A Functional Polymorphism in the EGF Gene Is Found with Increased Frequency in Glioblastoma Multiforme Patients and Is Associated with More Aggressive Disease

Deb A. Bhowmick,1,2 Zhengping Zhuang,1 Scott D. Wait,1 and Robert J. Weil1
1Surgical Neurology Branch, National Institutes of Neurological Disorders and Stroke, NIH, Bethesda, Maryland, and 2Baylor College of Medicine, Houston, Texas

Abstract
Glioblastoma multiforme, the most aggressive form of primary brain tumor in adults, is nearly universally fatal, with 5-year survivals of <5% (P. Kleihues and W. K. Cavenee, eds., pp. 1–314, Lyon: IARC, 2000). Alterations in the epidermal growth factor receptor (EGFR) are common events in many glioblastoma. We hypothesized that a polymorphism in the 5'-untranslated region of the epidermal growth factor (EGF) gene, a natural ligand of the EGFR, may play a role in the genesis of these malignant gliomas. We find that patients with the GA or GG genotype have higher tumoral levels of EGF, irrespective of EGFR status, that they are more likely to recur after surgery, and that they have a statistically significant shorter overall progression-free survival than patients with the AA genotype. These findings suggest that a single nucleotide polymorphism in EGF may play a role in the formation of glioblastomas, is a useful and powerful prognostic marker for these patients, and may be a target for tumor therapy.

Introduction
One characteristic of tumors is dysregulation of cell growth. Dysregulation may result, among other causes, from inherited or acquired genetic or epigenetic alterations in genes that influence signal transduction or control entry into the cell cycle.

In primary tumors of the brain, receptor tyrosine kinases, which are critical to normal cell proliferation and differentiation, appear to be important targets of gliomagenesis (1–3). Dysregulation of receptor tyrosine kinases may occur via gene overexpression, gene amplification, or through mutation that results in constitutive activation (1–3). Alterations of the epidermal growth factor (EGF) receptor (EGFR) occur in as many as half of all patients with glioblastoma multiforme (GBM), the most aggressive form of glioma (1–7). EGFR alterations are especially common in GBMs that arise de novo, as opposed to those that are the result of progression of a lower-grade astrocytoma, where alterations in cell cycle control genes, such as p53 and p16, are more common (1–3, 6–8).

In either type of GBM, activation of mitogenic signaling cascades downstream of receptor tyrosine kinases, whether EGFR alterations are present or absent, are common to both primary and secondary GBMs. In this setting, activation of autocrine growth factor/receptor loops creates a link between proliferation and the cell cycle machinery irrespective of the genetic abnormalities identified (1–3).

Recently, Shahbazi et al. (9) identified a novel, single nucleotide polymorphism in the 5' untranslated region (UTR) of the EGF gene that appears to have functional consequences. Substitution of guanine (G) for adenine (A) increased production of EGF by cultured peripheral blood mononuclear cells. In patients with melanoma, where altered EGFR mitogenic signaling is believed to play an important pathogenetic role, presence of the G allele conferred a 2.7-fold relative risk of melanoma (9). Patients with the GG genotype had a 4.9-fold increased relative risk compared with the normal population. Homozygous G genotype patients also were statistically more likely to have a thicker melanoma at presentation, a known risk factor for poorer prognosis, principally from loco-regional and systemic metastatic risk (9).

Because melanomas and GBMs share a common pathogenetic mechanism through EGFR-Ras-mitogen activated protein kinase pathway activation, we investigated a series of GBMs for the presence of this EGF polymorphism (1–3). To elucidate the potential mechanism by which the polymorphism may contribute to the formation of GBMs, levels of tumoral EGF and EGFR were examined. Finally, we correlated EGF genotype to tumor progression and overall survival.

Materials and Methods
Tissue and Clinical Information. Tissue samples from 42 consecutive, unselected patients with histologically confirmed GBM were collected fresh at surgery, snap-frozen in liquid nitrogen, and stored at ~80°C until analyzed. Clinical information and tissues were collected under Institutional Review Board-approved protocols at Vanderbilt University School of Medicine and the NIH. Patient clinical information was retrieved retrospectively in a blinded fashion. Data including patient age at diagnosis, sex, level of primary resection (near-total, 90–98% versus gross total, >98% preoperative magnetic resonance imaging contrast-enhanced tumor), and time to disease progression were collected.

