

Prognostic and Pathologic Significance of Quantitative Protein Expression Profiling in Human Gliomas¹

Arnab Chakravarti,² Meaghan A. Delaney, Elizabeth Noll, Peter McL. Black, Jay S. Loeffler, Alona Muzikansky, and Nicholas J. Dyson

Departments of Radiation Oncology [A. C., M. A. D., J. S. L.], Biostatistics [A. M.], and Molecular Oncology [N. J. D.], Massachusetts General Hospital, Harvard Medical School and Department of Neurosurgery [E. N., P. M. B.], Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

Purpose: Analysis of tumor-derived genetic lesions has provided insights into molecular pathogenesis of human gliomas. Because these changes represent only one of several mechanisms that alter gene expression during tumorigenesis, it is likely that further information will be obtained from a careful analysis of important regulatory proteins present in these tumors.

Experimental Design: We have quantified the levels of key cell cycle/signaling proteins in 94 prospectively collected, meticulously preserved, “snap frozen” glioma specimens and have compared these levels with histopathological data and patient outcome.

Results: The results of these experiments confirm that the levels of wild-type tumor suppressor proteins, such as p53, pRB, PTEN, p14^{ARF}, and p16^{INK4}, are lost or severely reduced in most gliomas, and that epidermal growth factor receptor, human telomerase reverse transcriptase, and cyclin-dependent kinase 4 are overexpressed frequently and with a few exceptions, almost exclusively, in glioblastomas. In addition, we report frequent underexpression of E2F-1 (in 55% of gliomas) and cyclin E overexpression (in 26% of gliomas), which have not yet been reported on the genomic level. Several of these markers significantly correlated with histopathological grade, and the levels of five proteins showed significant association with patient outcome. In particular, overexpression of epidermal growth factor receptor, human telomerase reverse transcriptase, cyclin-dependent kinase 4, and cyclin E was largely restricted to glioblastomas

and was significantly associated with reduced patient survivals.

Conclusions: We conclude that the quantitation of cell cycle/signaling proteins from meticulously preserved glioma specimens provides further insights into the molecular pathogenesis of human gliomas and yields valuable prognostic information.

INTRODUCTION

Gliomas are among the most aggressive and treatment refractory of all human tumors. Astrocytomas, oligodendrogliomas, and mixed tumors comprise over 80% of primary adult central nervous system tumors. With aggressive treatment, the 2-year survivals are 66, 45, and 9%, respectively, for low-grade astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme, according to the most recent Surveillance, Epidemiology, and End Results data (1).

The molecular pathogenesis of gliomas has been under active investigation as part of the effort to develop more effective therapeutic strategies for these tumors. Efforts have focused primarily on the genomic alterations within tumor cells. Reported chromosomal alterations have included loss of heterozygosity for 1p, 9p, 10, 13q, 17p, 19q, and 22q loci and gain of chromosome 7 (2–10). Cell cycle regulatory genes and genes encoding for growth factors are commonly affected. These include inactivation of *CDKN2A* (encoding for p16 and p14^{ARF}), *RB* (on 13q), *p53* (on 17p), *PTEN* (phosphatase and tensin homologue on 10q), and amplification of *EGFR*³ and *cdk4*, among others (11–18). Genetic features are thought to distinguish two broad groups of GBMs. Primary GBMs, or *de novo* GBMs, are diagnosed histopathologically as GBMs at first presentation and are characterized by *EGFR* gene amplifications without corresponding *p53* gene mutations. Secondary, or progressive GBMs, progress from lower-grade astrocytomas, seldom have *EGFR* gene amplifications, but usually do have *p53* gene mutations (4, 12).

However, genetic alterations represent only one of several mechanisms that alter gene expression in tumor cells. Changes in transcription or in protein stability, protein modification, or protein/protein interactions play important roles in tumor cell proliferation but are not detected in studies that rely solely on DNA analysis. Molecular studies of gene expression in gliomas, either at the RNA or protein level, are relatively sparse. Meticulous tissue handling and storage (snap freezing) are required to quantitatively examine mRNA or protein levels (or activation states). Because most centers preserve tissue in formalin-fixed paraffin, efforts to quantitatively analyze gene expression pat-

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² To whom requests for reprints should be addressed, at Massachusetts General Hospital, Laboratory of Molecular Oncology, 13th Street, Building 149, Room 7330, Charlestown, MA 02129. Phone: (617) 726-2241 (pager; i.d. 30601); Fax: (617) 726-5637; E-mail: achakravarti@partners.org.

³ The abbreviations used are: EGFR, epidermal growth factor receptor; cdk, cyclin-dependent kinase; GBM, glioblastoma multiforme; IHC, immunohistochemistry; hTERT, human telomerase reverse transcriptase.

