Published in final edited form as:

Am J Surg Pathol. 2012 August ; 36(8): 1186–1193. doi:10.1097/PAS.0b013e3182518e12.

Paradoxical relationship between degree of EGFR amplification and outcome in glioblastomas

Jonathan Hobbs, B.S.¹, Marina N. Nikiforova, M.D.², David W. Fardo, Ph.D.³, Stephanie Bortoluzzi, B.S.², Kathleen Cieply, M.S.L.², Ronald L. Hamilton, M.D.², and Craig Horbinski, M.D., Ph.D.¹

¹Department of Pathology, University of Kentucky, 800 Rose Street, Lexington, KY 40536

²Department of Pathology, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA 15213

³Department of Biostatistics, University of Kentucky, 800 Rose Street, Lexington, KY 40536

Abstract

Glioblastoma (GBM) is the most common primary brain tumor in adults and often has amplification of the *Epidermal Growth Factor Receptor (EGFR)* gene. The value of *EGFR* as a prognostic marker in GBMs is unclear; some studies have shown an adverse correlation, while others indicated a neutral or even favorable association with longer survival. Furthermore, *EGFR*-amplified GBMs are usually regarded as a single subgroup of tumors, though the range of *EGFR* copy number varies greatly. In this study, 532 glioblastomas were analyzed for *EGFR* amplification via fluorescence in situ hybridization at the time of initial diagnosis. While there was no difference in survival by *EGFR* amplification ($P = 0.33$), stratification by amount of *EGFR* amplification showed that, surprisingly, median survival was 39% longer in the high-amplifier group (*EGFR*: chromosome 7 ratio > 20) versus non-amplified GBMs ($P = 0.03$), and 43% longer versus GBMs with low-to-moderate *EGFR* amplification (*EGFR*: chromosome 7 ratio = 2–20, $P = 0.0007$). Stratifying by postsurgical treatment regimens, this difference was seen only when temozolomide (TMZ) was used; tumors without amplification and high *EGFR* amplification both responded better to TMZ than those with low-to-moderate amplification ($P = 0.01$), whereas those without adjuvant therapy or adjuvant therapy without TMZ showed no survival differences ($P = 0.63$ and 0.91 , respectively). These results suggest that glioblastomas with *EGFR* amplification are a heterogeneous group of tumors, and that behavior might differ according to degree of amplification, but not in a straightforward dose-response manner.

Keywords

EGFR; amplification; temozolomide; glioblastoma

Corresponding author: Craig Horbinski, M.D., Ph.D., MS-155, 800 Rose Street, Lexington, KY 40536, Phone 859-257-1937, Fax 859-323-2094, craig.horbinski@uky.edu.

Conflicts of Interest None of the authors have any conflicts of interest to declare.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase on chromosome 7p12 whose downstream signaling pathways modulate a wide range of cellular activities, including growth, migration, and survival (22). Since *EGFR* is a powerful oncogene, it is not surprising that neoplasms have developed multiple ways of enhancing its activity. Such strategies include upregulation of EGFR protein expression, inhibition/deletion of downstream pathway inhibitors, constitutively active EGFR (EGFRvIII), and *EGFR* amplification (1, 30), the latter producing an excess of *EGFR* genes in the tumor cell.

Amplification of *EGFR* (or other receptor tyrosine kinases) is a frequent event in many cancers, including those of the lung, breast, head and neck, gastrointestinal tract, and brain. In the brain, *EGFR* amplification is a hallmark of glioblastoma (GBM), the most common and lethal primary brain tumor in the adult population (23-25). About 40% of all GBMs have *EGFR* amplification, specifically those that arise *de novo* as GBMs. In contrast, GBMs that develop from lower-grade gliomas are much less likely to have *EGFR* amplification (4, 23-25).

In GBMs, EGFR signaling promotes cell division, tumor invasiveness, and resistance to radiation and chemotherapy (7, 10, 16, 20). A great deal of effort has thus been invested in developing targeted anti-EGFR therapies, with limited success thus far (6, 21, 28). Furthermore, multiple reports on the value of *EGFR* amplification as a prognostic marker have yielded conflicting results, with some studies showing no association with survival, others with negative impact, and some even suggesting a favorable impact on patient survival (2, 3, 13, 14, 17, 29, 30).

In such *EGFR*-based studies, the cutoff for amplification is generally at 2 or more copies of *EGFR* per copy of chromosome 7. Such a determination is usually made via fluorescence in situ hybridization (FISH), which uses fluorophore-labeled DNA probes to determine *EGFR* copy number and chromosome 7 (chr 7) ploidy. Yet an *EGFR*:chr 7 ratio of 2 or more encompasses a wide range of ratios, all of which are routinely pooled together when studying *EGFR*-amplified gliomas.

Herein we describe a prospective cohort of 532 GBMs, all of which had *EGFR* FISH performed at the time of initial diagnosis. Our results suggest that GBM behavior and response to therapy differs according to the degree of *EGFR* amplification, but not in a linear manner.

