, we compared median survival between immunopositive and immunonegative patients for each of the genetic variables; the results are summarized in Table 3. Figures 1-5 show the Kaplan-Meier survival plots paired with scatter diagrams for immunopositive *versus* immunonegative GBM for each of the 5 encoded gene products for patients stratified by age category.

Loss of p16 protein expression was previously characterized in 24 of the cases and was shown to correlate with homozygous deletion and inactivation of the p16 gene (38). The loss of p16 gene expression was found in 22 of 74 (30%) patients with GBM. Among those patients aged 61-80, a tendency for shorter median survival was observed with loss of p16 protein expression

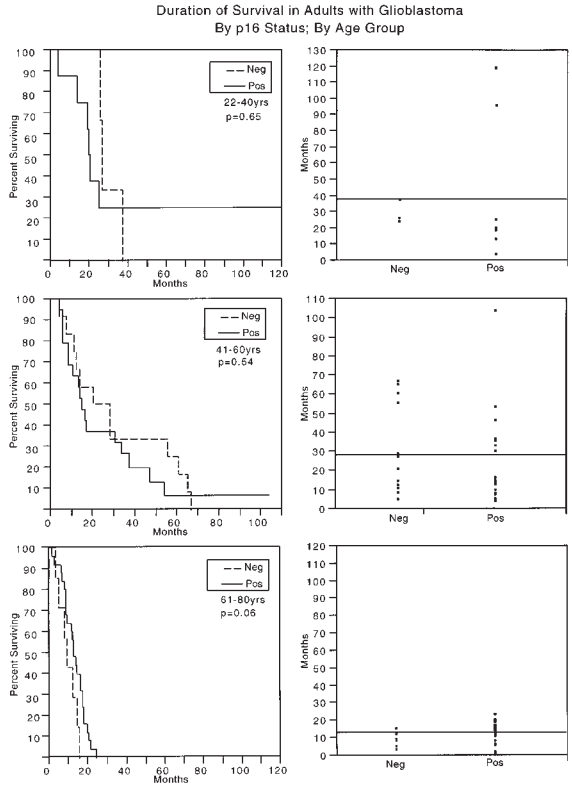

Figure 1. Kaplan-Meier and scatter plots for patient survival with GBM by p16 status by age group. Survival of patients aged 22-40 yrs with (N=8) or without (N=3) p16 expression; survival of patients aged 41-60 yrs with (N=19) or without (N=12) p16 expression; survival of patients aged 61-80 yrs with (N=24) or without (N=7) p16 expression.

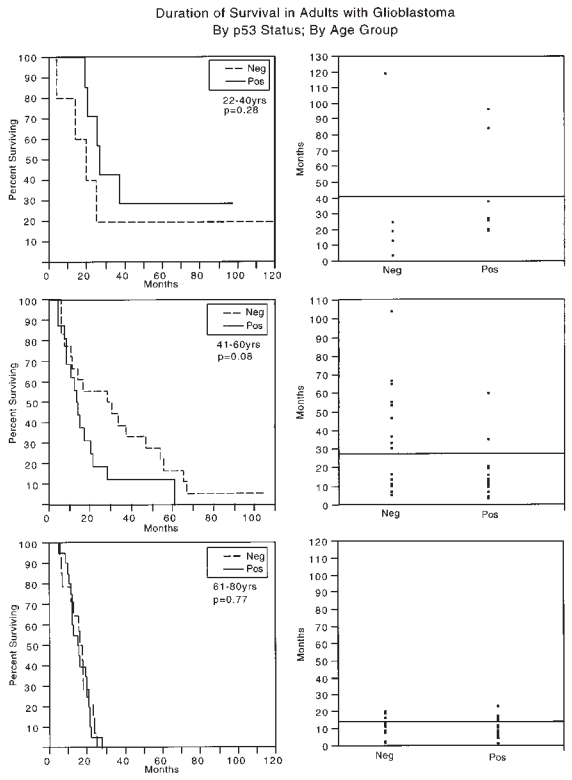

Figure 2. Kaplan-Meier and scatter plots for patient survival with GBM by p53 status by age group. Survival of patients aged 22-40 yrs with (N=7) or without (N=5) p53 expression; survival of patients aged 41-60 yrs with (N=16) or without (N=18) p53 expression; survival of patients aged 61-80 yrs with (N=20) or without (N=14) p53 expression.

but the difference was not significant (P=0.06). No significant differences in survival were detected among patients aged 22-60 with or without p16 protein expression by age category (Table 3). Figure 1 shows Kaplan-Meier survival curves paired with scatter diagrams for p16 immunopositive *versus* immunonegative patients by age category. The median survival of the patients aged 22-40 with normal expression of p16 was 20.3 months, compared with 27.1 months for those showing loss of p16 immunostaining (P=0.65). The median survival of the patients aged 41-60 with normal expression of p16 was 15.3 months, compared with 24.6 months for those showing loss of p16 immunostaining (P=0.54). In contrast, the median survival of the patients aged 61-80 with normal expression of p16 was 13.2 months compared, with 9.7 months for those showing loss of p16 immunostaining and this difference approached statistical significance (P=0.06).