Table 1 Histopathological identity of gliomas included in the study

Histology	No. of tumors
GBM	56
Anaplastic astrocytoma	13
Low-grade astrocytoma	12
Low-grade mixed glioma	3
Anaplastic oligodendroglioma	1
Low-grade oligodendroglioma	6
Ganglioglioma	2
Ependymoma	1
Total	94

terns at the RNA or protein level are effectively thwarted. IHC is often used to qualitatively assess protein expression on paraffin-embedded tissues. However, the interpretation of IHC results is subjective, and problems with antigen preservation and integrity mean that there is often substantial variation between tumor samples and between studies. Given these problems, it is not surprising that studies that rely solely on IHC data can be quite variable and can be inconsistent with the genomic information (13, 19). Given that proteins are the final effectors of gene function and that protein expression levels are not predicated solely on genomic alterations, questions of how changes in cell cycle/signaling genes at the protein level (determined quantitatively) impact upon the molecular pathogenesis of gliomas and affect patient outcome remain largely unanswered.

In an effort to obtain further insights into the molecular pathogenesis of gliomas, we have prospectively collected 100 “snap frozen” glioma specimens. We performed quantitative Western blot analysis to measure the levels of important regulatory proteins in gliomas. This method provides a rapid, quantitative, and accurate assessment of the role of cell cycle/signaling pathways in the molecular pathogenesis of gliomas and reveals whether these expression patterns are of prognostic value. We have quantitatively examined the levels of 12 proteins either in the pRB/p53 pathways or in signaling/immortalization pathways (EGFR, PTEN, and telomerase), most of which have been implicated in gliomagenesis at the genomic level.

The results of these experiments show a widespread pattern of loss of tumor suppressor proteins in gliomas and a more selective pattern of overexpressed oncoproteins. It becomes clear that aberrant expression of certain proteins are significantly interrelated, either directly or inversely. The results, described below, also show that the expression levels of five of these proteins are significant prognostic indicators of patient outcome.

MATERIALS AND METHODS

Tissue Processing. One hundred glioma specimens have been collected prospectively (at Brigham and Women’s Hospital) under an Institutional Review Board-approved protocol for the purposes of protein expression analysis of gliomas. The histopathological breakdown of these tumors is given in Table 1. Each specimen has been “snap frozen” after surgical removal, carefully sectioned, and histopathologically reviewed. Histopathological diagnosis was made by WHO (2000) classification. Tissue containing >95% tumor was provided for this analysis. Each tumor tissue was homogenized in RIPA buffer

using a Polytron homogenizer (Fisher) to generate protein lysates for Western blot analysis.

Western Blot Analysis. Bradford assays were performed to determine total protein concentrations, which were normalized to 1 $\mu\text{g}/\mu\text{l}$ for all samples. Samples were then prepared in sample buffer and heated to 95°C for 5 min. The percentage of polyacrylamide composition of gels was dependent on the molecular weights of the proteins to be isolated. Twenty μl of protein lysates in sample buffer from each tissue were loaded within each well. Gels were run at constant current (40 mA) for 3–4 h for maximum separation. Wet transfer was performed for 4 h at constant voltage (40 V) using polyvinylidene difluoride membrane presoaked in methanol. The membrane was then blocked for 1 h in 5% milk in 0.2% PVDF membrane (TBST). The membranes were then washed in 0.2% TBST three times for 15 min each. One of several internal controls (actinin, actin, and cdk1) was used to verify equivalent protein loading. The membranes were sectioned to separate the regions of the protein of interest from the internal controls. Positive controls from lysates of normal astrocytes obtained from 16-week-old fetuses were included in each gel. Because normal astrocytes do not express hTERT, positive controls for hTERT gels were from lysates obtained from an hTERT-expressing glioblastoma cell line established previously from a patient’s tumor. The membranes were then incubated overnight with primary antibodies for either p16 (JC-6), pRB (XZ-56), p14^{ARF} (SC8613), p130 (SC317), E2F-1 (KH 81, 95), PTEN (SC7974), EGFR (AB-4), cyclin D1 (HD 33,61), cyclin E (HE12), cdk4 (SC260), or hTERT (SC7214). The p16, pRB, E2F-1, cyclin D1, and cyclin E antibodies are monoclonal antibodies that were generated at the Massachusetts General Hospital Cancer Center and have been described previously (20–24). The p14^{ARF}, p130, PTEN, cdk4, and hTERT antibodies were purchased from Santa Cruz Biotechnology. The EGFR antibody was purchased from Calbiochem. Membranes were washed three times, incubated with secondary antibody, washed again, and incubated with chemiluminescence detection agent, after which film was exposed for detection. Three separate runs/sample/marker were performed to verify reproducibility. p53 levels were detected by immunoprecipitation-Westerns. The antibody used for the immunoprecipitation was specific for wild-type p53 only (oncogene AB-5). This sensitivity and specificity of this antibody were verified by detection of wild-type p53 in normal astrocytic lines and WI-38 cells and no detection of p53 in glioblastoma cell lines known to harbor mutations of p53 or known to lack p53 (LW5, WF, D384, SF126, U138, and U251), all of which were kindly supplied by Dr. Martin Haas (University of California, San Diego, CA; Ref. 25).