Materials and Methods

Cohort

Biopsy materials from 532 gliomas in patients 18-89 years were analyzed at the time of diagnosis for *EGFR* amplification at the University of Pittsburgh Medical Center from 2002-2010. Many also had EGFR expression, *MGMT* promoter methylation, and 10q loss of heterozygosity (LOH) analyses done (see below). Cases of recurrent or treated gliomas were excluded. Diagnoses were rendered according to standard World Health Organization (WHO) criteria (19). Clinical variables, including survival from the time of initial surgery, were obtained via the University of Pittsburgh Hillman Cancer Registry. All outcomes-based analyses were done according to University of Kentucky and University of Pittsburgh Institutional Review Board guidelines.

Fluorescence in situ hybridization

FISH for *EGFR* was performed as previously described (11). Briefly, 5- μ m sections from formalin-fixed, paraffin-embedded (FFPE) tissues were deparaffinized in xylene twice for 10 minutes, dehydrated twice with 100% ethanol, and then pretreated using the Vysis Paraffin Pretreatment Kit (Abbott Molecular, Des Plaines, IL). Slides were digested for 18 minutes in protease solution (0.5 mg/ml) at 37 °C. FISH was performed using probes for *EGFR* (7p12)/centromeric enumeration probe for chromosome 7 (CEP7) (Abbott Molecular, Des Plaines, IL). The target slide and probe were co-denatured at 95 °C for 8 minutes and incubated overnight at 37 °C in a humidified chamber. Post-hybridization washes were performed using 2XSSC/0.3% Igepal (Sigma, St. Louis, MO) at 72 °C for 2 minutes. Slides were air-dried in the dark and counterstained with DAPI (Abbott). Analysis was performed using a Nikon Optiphot-2 (Nikon, Inc, Melville, NY) and Quips Genetic Workstation equipped with Chroma Technology filter set with single band excitors for SpectrumOrange, FITC, and DAPI (uv 360 nm). In each case a minimum of 60 tumor cells were scored; *EGFR* amplification was defined as *EGFR*:CEP7 ratio ≥ 2.0 .

MGMT promoter methylation assay

DNA from FFPE-tissues was converted via sodium bisulfate reaction (EpiTect Bisulfite Kit, Qiagen, Valencia, CA). Real-time quantitative polymerase chain reaction (PCR) was done using bisulfite-specific primers for *MGMT* and *COL2A1* (internal control) using ABI 7900 instrument (Applied Biosystems, Carlsbad, CA). Methylation index (MI) was reported as previously described (31); tumors were considered negative for methylation when the MI was 0, low when the MI was between 0 and 4, and high when the MI was greater than 4. In addition, methylation specific PCR followed by agarose gel electrophoresis was performed as previously described with minor modifications (31) to confirm presence or absence of methylated products.

10q loss of heterozygosity (LOH) analysis

PCR-based LOH analysis on 10q was performed as previously described (11). DNA was isolated from FFPE tissues and PCR was done on 2 microsatellite markers on 10q23 (D10S520, D10S1173). PCR products were analyzed using capillary gel electrophoresis on GeneMapper ABI 3730 (Foster City, CA). Peak height ratios falling outside of 2 standard deviations beyond the mean of previously validated normal values were assessed as showing loss of heterozygosity. In the present study, LOH on either or both markers was scored as “positive” for 10q LOH.

EGFR immunohistochemistry

EGFR immunohistochemistry (IHC) was performed on FFPE tissue sections using EGFR primary antibody (Ventana 790-2988 / 3C6 / prediluted). Antibody labeling used the avidin-biotin complex method and was visualized with a horseradish peroxidase enzyme label and 2'-diaminobenzamide (DAB, Dako, Carpinteria, CA) as the substrate chromogen (brown).

EGFR IHC analysis was based on a semiquantitative scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), as per our previously published results which showed a direct correlation between this scale and the likelihood of *EGFR* amplification (12). Score was predicated on the strongest area of the tumor.

TCGA data

Affymetrix Genome-Wide Human SNP Array 6.0 *EGFR*, *LANCL2*, and *ECOP* copy number data on 372 GBMs was downloaded from The Cancer Genome Atlas (TCGA) Data Portal (<http://tcga-portal.nci.nih.gov/tcga-portal/AnomalySearch.jsp>). Either gene was

considered amplified when the normalized log₂ ratio ≥ 1.0 . Protected survival information was downloaded from the TCGA website after obtaining NCI approval.

Statistical analyses

Survival rates were compared via log-rank tests on Kaplan-Meier curves. The optimal survival-based cutoff between degrees of *EGFR* amplification was determined by log-rank testing (Figure 1); this analysis showed an optimal *EGFR*:*CEP7* ratio cutoff at 21.0, but because there was no substantial difference between 21.0 and 20.0, the latter was adopted for the sake of simplicity. This also corroborated our longstanding experiential observation that FISH ratio scores become less precise once the ratio exceeds 20.