Immunostaining for p53 protein accumulation and mutations in the p53 gene previously had been analyzed in 26 of the cases (36). Although a 1:1 correlation between p53 protein accumulation and the presence of mutations in exons 4-8 of the p53 gene is not observed in every GBM study (22, 43-48), in our experience >90% of the cases with immunohistochemically detectable p53 protein have detectable alterations in the p53 gene demonstrated by sequence analysis. Aberrant p53 protein accumulation was detected in 43 of 80 (54%) tumors and variant SSCP bands were present in 38 of 41 (93%) DNAs that could be analyzed from the 43 tumors. Among those patients aged 41-60, a tendency for shorter median survival was observed with p53 protein accumulation but the difference was not significant (P=0.08). No significant differences in survival were detected among patients aged 22-40 or 61-80 with or without p53 protein expression by age category (Table 3). Figure 2 shows Kaplan-Meier survival curves paired with scatter diagrams for p53 immunopositive *versus* immunonegative patients by age category. The median survival of the patients aged 22-40 with normal

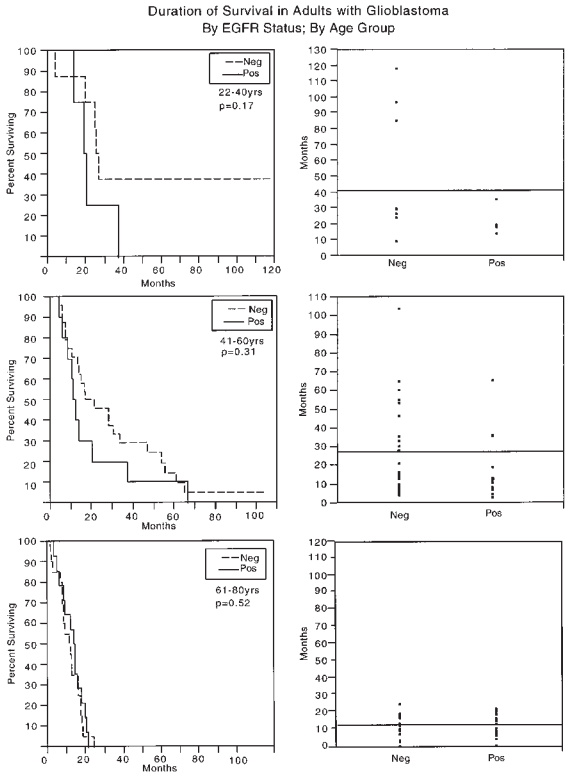

Figure 3. Kaplan-Meier and scatter plots for patient survival with GBM by EGFR status by age group. Survival of patients aged 22-40 yrs with (N=4) or without (N=8)EGFR expression; survival of patients aged 41-60 yrs with (N=10) or without (N=24) EGFR expression; survival of patients aged 61-80 yrs with (N=14) or without (N=19) EGFR expression.