Image Analysis. The images were then scanned using Scion image analysis software (PC version of NIH image). External calibration was used using a photographic step tablet. The integrated absorbance of each band was used to quantitatively assess protein expression levels. Optical densities of bands were compared with those of corresponding controls.

Patient Data. Histopathological diagnosis was provided by Brigham and Women’s Hospital pathologists. Clinical data were provided by the Brigham and Women’s Hospital tumor registry and included patient age at diagnosis, treatment type, treatment dates, and clinical/survival outcomes. Complete clinical data were available on 94 of the 100 patients. Data analysis

Table 2 Protein expression patterns

Protein	% of gliomas
A. Underexpression	
pRB	35% (33/94)
p16	54% (51/94)
p14 ^{ARF}	52% (49/94)
WTp53	49% (48/94)
p130	0% (0/94)
E2F-1	55% (52/94)
PTEN	18% (17/94)
EGFR	17% (16/94)
B. Overexpression	
EGFR	26% (24/94)
hTERT	18% (17/94)
Cyclin E	26% (24/94)
Cyclin D1	1% (1/94)
cdk4	16% (15/94)

was limited to these 94 patients. Patients with glioblastoma and anaplastic astrocytoma were generally managed by surgery and postoperative radiation and chemotherapy. Patients with low-grade gliomas were generally managed by surgery alone, with radiation deferred for progressive disease. Westerns were performed in a blinded manner, without prior knowledge of clinical outcome. Patient follow-ups were updated at the time of manuscript preparation.

Statistical Methods. The Fisher Exact test was used to determine association between overexpression in a marker and the type of glioma (GBM *versus* non-GBM). The results were considered significant if a two-sided P was <0.05 . Survival curves were calculated using the Kaplan-Meier method. Univariate analysis used the log-rank test to determine the significance of each prognostic factor. Multivariate models were fit using the Cox proportional hazards model. The comparison for the average number of anomalies between the GBM and the non-GBM group was done using t test.

RESULTS

Using 94 glioma specimens, which have been “snap frozen” after surgical removal, carefully sectioned, and histopathologically reviewed, we have examined the protein expression levels of each of the components of the pRB pathway that have been linked to tumorigenesis (p16^{INK4}, cyclin D1, cyclin E, cdk4, pRB, p130, and E2F-1). In addition, we examined components of the p53 pathway (p53 and p14^{ARF}), PTEN, and two oncoproteins that have been found to be misexpressed in some gliomas (EGFR and hTERT, a catalytic component of telomerase). The level of each protein was determined from three independent Western blots of each sample. The signal strength was quantified and compared with the level of expression in normal astrocytes, and an average was determined. The results of this analysis are summarized in Table 2. For this analysis, we use the term underexpression to refer to samples where there was no detectable protein, or where the level of expression was reduced 5-fold or more compared with controls (Fig. 1A). Overexpression was defined as samples where the protein level was at least 5-fold greater than the corresponding controls (Fig. 1B).

Insights into the Molecular Pathogenesis of Gliomas

Patterns of Aberrant Protein Expression. Most tumors underexpressed p16^{INK4} or pRB (68 of 94; 72%), the two best-characterized tumor suppressors in the RB pathway. All tumors had levels of p130 that were comparable with the wild-type controls, and only one tumor showed elevated levels of cyclin D1. Intriguingly, E2F-1, a pRB-binding protein that acts as a tumor suppressor in mice, was underexpressed in over half of the samples (52 of 94). In contrast, positively acting components of this pathway (cyclin D1, cyclin E, and cdk4), which drive the phosphorylation of pRB and behave as oncogenes, were overexpressed in 22% (21 of 94) of tumors. When these changes are considered together, pRB control of E2F1 is predicted to be lost or deregulated in 91% (86 of 94) of the tumors. Most tumors also displayed some deficiency in the p53 pathway (p53 or p14^{ARF} deficiency in 70 of 94 tumors; 74%). Fig. 2 illustrates that most gliomas demonstrate some deficiency in the pRB/p53 pathways at the protein level. Reduction in PTEN expression was seen in 18% of all gliomas (17 of 94). EGFR was overexpressed in 26% (24 of 94). Overexpression of hTERT was seen in 17 of 94 samples (18%).

Because GBMs have clinical courses that are characteristically more aggressive than their non-GBM counterparts, we compared the protein expression profiles of GBMs *versus* non-GBM tumors (Table 3, A and B). When the levels of PTEN, pRB, or E2F1 were examined, no significant difference was observed. However, altered levels of several other proteins showed a tight correlation with the histopathology of the tumor. For example, underexpression of p16^{INK4} and p14^{ARF} was significantly more common among GBMs compared with non-GBM tumors ($P = 0.02$). Conversely, more non-GBMs had loss of wild-type p53 expression compared with GBMs ($P = 0.01$).