Comparisons between *EGFR* amplification subgroups or *EGFR* expression categories were performed via Kruskal-Wallis with Dunn's post hoc test. Correlation between patient age versus *EGFR*:*CEP7* ratio, or *EGFR* amplification with WHO grade, was assessed via linear regression. Multivariate Cox proportional hazards regression was used to model survival. All the aforementioned analyses were done using GraphPad software (GraphPad Software, Inc., La Jolla, CA) and R statistical software (survival package) [<http://www.R-project.org>]; differences were considered significant if $P < 0.05$.

Results

Cohort characteristics

A total of 532 cases were prospectively analyzed, including 221 females and 311 males, with a median age of 63 years (range: 18-89 years). 452 patients (85.0%) were deceased at the time of analysis, with a median survival of 6.8 months (range: 0.1-75.7 months). Of the 80 still alive at the time of analysis, median followup interval was 19.1 months (range: 8.1-74.6 months). In 484 GBMs, tumor location (but not laterality) was known. 143 (26.9%) were in a frontal lobe; 133 (27.5%) were in a temporal lobe; 85 (17.6%) were in a parietal lobe; 17 (3.5%) were in an occipital lobe; 59 (12.2%) were in multiple lobes; 37 (7.6%) were in deep-seated regions of the cerebrum (e.g. basal ganglia, thalamus); 4 (0.8%) were in the brainstem; 3 (0.6%) were in the cerebellum; 3 (0.6%) were in the ventricular system.

Of the 495 cases in which surgery type was known, 342 (69.1%) underwent gross total or subtotal tumor resection, whereas 153 (30.9%) were only biopsied. Postoperative treatment data was obtained in 465 patients (87.4%). Of those, 218 (46.9%) received temozolomide (TMZ) and radiation therapy (RT); 87 (18.7%) received TMZ, RT, and some other chemotherapy (e.g. carmustine); 54 (11.6%) received RT alone; 16 (3.4%) received RT plus non-TMZ chemotherapy; 7 (1.5%) received TMZ alone; 2 (0.4%) received only non-TMZ chemotherapy; 81 (17.4%) received no adjuvant therapy.

Survival according to key clinical and molecular factors in GBMs

As expected, younger patients had significantly longer survival, as did patients undergoing tumor resection versus biopsy alone (Table 1). Regarding adjuvant therapy, the best median survival was 12.7 months in patients whose tumors were treated with TMZ (69% in conjunction with RT; 27.9% in conjunction with RT and other chemotherapy; 2.2% by TMZ alone). Those with adjuvant therapy not including TMZ (75% RT alone; 22.2% RT plus other chemotherapy; 2.8% chemotherapy alone) had the next best median survival at 6.7 months, followed by those receiving no adjuvant therapy at all (2.0 months).

Factors showing no significant survival differences on univariate analyses included gender, 10q status, *EGFR* amplification, and *EGFR* expression (Table 1). No significant difference

was seen in TMZ-treated GBMs by *MGMT* promoter methylation status (Supplemental Figure 1), though the total N for this subset was only 106 cases.

Stratification according to EGFR:CEP7 ratio alters prognosis in EGFR-amplified GBMs

FISH produces *EGFR* copy number results in the form of a ratio, using a centromeric enumeration probe for chromosome 7 (CEP7) as a ploidy control. To determine whether GBMs with differing *EGFR*:CEP7 ratios also showed different behavior, Kaplan-Meier survival curves were compared by grouping cases into three *EGFR*:CEP7 ratio bins (see Methods and Figure 1). The three subgroups included 301 cases (56.6%) without amplification (*EGFR*:CEP7 < 2); 71 tumors (13.3%) with low-to-moderate levels of amplification (*EGFR*:CEP7 = 2-20); and 160 (30.0%) with high-level amplification (*EGFR*:CEP7 > 20) (Figure 2A-C). Remarkably, GBMs with high-level amplification had a median survival of 11.0 months, compared to 7.9 months for non-amplified GBMs and 7.7 months for low-to-moderate amplifiers ($P = 0.006$) (Figure 2D). In other words, median survival was 39% longer in the high-amplifier group versus non-amplified GBMs ($P = 0.03$) and 43% longer versus low-to-moderately amplified GBMs ($P = 0.0007$).