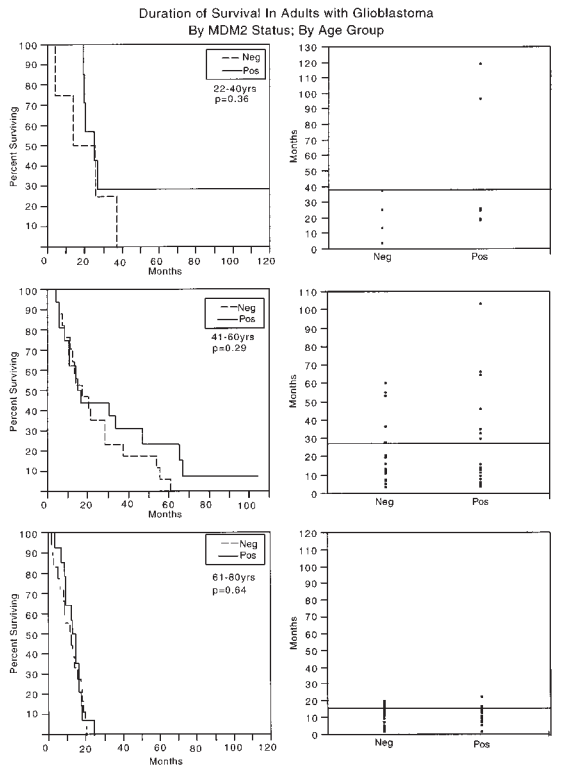

Figure 4. Kaplan-Meier and scatter plots for patient survival with GBM by MDM2 status by age group. Survival of patients aged 22-40 yrs with (N=7) or without (N=4) MDM2 expression; survival of patients aged 41-60 yrs with (N=16) or without (N=17) MDM2 expression; survival of patients aged 61-80 yrs with (N=14) or without (N=18) MDM2 expression.

expression of p53 was 20.1 months, compared with 27.1 months for those showing accumulation of p53 immunostaining (P=0.28). However, the median survival of the patients aged 41-60 with normal expression of p53 was 29.7 months, compared with 13.9 months for those showing accumulation of p53 immunostaining (P=0.08) and this difference approached statistical significance. Lastly, the median survival of the patients aged 61-80 with normal expression of p53 was 13.9 months, compared with 12.2 months for those showing accumulation of p53 immunostaining (P=0.77).

Overexpression of EGFR was characterized previously in a subset of 26 GBM and correlated with EGFR gene amplification (8). Overexpression of EGFR was detected in 28 of 80 (35%) tumors. No differences in survival based on EGFR status were detected in patients of different age groups (Table 3). Figure 3 shows Kaplan-Meier survival curves paired with scatter diagrams for EGFR immunopositive *versus* immunonegative patients by age category. The median survival of the patients aged 22-40 without overexpression of EGFR was 26.4 months, compared with 19.9 months for those with EGFR immunostaining (P=0.17). The median survival of the patients aged 41-60 without overexpression of EGFR was 19.5 months, compared with 12.2 months for those with EGFR immunostaining (P=0.31). The median survival of the patients aged 61-80 without overexpression of EGFR was 12.1 months, compared with 14.5 months for those with EGFR immunostaining (P=0.52).

MDM2 protein expression is undetectable in normal astrocytes but upregulation of its expression can occur both with or without gene amplification (22, 32, 33, 49). Overexpression of the MDM2 protein was detected in 37 of 76 (49%) tumors. No differences in survival were detected among patients with or without MDM2 protein expression by age category (Table 3). Figure 4 shows Kaplan-Meier survival curves paired with scatter diagrams for MDM2 immunopositive *versus* immunonegative patients by age category. The median survival of the

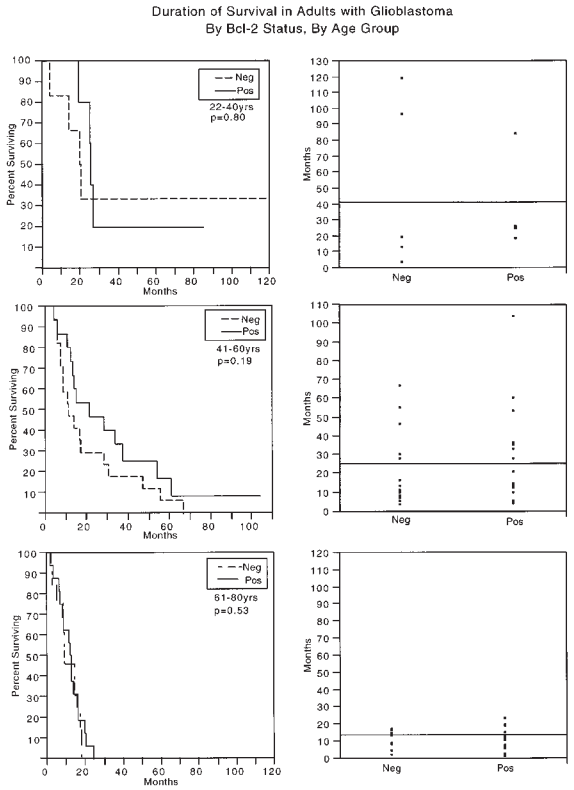

Figure 5. Kaplan-Meier and scatter plots for patient survival with GBM by Bcl-2 status by age group. Survival of patients aged 22-40 yrs with (N=5) or without (N=6) Bcl-2 expression; survival of patients aged 41-60 yrs with (N=15) or without (N=17) Bcl-2 expression; survival of patients aged 61-80 yrs with (N=16) or without (N=13) Bcl-2 expression.