Strikingly, the overexpression of cyclin E, EGFR, cdk4, and hTERT was significantly more common among GBMs compared with their non-GBM counterparts ($P < 0.0001$). Indeed, the overexpression of one or more of these proteins was observed, in conjunction with deficits in one or more tumor suppressor genes, in 73% of GBMs compared with 11% of the non-GBMs ($P < 0.0001$; Table 3C). This raises the possibility that the loss of expression of tumor suppressor genes may be an early event in gliomagenesis, whereas oncogene amplification may provide additional growth and survival advantages for high-grade gliomas during the latter stages of tumorigenesis. When the complete panel of proteins is considered, the average number of expression anomalies was higher in GBM *versus* non-GBM tumors (4.107 *versus* 2.6053; $P < 0.0001$), a result that may reflect increased genomic instability in the higher grade tumors (Fig. 3).

Interrelationship of Protein Expression Anomalies in Gliomagenesis. The data set was analyzed for significant associations among patterns of alterations in protein expression (Table 4). This revealed an inverse correlation between overexpression of EGFR and the loss of wild-type p53 ($P = 0.03$), when all tumors were considered (4). As expected from the close proximity of their genes, a strong and significant association was observed between the levels of p16^{INK4} and p14^{ARF} ($P < 0.0001$) expression. However, in two tumors, loss of p16^{INK4} expression was not accompanied by loss of p14^{ARF},