EGFR amplification subgroups according to key clinical and molecular variables

Among the three *EGFR* subgroups (not amplified, low-moderate amplification, and high amplification) there was no significant difference in patient age, gender, or surgery type (Table 2). The proportion of non-amplified, low-to-moderate, and high-amplified tumors also did not significantly vary by primary tumor location ($P = 0.24, 0.44$, and 0.21 , respectively). Non-amplified GBMs had significantly lower *EGFR* expression than the amplified subgroups and a significantly lower rate of 10q LOH compared to high-amplifiers, but not compared to low-moderate amplifiers. Interestingly, there was also a significant difference in the treatment groups between non-amplified and high-amplified GBMs, wherein the former had a higher rate of no adjuvant therapy while the latter had a higher rate of TMZ treatment. In contrast, none of the variables significantly differed between the low-moderate amplifiers and the high amplifiers. There was also no significant difference between non-amplified GBMs and low-moderate amplified GBMs according to treatment or 10q LOH. In the subset with *MGMT* promoter methylation data, there was no difference in methylation frequency between the *EGFR* subgroups.

Response to temozolomide differs according to the degree of EGFR amplification

Patient survival was stratified according to three main postsurgical treatment subsets: those that received TMZ as part of the adjuvant therapeutic regimen, those receiving adjuvant therapies without TMZ, and patients who received no adjuvant therapy at all (see Table 1). Survival was equally poor among the three *EGFR* subgroups when no adjuvant therapy was implemented; median survival in non-amplified GBMs was 2.3 months ($N = 56$), 1.9 months in low-to-moderate amplified tumors ($N = 9$), and 1.1 months in tumors with high *EGFR* amplification ($N = 17$) (overall $P = 0.63$, Figure 3A).

Compared to untreated cases, patients exposed to adjuvant therapy but without TMZ showed longer median survivals in all three *EGFR* subsets (6.0 months for non-amplified tumors, $N = 43$; 7.3 months for low-to-moderate amplification, $N = 9$; 6.7 months for high-amplifiers, $N = 19$), but again there was no significant differences between the subgroups ($P = 0.91$, Figure 3B).

Most cases in the cohort included TMZ as part of the postsurgical therapy. Under such treatment conditions, longer median survival was seen in both non-amplified GBMs (12.7 months, $N = 162$) and high-amplified GBMs (15.3 months, $N = 110$) compared to GBMs with low-to-moderate amplification (9.1 months, $N = 40$) (overall $P = 0.01$, Figure 3C). The

difference between the non-amplified and high-amplified groups was not significant ($P = 0.35$). In the low-to-moderate *EGFR*-amplified GBMs, median survival was only 25% higher in the TMZ-treated cases compared to those without TMZ, and this difference was not significant ($P = 0.55$). On the other hand, non-amplified GBMs treated with TMZ had a 2.1-fold longer median survival than non-amplified tumors without TMZ ($P < 0.0001$); likewise, high amplifiers had a 2.3-fold longer median survival than high amplifiers without TMZ ($P = 0.003$).

Multivariate analyses

All variables that were significant on univariate analyses (Table 1) were also shown to be independent prognostic factors (Table 3). The presence of low-to-moderate *EGFR* amplification was also found to be an independent adverse prognostic variable (hazard ratio = 1.42, 95% CI = 1.07-1.89, $P = 0.017$). This association was persistent in the subset of cases that were treated with TMZ (hazard ratio = 1.56, 95% CI 1.10-2.22, $P = 0.013$). Neither the presence of high-level *EGFR* amplification (hazard ratio = 0.85, $P = 0.15$) nor the absence of amplification (hazard ratio = 0.99, $P = 0.91$) were independent prognostic markers in similar models (not shown).

Discussion

EGFR is associated with WHO grade IV GBMs and is well-studied, yet our understanding of the mechanisms of amplification and its impact on tumor cell biology is incomplete. Originally, it was anticipated that higher levels of *EGFR* amplification in this cohort might correlate with poorer response to adjuvant therapy and thus have the worst outcomes. Yet the relationship between *EGFR* amplification and outcome was paradoxical, such that lower levels of amplification correlated with worse response to TMZ-containing adjuvant therapeutic regimens compared to GBMs with high amplification or none at all. Since amplification of *EGFR* and other receptor tyrosine kinases is seen in a variety of cancers, this phenomenon may apply to a broader range of neoplasms beyond the brain.

These results may help explain the incongruities seen in other studies evaluating *EGFR* amplification as a glioma biomarker. Some have shown *EGFR* amplification to be a negative prognostic marker in GBMs (14, 29, 30), but others have not (3, 15, 17); some have even suggested it could be a favorable marker, especially in older patients (2, 13, 29). In our large cohort, pooling all *EGFR*-amplified GBMs together and comparing them to non-amplified GBMs showed no difference in survival (Table 1). It was only when the *EGFR*-amplified tumors were stratified according to degree of amplification that significant survival differences were unmasked (Figure 2). Further unmasking was done via subgroup analyses according to postsurgical adjuvant therapy, which suggested that there was a survival difference only if TMZ was part of the regimen, and that low-to-moderately-amplified GBMs did not respond as well to TMZ (Figure 3). Thus, it is possible that the discrepancies between previous cohorts may have been due to varying proportions of these subgroups of *EGFR*-amplified GBMs as well as to treatment confounders. Since only 16 cases in the entire cohort were treated with other chemotherapies but without any TMZ, it was difficult to determine whether the adverse effects of low-to-moderate *EGFR* amplification might be exclusive to TMZ or also be a feature of other chemotherapies. Still, low-to-moderate *EGFR* amplification was an independent adverse prognostic factor in a model with TMZ versus no TMZ, as well as a model focusing only on TMZ-treated cases (Table 3).