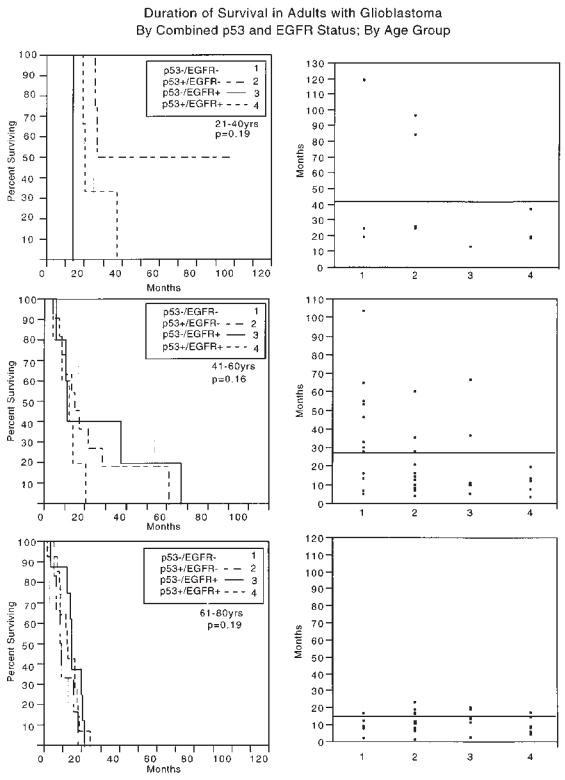

Figure 6. Kaplan-Meier and scatter plots for patient survival with GBM by combined p53 and EGFR status by age group. Survival of patients aged 22-40 yrs with $p53^-/EGFR^-$ GBM (N=4) or $p53^+/EGFR^-$ GBM (N=4) or $p53^-/EGFR^+$ GBM (N=1) or $p53^+/EGFR^+$ GBM (N=3); survival of patients aged 41-60 yrs with $p53^-/EGFR^-$ GBM (N=13) or $p53^+/EGFR^-$ GBM (N=11) or $p53^-/EGFR^+$ GBM (N=5) or $p53^+/EGFR^+$ GBM (N=6); survival of patients aged 61-80 yrs with $p53^-/EGFR^-$ GBM (N=6) or $p53^+/EGFR^-$ GBM (N=14) or $p53^-/EGFR^+$ GBM (N=8) or $p53^+/EGFR^+$ GBM (N=6).

patients aged 22-40 without MDM2 expression was 20.0 months compared with 25.6 months for those with MDM2 immunostaining (P=0.36). The median survival of the patients aged 41-60 without MDM2 expression was 17.2 months compared with 16.1 months for those with MDM2 immunostaining (P=0.29). Similarly, the median survival of the patients aged 61-80 without MDM2 expression was 12.3 months, compared with 13.8 months for those with MDM2 immunostaining (P=0.64).

Expression of Bcl-2 had been characterized previously in 22 of the GBM (13). Overexpression of the Bcl-2 protein was detected in 36 of 72 (50%) tumors. No differences in survival were detected among patients with or without Bcl-2 protein expression by age category (Table 3). Figure 5 shows Kaplan-Meier survival curves paired with scatter diagrams for Bcl-2 immunopositive *versus* immunonegative patients by age category. The median survival of the patients aged 22-40 without Bcl-2 expression was 20.3 months compared with 25.8 months for those with Bcl-2 immunostaining (P=0.80). The median survival of the patients aged 41-60 without Bcl-2 expression was 11.7 months compared with 21.8 months for those with Bcl-2 immunostaining (P=0.19). Similarly, the median survival of the patients aged 61-80 without Bcl-2 expression was 11.4 months, compared with 12.6 months for those with Bcl-2 immunostaining (P=0.53).

Patient survival by glioblastoma variant stratified for age. Subsets of GBM have been classified broadly into two genetic groups by DNA analysis for p53 gene mutation and immunohistochemical analysis for EGFR protein accumulation as progressive ($p53^+/EGFR^-$) and *de novo* ($p53^-/EGFR^+$) (8, 11, 21, 23). However, genotype analysis for p53 gene mutation and immunopheno-

type for EGFR expression distinguishes four tumor variants. Table 4 summarizes the distribution of the four GBM variants ($p53^-/EGFR^-$; $p53^+/EGFR^-$; $p53^-/EGFR^+$; $p53^+/EGFR^+$) among 80 patients according to age category. Figure 6 shows the Kaplan-Meier survival plots together with paired scatter diagrams for each of the GBM variants by age group. No significant differences in survival were observed between any of the four GBM variants within any of the age categories. With respect to the so called progressive ($p53^+/EGFR^-$) or *de novo* ($p53^-/EGFR^+$) GBM variants, patients aged 41-60 and 61-80 had similar median survivals. The median survivals for 25 patients with the progressive GBM variant aged 41-60 or 61-80 were 15.2 and 12.7 months, respectively. Among the 13 patients with the *de novo* GBM variant the median survivals for patients aged 41-60 or 61-80 were 11.7 and 14.7 months, respectively.