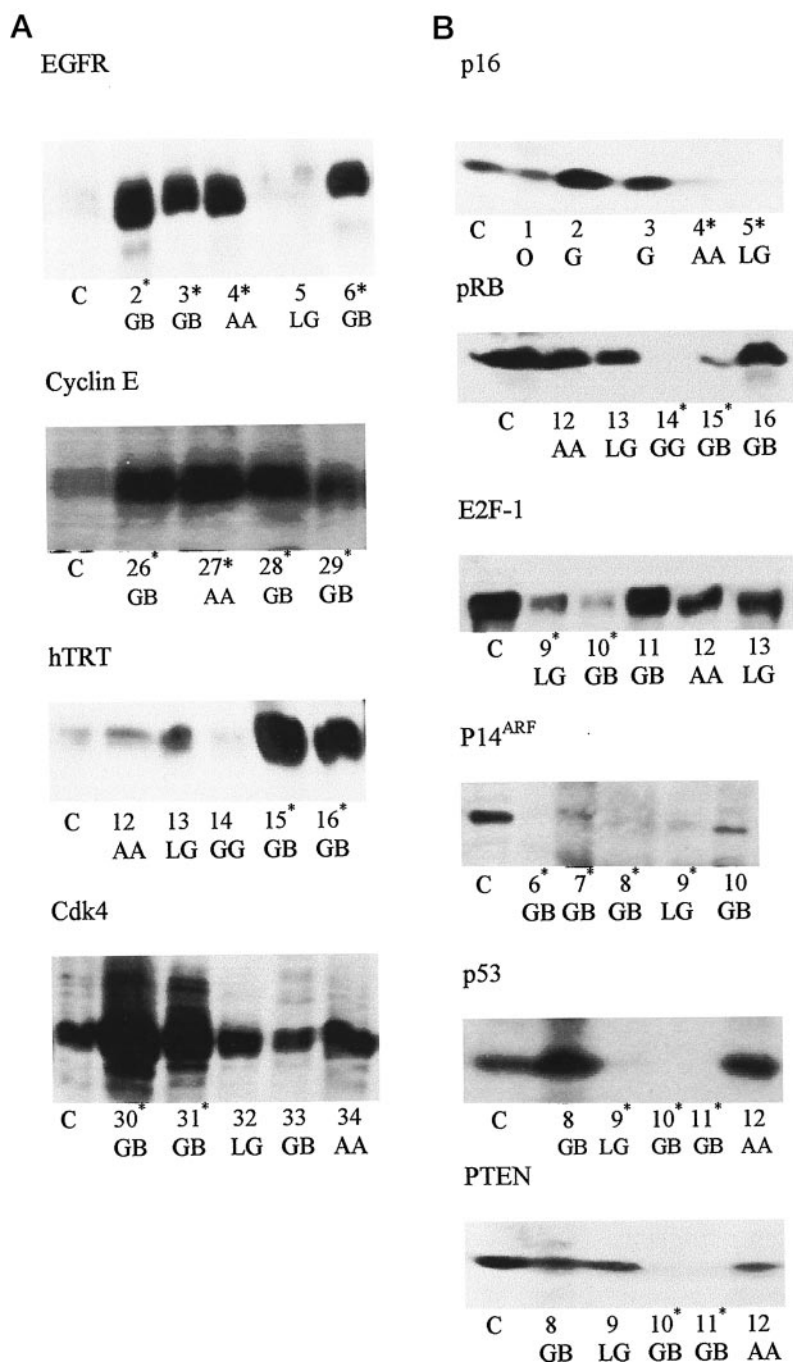


Fig. 1 A, representative Western blots of proteins commonly overexpressed in gliomas. *, lanes where overexpression was scored. Numbers at the bottom of lanes refer to patient numbers. Below the patient numbers are the histopathological diagnosis. GB, glioblastoma multiforme; AA, anaplastic astrocytoma; LG, low-grade astrocytoma; O, oligodendroglioma; GG, ganglioglioma. Each run included positive control lysates (C; first lane of each Western). Positive controls for each marker, with the exception of hTERT, represented lysates from 16-week-old fetal astrocytes. For hTERT, positive controls were obtained from lysates of an hTERT-expressing glioblastoma cell line established from a patient's tumor. Of note, the majority of cases of overexpression shown here represented glioblastomas. B, representative Western blots of proteins commonly underexpressed in gliomas. As in the top panel, patient numbers and histopathological diagnosis are provided. *, lanes demonstrating underexpression. Note that underexpression of these regulatory proteins is more evenly represented among GBM and non-GBM tumors. C, representative Western blots of proteins not commonly over- or underexpressed in gliomas. Cyclin D1 and p130 were expressed at relatively constant levels in most patient tumors.

and these may be examples in which selective methylation of the p16^{INK4} promoter has occurred (26). No other combinations of changes were found that were statistically significant, either among any given histopathological variant of glioma or for the tumors as a whole.

Surprisingly, these tumors showed no significant mutual exclusivity between underexpression of p16^{INK4} and pRB ($P = 0.52$) or between the lack of wild-type p53 and p14^{ARF} deficiency ($P = 0.53$), as has been suggested previously (12, 27). In

some tumors, these genes are mutated in a mutually exclusive manner, consistent with the idea that they form linear pathways (12, 27, 28). However, in this set of tumors, 16 tumors showed underexpression of both p16^{INK4} and pRB, whereas 22 tumors had deficits in both p53 and p14^{ARF}. Mutations of both p16^{INK4} and pRB or of mutation of p53 and p14^{ARF} in tumors have been reported, and these results are consistent with recent evidence that p16^{INK4} can have functions that are not mediated through pRB, and that p14^{ARF} can act independently of p53 (26, 29–33).

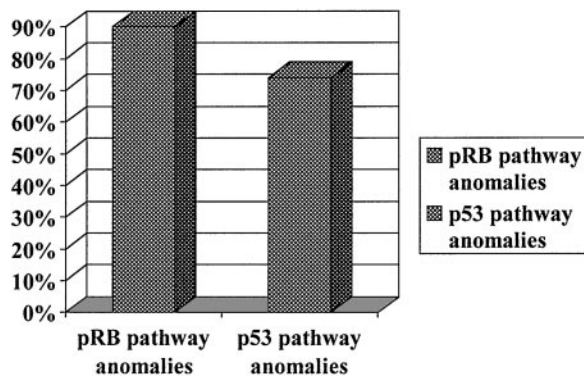


Fig. 2 pRB and p53 pathway anomalies are frequent events in gliomas. The columns demonstrate the percentage of gliomas with pRB pathway (underexpression of pRB, p16, E2F-1, and/or amplification of cyclin D1, cyclin E, and cdk4) and p53 pathway (lack of wild-type p53 and/or p14^{ARF} underexpression) anomalies.

Table 3 Frequency of protein expression in GBM versus non-GBM tumors

A. Frequency of underexpression of proteins in GBM vs. non-GBM tumors			
Underexpressed protein	Frequency of underexpression in GBM tumors	Frequency of underexpression in non-GBM tumors	P
pRB	34% (19/56)	37% (14/38)	NS
p16	63% (35/56)	42% (16/38)	0.06
p14 ^{ARF}	63% (35/56)	37% (14/38)	0.02
p130	0% (0/56)	0% (0/38)	NS
E2F-1	61% (34/56)	47% (18/38)	NS
Lack of wild-type p53	38% (21/56)	66% (25/38)	0.01
PTEN	18% (10/56)	18% (7/38)	NS ^a
B. Frequency of overexpression of proteins in GBM vs. non-GBM tumors			
Overexpressed protein	Frequency of overexpression in GBM tumors	Frequency of overexpression in non-GBM tumors	P
EGFR	41% (23/56)	3% (1/38)	<0.0001
Cyclin E	38% (21/56)	8% (3/38)	0.0014
cdk4	25% (14/56)	3% (1/38)	0.0035
hTRT	30% (17/56)	0% (0/38)	<0.0001
Cyclin D1	2% (1/56)	0% (0/38)	NS
C. Frequency of overexpression of proteins in combination with tumor suppressor deficits in GBM vs. non-GBM tumors			
GBM	Non-GBM	P	
73% (41/56)	11% (4/38)	<0.0001	

^a NS, not significant.