The significantly better survival seen in GBMs with high *EGFR* amplification compared to non-amplified tumors (Figure 2) may have been due to nonrandom enrichment of the latter group with GBMs not exposed to adjuvant treatment, with a concomitant reduction in the

relative proportion of non-amplified cases treated with TMZ (Table 2). Why this happened is not clear, since 94% of all cases were obtained from 2005 and later when TMZ was standard-of-care in GBM treatment. When reasons for withholding TMZ were retrievable, the most common were adverse systemic reactions, poor overall patient health, voluntary patient/family decisions, and inability to cover treatment costs. None of these intuitively correlate with the presence or degree of *EGFR* amplification. Therefore, our data as a whole suggest that high *EGFR* amplification might not be a favorable marker per se, but rather that low-to-moderate amplification is an adverse marker, especially in TMZ-treated cases. Other well-known biomarkers of GBM outcome appeared to be evenly distributed between non-amplified, low-to-moderate, and high-amplified GBMs. Although advanced patient age is a powerful adverse prognostic marker (25), there was no difference in patient age, gender, or surgery between non-amplified, low-to-moderate, and high-level *EGFR*-amplified GBMs (Table 2). As expected, *EGFR* expression and frequency of 10q LOH were higher in the amplified versus non-amplified tumors, but there was no significant difference between the low-to-moderate and high amplifier subsets in any of the measured parameters. *MGMT* promoter methylation showed no significant difference between amplification subtypes (Table 2), so while the number and followup interval of methylation-tested cases was not powerful enough for univariate and multivariate survival analyses, it is unlikely to be a confounding variable.

Although our understanding of gene amplification is rudimentary, some mechanistic insights have been discovered that allow for testable hypotheses. It was recently demonstrated that, of GBMs with *EGFR* amplification, those with lower *EGFR* copy numbers tended to feature interstitial amplification, wherein all the *EGFR* genes were located exclusively within chromosome 7. In contrast, *EGFR* in GBMs with higher copy numbers were mostly located in fragments of extrachromosomal DNA called double minutes (18). Double minutes have been known to exist in a variety of cancers, arising through defects in DNA reproduction and faulty repair machinery (9, 32). Perhaps GBMs with high levels of *EGFR* simply have more fragile genomes, thus responding better to DNA-damaging TMZ and offsetting the otherwise deleterious effects of *EGFR* amplification. Indeed, other recent work has suggested that high chromosomal fragility can actually be a favorable prognostic marker in various carcinomas (5) and even in other brain tumors like ependymomas (33).

Since the size of the 7p12 amplicon varies, other nearby genes are often co-amplified with *EGFR* and could impact tumor biology. *LANCL2*, for example, is co-amplified and over-expressed in about 50% of *EGFR*-amplified GBMs (8). *LANCL2* encodes Lanthionine Synthetase C-like 2, which may promote sensitivity to chemotherapy (27). About one-third of *EGFR*-amplified GBMs also co-amplify *ECOP*, encoding *EGFR*-Coamplified and Overexpressed Protein, which upregulates the antiapoptotic activity of NF- κ B (26). The FISH probe used in this cohort for clinical testing covers the entire *EGFR* gene and extends beyond its 3' centromeric end, but does not reach *LANCL2* or *ECOP* (Supplemental Figure 2). However, analysis of TCGA GBMs showed no difference in survival by co-amplification of *LANCL2* (Supplemental Figure 3) or by co-amplification of *ECOP* ($P=0.61$, not shown).

Studying additional retrospective cohorts will be of high interest given the nature of these findings. Analysis of TCGA GBMs weakly trended toward a similar paradoxical relationship between degree of amplification and outcome (Supplemental Figure 4), though there are several possible reasons why a stronger relationship was not found. First, the TCGA cohort is 30% smaller than the current study. Second, the TCGA dataset is inherently biased toward cases in which large resections of highly viable tumors was possible. This cohort, in contrast, studied all cases regardless of sample size and degree of necrosis, as long as at least 60 scoreable tumor cells were in the specimen. Third, copy number in the TCGA was determined by SNP arrays and reported as log2 ratios (see Methods). While in principle this

is analogous to FISH, in reality it proved very difficult to precisely extrapolate the FISH cutoffs to SNP array data. Still, the TCGA cohort at least suggests that the current cohort data is valid.