Associations of altered gene expression in glioblastoma. Each immunohistochemical marker was tested in a pairwise comparison for any positive (synergistic) or negative (mutually exclusive) association using Chi-square analysis. In order to protect the analysis from a false-positive (Type 1) error caused by the multiple comparison of data, the Bonferroni procedure was used to correct the critical level of the reported P value for statistical significance (43). There were no significant differences in positive or negative associations between the genetic variables (Table 5). In particular, we did detect EGFR expression in some GBM showing abnormalities of p53 expression in contrast to the report by Watanabe et al (21). With regard to these "double positive" GBM, we used immunohistochemistry data and compared it with data obtained by SSCP for p53 alterations followed by sequence analysis. We found no major changes in the distribution of p53 positive or negative and EGFR positive or negative tumors (Table 5).

However, there was one association that approached statistical significance. Tumors with normal p16 expression tended to show accumulation of MDM2 protein (N=29) and this association approached statistical significance (observed P=0.04; Bonferroni threshold for significance with four comparisons P=0.01). Because the number of these observations was small, further experiments will be required to confirm this association.

Discussion

A goal of molecular neuro-oncology is to establish relationships between histopathologic criteria used for grading astrocytoma, the molecular alterations associated with that grade and clinical outcome. GBM are a heterogeneous group of neoplasms having a wide range of histological and biological features such that attempts to relate a particular factor with clinical outcome have largely been unsuccessful. Presently, the only standard prognostic criteria consistently related to clinical outcome are younger age at diagnosis, grade and performance status (1, 2, 16, 26-32). In our study all patients had astrocytic WHO grade IV tumors. An inverse association was found between younger age and survival as expected and is similar to our previous experience (13).

One aim of this study was to evaluate genetic alterations in GBM and relate the genetic findings stratified by age with patient survival. We evaluated 80 astrocytic GBM for alterations in the growth control genes p16, p53, EGFR, MDM2 and Bcl-2 to determine their relevance as prognostic factors. A comparison of patient age and frequency of gene expression showed no significant differences (Table 2). The results indicate that for these 5 genetic variables there was no indication that GBM arising in younger *versus* older patients had different genetic profiles.

Among immunopositive *versus* immunonegative GBM, the median survivals by Kaplan-Meier analyses stratified by age for each genetic variable were similar within each age group for each genetic variable. While statistically significant differences in survival based on gene expression were not detected in our study, we did identify two associations that showed survival differences and which approached statistical significance. Among patients aged 61-80 with GBM showing loss of p16 protein expression a shorter median survival (9.7 months) was observed compared to that for patients with p16 positive GBM (13.2 months) (P=0.06).

Another association was uncovered among patients aged 41-60. Those patients with p53 positive GBM tended to have a shorter median survival (13.9 months) compared to that for patients with p53 negative GBM (29.7 months) (P=0.08). Some studies have shown an association between p53 gene mutation and protein accumulation and poor survival (18, 27, 32, 33), while others have not found such a correlation (19, 30, 31, 47). The predictive role of p53 gene status in GBM has not shown any strong correlation with survival to date and may relate to the fact that most studies have not assessed patient survival by p53 gene status according to age category. Given the current use of molecular genetics to attempt to subtype GBM into p53 positive or progressive GBM *versus* p53 negative or *de novo* GBM, it is important to consider the very strong association age has on survival in patients with GBM (1, 2). The progressive GBM which arise from previous low-grade tumors are

known to occur in younger patients (14, 15). However, in this instance, better prognosis has not been associated with the presence of p53 gene mutation in young adult patients (23). Indeed, one report indicated that p53 positive low-grade tumors that progressed recurred more quickly than those tumors without p53 protein accumulation (34). Future studies are needed to clarify the role of p53 gene alterations in GBM stratified by age.