Prognostic Information

When the tumor suppressor proteins were examined by univariate analysis, only p14^{ARF} expression had significant prognostic value with low or no expression of p14^{ARF} predicting for reduced survival ($P = 0.04$). This correlation is seen for gliomas as a whole but is not significant when GBMs are considered alone. Deficient expression of p16^{INK4}, pRB, E2F-1,

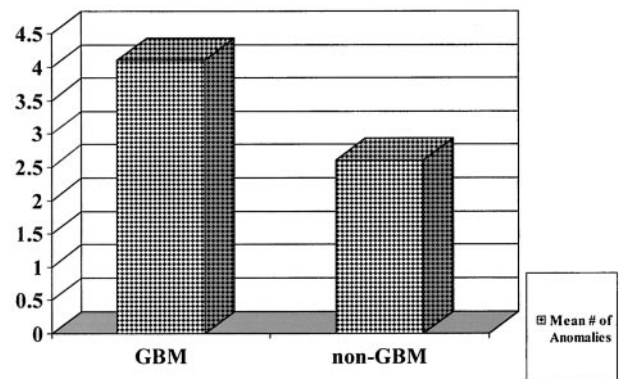


Fig. 3 GBMs demonstrate a greater number of protein expression anomalies than their non-GBM counterparts.

Table 4 Significant and notable non-significant associations among protein expression anomalies

Association	Significance
Overexpression of EGFR and loss of wild-type p53	$P = 0.03$
Underexpression of p14 ^{ARF} and p16	$P < 0.0001$
Mutual exclusivity of underexpression of both pRB and p16	$P = 0.52$
Mutual exclusivity of lack of wild-type p53 and underexpression of p14 ^{ARF}	$P = 0.53$

p53, or PTEN tumor suppressors showed no significant correlation with patient survival. Interestingly, overexpression of cyclin D1 was seen in only 1 patient and does not appear to be a major factor in gliomagenesis.

Overexpression of either EGFR, cyclin E, cdk4, or hTRT was strongly associated with aggressive tumors. Overexpression of any one of these proteins predicted for worse survival on univariate analysis, as did advanced age ($P < 0.0001$). Fig. 4A illustrates the actuarial survival of patients with tumors overexpressing one or more of these four oncogenes compared with all other tumors. Multivariate analysis (including all significant variables on univariate analysis) indicates that overexpression of EGFR ($P < 0.0001$), cyclin E ($P = 0.0042$), cdk4 ($P = 0.0085$), and hTRT ($P < 0.0001$) correlates with reduced survival. Advanced age did not reach significance in the multivariate model.

Although survival is quite limited for most GBM patients, the molecular profiles were still able to distinguish the more treatment-refractory, aggressive tumors from those that run a more indolent course. GBMs that had overexpression of either EGFR, cyclin E, cdk4, or hTRT, in combination with underexpression of one or more tumor suppressor proteins had reduced median survivals of 8 months, compared with 26.5 months for tumors that lacked such features ($P < 0.0001$). Fig. 4B illustrates actuarial survival of GBM patients with tumors displaying overexpression of one or more oncoproteins compared with GBMs without overexpression. The overexpressed proteins that were associated with reduced survival for GBMs were EGFR ($P = 0.0011$), cdk4 ($P < 0.0001$), and hTRT ($P < 0.0001$).