This study has key advantages of a very large cohort and prospective nature of the molecular data. Yet the survival analysis was retrospective, with all the attendant limitations thereof. Specifically, the correlation between low-to-moderate *EGFR* amplification and poorer TMZ response should be validated prospectively, and could readily be incorporated into any number of ongoing clinical trials. Nevertheless, this study provides the first large-scale evidence that the degree of *EGFR* amplification may impact the biological behavior of GBMs, though in a counterintuitive manner. This could account for conflicting results in prior outcome-based studies of *EGFR* amplification, and suggests that there might be more biologically relevant heterogeneity in *EGFR*-amplified tumors than has been previously assumed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Sanja Dacic, M.D., Ph.D., and the In Situ Hybridization Laboratory at the University of Pittsburgh Medical Center for their expertise and assistance with FISH imaging. Thanks also to Dr. Natasha Kyprianou at the University of Kentucky for her review of the manuscript and thoughtful suggestions, as well as to Sharon Winters of the University of Pittsburgh Hillman Cancer Registry for assistance with clinical data retrieval.

Sources of Funding CH was supported by a National Institute of Health K08CA155764-01A1 grant and a University of Kentucky College of Medicine Physician Scientist Fellowship.

References

1. Aldape KD, Ballman K, Furth A, et al. Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. *J Neuropathol Exp Neurol.* 2004; 63:700–707. [PubMed: 15290895]
2. Batchelor TT, Betensky RA, Esposito JM, et al. Age-dependent prognostic effects of genetic alterations in glioblastoma. *Clin Cancer Res.* 2004; 10:228–233. [PubMed: 14734474]
3. Benito R, Gil-Benso R, Quilis V, et al. Primary glioblastomas with and without EGFR amplification: relationship to genetic alterations and clinicopathological features. *Neuropathology.* 2009; 30:392–400. [PubMed: 20051017]
4. Biernat W, Tohma Y, Yonekawa Y, et al. Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastomas. *Acta Neuropathol.* 1997; 94:303–309. [PubMed: 9341929]
5. Birkbak NJ, Eklund AC, Li Q, et al. Paradoxical Relationship between Chromosomal Instability and Survival Outcome in Cancer. *Cancer Res.* 2011; 71:3447–3452. [PubMed: 21270108]
6. Brandes AA, Franceschi E, Tosoni A, et al. Epidermal growth factor receptor inhibitors in neuro-oncology: hopes and disappointments. *Clin Cancer Res.* 2008; 14:957–960. [PubMed: 18281526]
7. Chakravarti A, Chakladar A, Delaney MA, et al. The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. *Cancer Res.* 2002; 62:4307–4315. [PubMed: 12154034]
8. Eley GD, Reiter JL, Pandita A, et al. A chromosomal region 7p11.2 transcript map: its development and application to the study of EGFR amplicons in glioblastoma. *Neuro Oncol.* 2002; 4:86–94. [PubMed: 11916499]
9. Gibaud A, Vogt N, Hadj-Hamou NS, et al. Extrachromosomal amplification mechanisms in a glioma with amplified sequences from multiple chromosome loci. *Hum Mol Genet.* 2010; 19:1276–1285. [PubMed: 20056677]