Using molecular genetic approaches several studies have shown, using unselected series' of tumors, that GBM could be grouped into one of three genetic variants based on the spectrum of genetic alterations (8, 12, 17, 25, 27, 47). One variant generally contains mutations of the p53 gene associated with accumulation of the p53 protein but most often lacks amplification and overexpression of the EGFR gene. A second variant is more often associated with amplification and overexpression of the EGFR gene and retains the wildtype p53 gene (inferred by lack of p53 protein accumulation). A third variant appears to have normal p53 and EGFR genes but has frequent deletion of the p16 gene (12).

Genotyping GBM for p53 gene mutations by SSCP analysis and immunophenotyping for p53 and EGFR expression also identifies a fourth group of tumors consisting of the "double positive". In this study of 80 GBM we found that 29% were negative for both p53 and EGFR expression, 36% were positive for p53 protein accumulation only, 18% were positive for EGFR overexpression only, while 18% were "double positive" for both p53 and EGFR expression. The frequency was similar when p53 positivity was restricted to those demonstrating p53 gene mutations. In an analysis of 70 unselected GBM by Hayashi et al (12) 30% were negative for both p53 mutations and EGFR gene amplification, 30% were positive for p53 gene mutations, 40% showed amplification of the EGFR gene, while none were double positive. Another study of 46 unselected adult GBM by Rasheed et al (45) found 53% were negative for both p53 mutations and EGFR gene amplification, 16% were positive for p53 gene mutations, 29% showed amplification of the EGFR gene, while 2% were double positive.

More recently, GBM selected on the criteria of a clinical history <3 months were analyzed for frequency of p53 and EGFR gene alterations (21). Results from these studies confirmed that GBM arise *via* different genetic pathways referred to as "primary" (here "*de novo*"), and "secondary" (here "progressive"). In addition this study extended the results of the genetic analysis reported previously for p53 and EGFR alterations, demonstrating in their selected tumor series that mutations in the p53 gene, documented by positive immunostaining and positive SSCP analysis together with overexpression of the EGFR gene were largely mutually exclusive genetic alterations, i.e. genotyping separated the "secondary" ($p53^+/EGFR^-$) from the "primary" ($p53^-/EGFR^+$) GBM. Among the 19 primary GBM analyzed by Watanabe et al, (21), the distribution of the patients by age was similar to our current study showing 15% of patients aged <40, 47% aged 41-60 and 37% aged 61-80. The distribution of genetic variants among this series of primary GBM was 16% negative for both p53 and EGFR expression, 21% positive for p53 protein accumulation only, 47% positive for EGFR overexpression only, while 16% were double positive for both p53 and EGFR expression. The relative distribution of the four GBM variants among the selected series of GBM is comparable to the distribution reported in our current study among unselected GBM.

Deletions of the p16 gene assessed in primary GBM, again selected on the basis of clinical history and presumably positive for overexpression of EGFR, was 36% (50). This frequency of p16 inactivation in a selected series of GBM is similar to the incidence of 44% observed in our study using unselected cases but contrasts with the much higher incidence of 71% reported by Hayashi et al (12).

The second aim of this study was to determine whether genetically defined GBM representing the *de novo* ($p53^-/EGFR^+$) or progressive ($p53^+/EGFR^-$) tumor variants differed in their biological behavior in patients stratified according to age category using patient survival as the clinical outcome. Because younger age is a strong prognostic factor associated with favorable outcome in patients with GBM, we stratified our analysis of the different GBM variants by age. No significant differences in survival were observed within each age group for a given GBM tumor variant.

Although there were no statistical differences in survival, there were a couple of interesting observations. In this study, 13 patients aged 41-60 with GBM that retained normal p53 and EGFR expression had a median survival of 31 months compared to shorter survival for all other GBM variants within that age category. Similar to the findings noted above for p53, some studies have shown an association between EGFR amplification and poor survival (17, 51, 52), while others have not found such a correlation (27, 29, 33, 53). Again, our numbers are limited and further studies using a larger series of genetically defined GBM from patients in this age category will be needed to determine whether the

GBM variant lacking alterations in both p53 and EGFR is associated with better survival outcome.

Among patients aged 61-80 similar patterns of poor survival were detected for all GBM variants. However, we did observe that loss of p16 expression among this patient population was associated with decreased median survival compared to those patients that retained p16 gene expression (P=0.06). Overall our results suggest that age >61 may be a stronger prognostic factor for poor survival in patients with GBM rather than the presence or absence of any one or combination of genetic alterations.