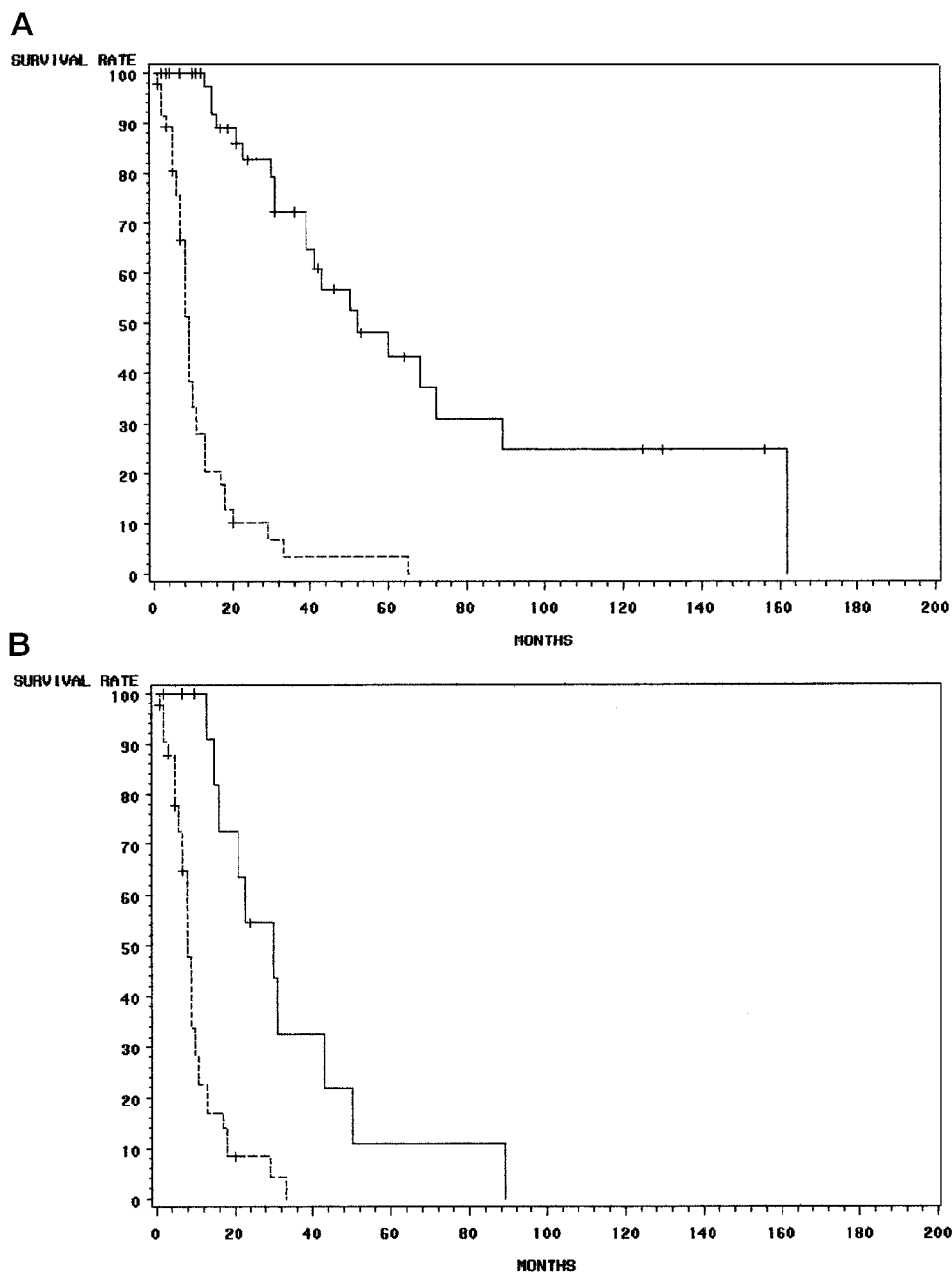


Fig. 4 A, overexpression of EGFR, hTRT, cyclin E, and/or cdk4 is associated with reduced survival for glioma patients taken as a whole. This figure demonstrates actuarial survival of all glioma patients with overexpression of either EGFR, hTRT, cyclin E, or cdk4 (----) compared with glioma patients without overexpression of these proteins (——). B, overexpression of either EGFR, hTRT, cyclin E, or cdk4 is associated with reduced survivals for GBM patients. This figure demonstrates actuarial survival of GBM patients with overexpression of either EGFR, hTRT, cyclin E, or cdk4 (----) compared with GBM patients without overexpression of these proteins (——).

Cyclin E amplification was of borderline significance ($P = 0.088$) in predicting survival among GBM patients.

DISCUSSION

The results of this study highlight four important points:

(a) The results obtained by quantitative Western analysis of protein levels in carefully preserved glioma specimens are, for the most part, consistent with previous DNA-based studies with a few exceptions. For example, consistencies include the observation abnormal levels of key cell cycle regulators are found in almost all tumors, which supports the idea that deregulation of the cell cycle may be an obligatory event for gliomagenesis.

Previous DNA-based studies have found allelic loss of 13q at the *RB* gene, *RB* gene deletions, and *RB* gene mutations in ~30% of gliomas (28, 34). In this study, we observed that a similar percentage of tumors, 35%, had either no detectable wild-type protein or had levels that were at least 5-fold lower than wild-type. In a similar way, the frequency of p16 underexpression detected in this protein analysis (56%) closely matches the rate of homozygous deletion of the *CDKN2A* locus reported in previous DNA-based studies (13, 28, 35). In this study, 89% of gliomas had deficiencies of one or more tumor suppressor genes in the pRB pathway, and 22% had overexpression of one or more oncogenes in the pRB pathway. Other

relationships such as the significant association between loss of both p16 and p14^{ARF} and the inverse relationship of EGFR amplification and p53 mutation/loss were also consistent with previous reports at the genomic level.

The results also contained a few differences with DNA-based studies. We observed tumors that had low levels of both pRB and p16 or that had low levels of p14^{ARF}, together with wild-type p53. Previous reports of genomic changes in gliomas had suggested that inactivation of these genes is mutually exclusive (4). However, there is evidence that these proteins have certain functions that are independent from one another. It is possible that in some situations, deficiencies in both (loss of both pRB and p16 protein expression and loss of both p53 and p14^{ARF}) may be advantageous for gliomagenesis (24, 27–31).