10. Hatanpaa KJ, Burma S, Zhao D, et al. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia*. 2010; 12:675–684. [PubMed: 20824044]
11. Horbinski C, Hamilton RL, Nikiforov Y, et al. Association of molecular alterations, including BRAF, with biology and outcome in pilocytic astrocytomas. *Acta Neuropathol*. 2010; 119:641–649. [PubMed: 20044755]
12. Horbinski C, Hobbs J, Cieply K, et al. EGFR Expression Stratifies Oligodendroglioma Behavior. *Am J Pathol*. 2011:11.
13. Houillier C, Lejeune J, Benouaich-Amiel A, et al. Prognostic impact of molecular markers in a series of 220 primary glioblastomas. *Cancer*. 2006; 106:2218–2223. [PubMed: 16568472]
14. Korshunov A, Sycheva R, Golanov A. The prognostic relevance of molecular alterations in glioblastomas for patients age < 50 years. *Cancer*. 2005; 104:825–832. [PubMed: 15981281]
15. Layfield LJ, Willmore C, Tripp S, et al. Epidermal growth factor receptor gene amplification and protein expression in glioblastoma multiforme: prognostic significance and relationship to other prognostic factors. *Appl Immunohistochem Mol Morphol*. 2006; 14:91–96. [PubMed: 16540738]
16. Li L, Dutra A, Pak E, et al. EGFRvIII expression and PTEN loss synergistically induce chromosomal instability and glial tumors. *Neuro Oncol*. 2009; 11:9–21. [PubMed: 18812521]
17. Liu L, Backlund LM, Nilsson BR, et al. Clinical significance of EGFR amplification and the aberrant EGFRvIII transcript in conventionally treated astrocytic gliomas. *J Mol Med*. 2005; 83:917–926. [PubMed: 16133418]
18. Lopez-Gines C, Gil-Benso R, Ferrer-Luna R, et al. New pattern of EGFR amplification in glioblastoma and the relationship of gene copy number with gene expression profile. *Mod Pathol*. 2010; 23:856–865. [PubMed: 20305620]
19. Louis, DN.; Ohgaki, H.; Wiestler, OD., et al., editors. WHO Classification of Tumors of the Central Nervous System. IARC; Lyon: 2007.
20. Mazzoleni S, Politi LS, Pala M, et al. Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. *Cancer Res*. 2010; 70:7500–7513. [PubMed: 20858720]
21. Mellinger IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med*. 2005; 353:2012–2024. [PubMed: 16282176]
22. Nagane M, Coufal F, Lin H, et al. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res*. 1996; 56:5079–5086. [PubMed: 8895767]
23. Ohgaki H, Dessen P, Jourde B, et al. Genetic pathways to glioblastoma: a population-based study. *Cancer Res*. 2004; 64:6892–6899. [PubMed: 15466178]
24. Ohgaki H, Kleihues P. Genetic alterations and signaling pathways in the evolution of gliomas. *Cancer Sci*. 2009
25. Ohgaki H, Kleihues P. Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *J Neuropathol Exp Neurol*. 2005; 64:479–489. [PubMed: 15977639]
26. Park S, James CD. ECop (EGFR-coamplified and overexpressed protein), a novel protein, regulates NF-kappaB transcriptional activity and associated apoptotic response in an IkappaBalpha-dependent manner. *Oncogene*. 2005; 24:2495–2502. [PubMed: 15735698]
27. Park S, James CD. Lanthionine synthetase components C-like 2 increases cellular sensitivity to adriamycin by decreasing the expression of P-glycoprotein through a transcription-mediated mechanism. *Cancer Res*. 2003; 63:723–727. [PubMed: 12566319]
28. Prados MD, Chang SM, Butowski N, et al. Phase II study of erlotinib plus temozolomide during and after radiation therapy in patients with newly diagnosed glioblastoma multiforme or gliosarcoma. *J Clin Oncol*. 2009; 27:579–584. [PubMed: 19075262]
29. Shinojima N, Tada K, Shiraishi S, et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res*. 2003; 63:6962–6970. [PubMed: 14583498]

30. Smith JS, Tachibana I, Passe SM, et al. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. *J Natl Cancer Inst.* 2001; 93:1246–1256. [PubMed: 11504770]
31. Vlassenbroeck I, Califice S, Diserens AC, et al. Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma. *J Mol Diagn.* 2008; 10:332–337. [PubMed: 18556773]
32. Vogt N, Lefevre SH, Apiou F, et al. Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. *Proc Natl Acad Sci U S A.* 2004; 101:11368–11373. [PubMed: 15269346]
33. Witt H, Mack SC, Ryzhova M, et al. Delineation of two clinically and molecularly distinct subgroups of posterior fossa ependymoma. *Cancer Cell.* 2011; 20:143–157. [PubMed: 21840481]

Figure 1. Optimal outcome-based EGFR:CEP7 cutoff between low-moderate and high-amplified GBMs

532 GBMs with known survival times were analyzed for EGFR amplification by FISH (see Methods). This plot displays results from a log-rank test comparing the survival curves of patients with EGFR ratios below the cut-off to those above it. The x-axis varies cut-off values and the y-axis shows the P-value from the resulting log-rank test. The vertical black line denotes the EGFR ratio cut-off with the minimum P-value, i.e. maximal separation between the two groups, equating to an optimal cut-off of 21.0 (for the sake of simplicity a cutoff of 20, which generated comparable P value, was used henceforth).

Figure 2. GBM survival varies according to degree of EGFR amplification

532 GBMs were pooled into three groups: (A) no *EGFR* amplification ($EGFR:CEP7 < 2$), (B) low-to-intermediate level of amplification ($EGFR:CEP7 = 2-20$), (C) high-level amplification ($EGFR:CEP7 > 20$). (D) Surprisingly, tumors with low-to-intermediate *EGFR* amplification had the shortest overall survival, followed by non-amplifiers and high-level amplifiers ($P < 0.006$ overall; $*P = 0.0007$ versus $EGFR:CEP7 = 2-20$ and $P = 0.03$ versus non-amplified. $P = 0.09$ between non-amplified and $EGFR:CEP7 = 2-20$). In (A-C), red signals = *EGFR* and green signals = centromeric enumeration probe for chromosome 7. GBM = glioblastoma.

Figure 3. Response to temozolomide differs according to degree of EGFR amplification
 (A) Overall survival was equally poor among all three subgroups when no postsurgical adjuvant therapy was administered ($P = 0.63$). (B) Likewise, survival did not differ when adjuvant therapy was given but did not include temozolomide ($P = 0.91$). (C) However, when temozolomide was part of the adjuvant regimen, patients whose GBMs had low-to-moderate EGFR amplification had worse survival than non-amplified GBMs ($P = 0.02$) and GBMs with high EGFR amplification ($P = 0.003$).