Using a pairwise comparison we asked whether certain genetic alterations were more likely to occur together or not. We wanted to test the hypothesis that certain alterations that have been reported to occur more frequently in "primary" GBM such as overexpression of EGFR and MDM2 and lack of p53 mutations in tumors selected on the basis of clinical presentation (21) would hold for GBM analyzed for similar alterations that were unselected. Similar patterns of altered genes in GBM variants regardless of selection bias, if associated with clinical outcome, could be used for patient risk assessment and management. As noted in other reports and in the current study, four GBM variants can be distinguished by p53 and EGFR alterations (21, 27, 32, 47). In our series of 80 GBM, we did not detect an inverse correlation between EGFR positive *versus* p53 positive GBM as has been reported by others (12, 21). The status of the p53 gene was demonstrated using immunohistochemistry as well as sequence analysis. Equal numbers of immunopositive or immunonegative EGFR tumors were positive or negative for p53 protein expression or p53 mutations (Table 5). Similarly, *de novo* or primary GBM have been reported to frequently overexpress EGFR as well as MDM2 (22). If this were the case, a large number of GBM would be expected to be double positive for EGFR and MDM2 and lack p53 gene mutations. In this series of 80 unselected GBM equal numbers of EGFR or p53 immunopositive or immunonegative tumors were positive or negative for MDM2 protein expression (Table 5). Co-expression of p53 and MDM2 in unselected series of GBM also has been reported by Korkolopoulou et al (32) and Rainov et al confirming our observation (33). However, overexpression of MDM2 was associated with poor survival in GBM in these studies which differs from our finding (32, 33). Clearly, future studies will be needed to further clarify the association between aberrant overexpression of EGFR and MDM2 and MDM2 and p53 in variants of GBM and survival outcome.

The observation that GBM with normal expression for the p16 gene tended to show aberrant accumulation for the MDM2 oncoprotein (N=29) approached statistical significance (P=0.04) (see methods). Disruption of the Rb cell cycle control pathway occurs in >80% of GBM through loss of p16 function or amplification of CDK4 or mutation of Rb (50, 54-56). However, it is now known that the p16 gene locus can encode two separate proteins, $p19^{ARF}$ and $p16^{INK4a}$, through differential splicing (57). Recent reports have shown that the $p19^{ARF}$ gene product is a potent tumor suppressor protein which can complex with MDM2 inhibiting the ability of MDM2 to induce degradation of the p53 protein (58, 59). The expression of p53 and MDM2 serve in an autoregulatory feedback loop important for controlling their respective activities (60). Loss of p16 function would selectively disrupt the p16/cyclin D-CDK4/Rb pathway, while loss of the entire $p19^{ARF}$-$P16^{INK4a}$ locus would serve to inactivate both the Rb and $p19^{ARF}$/MDM2/p53 pathways (59).

Approximately one-third of GBM in this study showed up-regulation of MDM2 gene expression without loss of the p16 gene. At present the mechanism by which MDM2 expression is up-regulated in 50% of GBM in the absence of gene amplification is unknown. One possibility is the presence of short alternatively spliced MDM2 transcripts reported in >65% of GBM (61). However, the correlation between aberrant MDM2 transcripts with expression for MDM2 protein in the same tumor samples was not assessed (61). Clearly there is mounting evidence to suggest that deregulation of the MDM2 gene may play an important role in the development of GBM (22, 32, 50, 61). The recent report by Pomerantz et al (58) emphasizes the importance of analyzing multiple genetic changes such as $p19^{ARF}$, $p16^{INK4a}$, MDM2 and p53 in the same tumor sample in order to distinguish common from alternative genetic pathways in the development of cancers. Accordingly, mutations in tumor suppressor genes which are mutually exclusive, such as inactivation of p16 or Rb, argue for the disruption of a common genetic pathway through which both tumor suppressor genes exert their activity, a common event in GBM (50, 54-56). It is tempting to speculate that the GBM variant lacking alterations in the p16 gene may represent a variant in which the $p19^{ARF}$ gene itself or the $p19^{ARF}$/MDM2/p53 pathway may be deregulated. Future studies will be required to determine whether inactivation of the $p19^{ARF}$ gene is associated with deregulated expression of the MDM2 gene in GBM

and inactivation of the $p19^{ARF}$/MDM2/p53 pathway.