(b) The expression patterns detected in this study illustrate the high degree of heterogeneity between gliomas. In this study, we examined just 12 proteins that are known to play important roles in cell cycle control, apoptosis, cell immortalization, and cell proliferation. These products have been shown to be causally involved in tumorigenesis, and it seems likely therefore that drastic changes in the activity of these proteins will have a significant impact on the properties of the tumor cells. It is striking that the levels of these key regulators were altered in many different permutations in this collection of tumors, with the most aggressive tumors containing the largest number of changes. Lower grade tumors, such as oligodendrogliomas, are reported to demonstrate infrequent microsatellite instability and generally fewer genomic alterations than their higher grade counterparts (36). Potentially, the changes in expression may be markers of genome instability, with the most aggressive tumors being the most unstable. Alternatively, it is possible that gliomas may arise through multiple different routes, with different combinations of changes driving the tumorigenic process in different tumors.

(c) Only a small subset of these 12 proteins are useful for distinguishing between GBMs and non-GBMs. In this regard, the overexpression of proteins, such as EGFR, hTERT, cdk4, and cyclin E, is far more informative than the loss of expression of well-studied tumor suppressors such as pRB, p16, and p53. Overexpression of EGFR was observed in 23 of 56 GBMs (41%) compared with only 1 of 38 non-GBM tumors (2.6%; $P < 0.0001$). This finding is highly consistent with the frequency of *EGFR* gene amplification in GBMs versus non-GBMs from previous reports (4, 37). In the present series, there appeared to be an inverse correlation of EGFR overexpression with loss of wild-type p53 protein ($P = 0.03$), but no significant association was found between EGFR overexpression and the underexpression of p14^{ARF}. Overexpression of hTERT, the catalytic component of telomerase, was present in 17 of 56 (30%) GBMs, compared with 0 of 38 non-GBM tumors ($P < 0.0001$). Overexpression of cyclin E was found in 21 of 56 GBMs (38%) versus only 2 of 38 (5%) non-GBMs ($P < 0.0001$). Overexpression of cyclin E is a frequent event in breast tumors but has not been reported previously for gliomas (38–40). Overexpression of cdk4 protein expression was evident in 13 of 56 GBMs (23%) compared with only 1 of 38 (3%) non-GBMs ($P < 0.0001$). This frequency is similar to the 15% *cdk4* gene amplification rate reported previously (4). When these four markers are considered together, overexpression of as EGFR, hTERT,

cdk4, or cyclin E was observed in 73% of GBM versus 13% of non-GBM tumors ($P < 0.0001$).

(d) Perhaps most importantly, the expression levels of 5 of these 12 markers provided clear prognostic information. Increased levels of EGFR, cyclin E, cdk4, or hTERT in gliomas are strong predictors of poor survival ($P < 0.0001$). Of all of the tumor suppressor genes examined, only underexpression of p14^{ARF} showed a clear correlation with poor survival ($P = 0.03$). Previous studies have disagreed over the prognostic value of *EGFR* amplification (37, 41, 42). One study suggests that *EGFR* gene amplification is not an important prognostic indicator (37), but two studies that examined EGFR immunoreactivity on the protein level suggest that elevated levels of EGFR is prognostically significant (41, 42). Our study provides further evidence that the overexpression of EGFR protein strongly correlates with reduced survival in glioma patients and underscores the importance of complementing genomic data with protein/gene expression data. Likewise, there has been conflicting data on the prognostic significance of telomerase activity with clinical outcome. Several studies using telomeric repeat amplification protocol assays in determining telomerase activity failed to demonstrate enhanced telomerase activity correlating with higher tumor grade or outcome (43–45). Other studies suggest that enhanced telomerase activity as determined by telomeric repeat amplification protocol correlates with enhanced malignant potential of gliomas (46–48). The results described here indicate that the levels of hTERT expression serve as an informative marker, and that high levels of hTERT strongly correlate with reduced patient survival.

In summary, these data suggest that expression levels of cell cycle/signal transduction proteins can provide valuable insights into the molecular pathogenesis and clinical behavior of gliomas. Protein expression anomalies were present in 92 of the 94 glioma specimens (98%) in this series, indicating that cell cycle/signaling anomalies at some level are obligatory in gliomagenesis. Because GBMs are routinely managed by adjuvant therapy (e.g., radiation and chemotherapy), the observation that increased levels of EGFR, cdk4, hTERT, and cyclin E strongly correlate with reduced survival may suggest that these proteins are associated with resistance to DNA-damaging agents. Further investigations are needed to better understand how these oncogene amplifications are related to specific upstream signal transduction pathways and how they can be manipulated to influence the natural history of the disease and treatment outcome.

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