Table 1
Univariate analysis of clinical and molecular factors in GBMs

Overall survival in 532 GBMs was stratified according to key clinical and molecular factors. Of note, not all variables were retrievable in all 532 cases. * $P < 0.0001$ for all three intergroup comparisons. GBM = glioblastoma; TMZ = temozolomide; LOH = loss of heterozygosity; *EGFR* = epidermal growth factor receptor

variable	# (%)	median survival (months)	<i>P</i>
age ≤ 45 years	58 (10.9)	19.4	< 0.0001
age > 45 years	474 (89.1)	7.6	
male	311 (58.5)	9.1	0.78
female	221 (41.5)	8.1	
biopsy only	153 (30.9)	3.8	< 0.0001
resection	342 (69.1)	12.0	
no adjuvant therapy	81 (17.4)	2.0	< 0.0001*
adjuvant therapy without TMZ	72 (15.5)	6.7	
adjuvant therapy with TMZ	312 (67.1)	12.7	
10q intact	70 (18.0)	8.6	0.81
10q LOH	318 (82.0)	9.1	
<i>EGFR</i> not amplified	301 (56.6)	7.9	0.33
<i>EGFR</i> amplified	231 (43.4)	9.8	
negative <i>EGFR</i> expression	10 (2.0)	8.2	0.59
weak <i>EGFR</i> expression	63 (12.6)	8.0	
moderate <i>EGFR</i> expression	147 (29.4)	7.6	
strong <i>EGFR</i> expression	280 (56.0)	9.4	

Table 2
Clinical and molecular variables stratified according to EGFR amplification subgroup

Data are expressed as means $\pm$ SEM. LOH = loss of heterozygosity. IHC = immunohistochemistry. * $P < 0.05$ and ** $P < 0.001$ between no amplification and EGFR:CEP7 > 20 . ***In EGFR IHC expression, $P < 0.001$ between no amplification and in any of the other variables. TMZ = temozolomide; EGFR = epidermal growth factor receptor; IHC = immunohistochemistry; LOH = loss of heterozygosity; MGMT = O-6-methylguanine-DNA methyltransferase.

Variable	EGFR not amplified	EGFR: CEP7 = 2-20	EGFR: CEP7 > 20	Overall P
age (years)	64.1 $\pm$ 0.9	63.5 $\pm$ 1.7	62.2 $\pm$ 0.9	0.38
% male	56.8 $\pm$ 2.9	64.8 $\pm$ 5.7	58.8 $\pm$ 3.9	0.47
% resected (versus biopsy alone)	67.5 $\pm$ 2.8	69.8 $\pm$ 5.8	71.7 $\pm$ 3.7	0.66
% no adjuvant therapy	21.5 $\pm$ 2.5	15.5 $\pm$ 4.8	11.6 $\pm$ 2.7	0.04*
% adjuvant therapy without TMZ	16.5 $\pm$ 2.3	15.5 $\pm$ 4.8	13.0 $\pm$ 2.8	0.65
% adjuvant therapy with TMZ	62.1 $\pm$ 3.0	69.0 $\pm$ 6.1	75.3 $\pm$ 3.6	0.02*
EGFR IHC expression score	2.0 $\pm$ 0.05	2.8 $\pm$ 0.05	2.9 $\pm$ 0.02	$< 0.0001^{***}$
% with 10q LOH	75.3 $\pm$ 2.9	83.6 $\pm$ 5.0	94.5 $\pm$ 2.1	$< 0.0001^{**}$
% with no MGMT promoter methylation	34.0 $\pm$ 4.8	30.0 $\pm$ 10.5	32.2 $\pm$ 6.1	0.93

Table 3**Multivariate analyses**

Modeling according to clinical factors significant on univariate analysis showed that low-to-moderate *EGFR* amplification was an independent adverse prognostic factor for overall survival, both in all cases with known adjuvant therapy status (upper) and the subset treated with TMZ (lower). HR = hazard ratio, CI = confidence interval, *EGFR* = epidermal growth factor receptor; CEP7 = centromeric enumeration probe for chromosome 7, TMZ = temozolomide.

All cases with known adjuvant therapy status (N = 465)			
Variable	HR	95% CI	P
Patient age > 45 years versus ≤ 45 years	2.46	1.79-3.38	< 0.0001
Resection versus biopsy	0.42	0.34-0.52	< 0.0001
TMZ versus no TMZ	0.39	0.30-0.46	< 0.0001
EGFR:CEP7 ratio = 2-20 versus non-amplified or ratio > 20	1.42	1.07-1.89	0.017
Only cases treated with TMZ (N = 312)			
Variable	HR	95% CI	P
Patient age > 45 years versus ≤ 45 years	2.09	1.41-3.09	0.0002
Resection versus biopsy	0.39	0.29-0.51	< 0.0001
EGFR: CEP7 ratio = 2-20 versus non-amplified or ratio > 20	1.56	1.10-2.22	0.013