In summary, we have shown that deregulated expression of several critical growth control genes p16, p53, EGFR, MDM2 and Bcl-2 frequently occurs in GBM, but none serve as independent prognostic factors to predict biologic behavior for GBM and patient survival. This suggests that other genes, presently unidentified, may play a significant role in malignant transformation and may be more closely correlated with clinical outcome. In addition, our analysis by age category has shown that genetically defined variants of GBM including the progressive ($p53^+/EGFR^-$) or *de novo* ($p53^-/EGFR^+$) GBM variants have similar clinical outcomes. Our findings are supported by a recent case control study which evaluated the survival of patients whose GBM began as low-grade astrocytomas that progressed to higher grade *versus* those patients whose tumors arose as *de novo* GBM and found no significant differences (15). In this study, patients were matched for tumor histology which included GBM, anaplastic oligodendroglioma and mixed anaplastic glioma, age at diagnosis (median 41 years), Karnofsky performance score (mean 83-86), and type of surgery that confirmed the tumor histology. The overall median survival for 68 patients with GBM beginning as low grade tumors was 19.7 months compared with 22.0 months for 68 patients with matched *de novo* tumors (15). Although this series of tumors has not been evaluated for genetic alterations, the authors concluded "that gliomas with a given malignant phenotype have similar clinical behavior even though they arose by several distinct genetic pathways" (15). The challenge for molecular neuro-oncologists will be to catalogue multiple genetic alterations in a single GBM specimen in order to distinguish common from alternative genetic pathways and to ultimately refine genotype analysis of GBM variants so that they may be associated with better patient management and increased survival.

Acknowledgments

This work was generously supported by a grant from The Brain Tumor Society, Boston, MA, USA and by funding from the Kaplan Cancer Center and its NIH Grant CA 16087 to E.W.N.

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EDITORIAL

Subsets of Glioblastoma: Clinical and Histological vs. Genetic Typing.

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The glioblastoma is difficult to beat. It has resisted a variety of therapeutic approaches and although some patients show remission and longer survival, attempts to identify predictive factors for response to therapy have largely failed. This includes histopathological features, none of which have been found to reliably predict individual prognosis.

During the past decade, a wealth of information has accumulated on genetic alterations associated with the evolution of glioblastomas. Additionally, putative suppressor genes on chromosomes 10 and 19 are likely to be identified in the near future. As early as 1993, evidence began to accumulate that there are different pathways leading to the glioblastoma as the common phenotypic endpoint (2, 7) and more recently, patterns of genetic alterations have been assigned to clinically and histologically defined entities (1, 8). It has long been recognised that glioblastomas may develop after a short clinical history *de novo*, i.e. without an identifiable, less malignant precursor lesion. They have been termed "primary glioblastomas," affect older patients, and typically contain *EGFR* overexpression, *PTEN* mutations, *p16* deletions, and less frequently, *MDM2* amplification. "Secondary glioblastomas" develop through progression from low-grade or anaplastic astrocytoma, affect younger patients, and commonly contain a *p53* mutation which is typically already present in the respective precursor lesion.

The paper by Newcomb *et al* in this issue of *Brain Pathology* addresses a problem of considerable clinical

importance in attempting to correlate the patterns of genetic alterations in glioblastomas with clinical outcome. They assessed altered expression of *p16*, *p53*, *EGFR*, *MDM2*, and *Bcl-2* genes but found that survival of patients with or without altered gene expression showed no significant difference by age group or gene expression. This lack of predictive value may seem disappointing, but there are several factors to be considered. I humbly disagree with the authors' approach to type glioblastoma subsets exclusively on the basis of gene expression profiles. The genetic pathways leading to the evolution of primary and secondary glioblastomas outlined above have been largely derived from cohorts of patients stringently selected on the basis of clinical history and sequential biopsies. The observation that glioblastomas from patients with histologically proven progression from low-grade astrocytoma typically contain a *p53* mutation does, in my experience, not allow the conclusion that *all* glioblastomas with a *p53* mutation have progressed from a prior low-grade glioma. Similarly, two thirds of *de novo* glioblastomas with a very short clinical history contain an EGFR amplification/overexpression, but there are no data showing that *all* glioblastomas with this genetic alteration have developed *de novo*. Thus, the classification by the authors of p53+/EGFR- glioblastomas as secondary (or, in their terminology, progressive) and p53-/EGFR+ lesions as primary glioblastomas is presumptive. More subtypes of glioblastoma may exist with intermediate clinical and genetic profiles (3). This is exemplified by the giant cell glioblastoma, a histologically distinct variant that shares with primary (*de novo*) glioblastomas a short clinical history, the absence of a less malignant precursor lesion, and a 30% frequency of *PTEN* mutations (6). With secondary glioblastomas it has in common a younger patient age and a high frequency of *p53* mutations (4, 5). Thus, the currently available data are insufficient for a substitution of clinical and histological classification by genetic typing. More work in this exciting research field may eventually lead to the identification of combined clinical, histological, and genetic profiles of astrocytic tumors, which will hopefully be predictive for response to therapy and survival.

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