Thirty-one tumors were primary GBMs, and 11 had progressed from lower grade astrocytomas. All of the patients had postoperative radiation therapy (average, ~6000 rads) and adjuvant chemotherapy after surgery for GBM. Thirty-five patients received carmustine (BCNU; 9 AA, 14 GA, and 12 GG) and 7 received temozolomide (2 AA, 2 GA, and 3 GG) as the first chemotherapeutic agent; there were no statistical differences in survival based on chemotherapy. Disease progression was defined as clear radiographic evidence of tumor growth at a new location or local recurrence at the resection site. Two patients lost to follow-up were excluded from the survival analysis; all of the others were followed until death. Three control samples of non-neoplastic brain from epilepsy surgery were also collected as tissue controls. To determine the normal prevalence of the 5'UTR polymorphism, DNA from 78 normal controls was obtained from consenting blood donors at the NIH Clinical Center, under an Institutional Review Board-approved protocol.

Total RNA and DNA were extracted from 20 mg of frozen tumor using Qiagen RNA/DNA extraction kits (Valencia, CA) according to the manufacturer's instructions. RNA from normal control blood samples was purified using Qiamp DNA blood extraction microcolumns (Qiagen, Valencia, CA), after the manufacturer’s instructions.

Genotyping and Expression Analysis. The polymorphism was identified by PCR amplification of the EGF 5'UTR and demonstrated by the presence of a restriction site obliterated by the 61G allele. One hundred ng of tumor and control genomic DNA were subjected to 35 cycles of PCR (initial denaturation of 95°C for 5 min, then 95°C for 30 s, 51°C for 30 s, and 72°C for 1 min) to
amplify the nucleotide positions -78 to +164 of the EGF gene. The following primers were used: forward: TGTCACTAAAGGAAAGGA and reverse: TTCAAGAGTTAAGCAGCCC. The samples were then digested using 2 units of AluI for 14 h at 37°C, and subsequently subjected to electrophoresis on a 3% agarose gel and stained by ethidium bromide. 61G alleles were delineated from 61A alleles by visualization of a single 193-bp fragment instead of 91- and 102-bp fragments.

EGF and EGFR gene expression were analyzed by quantitative reverse transcription-PCR techniques, using appropriate intron spanning primers and radioactive nucleotide labeling. First, 250 nmol of total RNA from each tumor was used to create cDNA by single strand reverse transcription using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). An average quantitative standard was created by pooling all of the sample cDNAs. The pooled sample was subjected to 10–35 cycles of PCR with appropriate intron spanning primers for EGF, EGFR, and GAPDH transcripts in the presence of 1 μCi/μL α-32P-labeled dCTP. A standard amplification curve was then created for EGF, EGFR, and GAPDH transcripts by quantifying radiolabeled PCR product at every second cycle. A range of six cycles was then determined to have linear amplification for each primer set in the pooled sample. Each tumor cDNA sample was then amplified in the presence of 1 μCi/μL α-32P-labeled dCTP over the range of six cycles for each primer set, run on a 6% denaturing polyacrylamide gel, and quantified after a 10-h exposure to a 32P phosphor screen. Linearity of PCR amplification over the six cycles was confirmed for each tumor sample, and quantification of EGF and EGFR mRNA was determined as a ratio of EGF and EGFR radiolabeled PCR product to GAPDH PCR product. All of the reactions were done in triplicate. The following primer sequences were used for PCR amplification of cDNA, EGF-forward: CAGGTAAATGGAAGAGGCTTCTCAT, -reverse: GAGTTAATGCTACTGATCT.C; EGFR-forward: TTCCTTACACTTGCG; GAPDH-forward: GAGGCCACATCGCTGAC, -reverse: GTCTCAGTGGTGTGAAG. EGF amplifications was assessed as described in Smith et al. (2)

**Results**

Frequencies of EGF 5′UTR genotypes are illustrated in Table 1. GBM patients were more likely than normal control patients to have either a G/A or G/G genotype (P = 0.03, two-tailed χ² test). Furthermore, the total frequency of the G allele in GBM patients was significantly greater than in normal controls (P < 0.001, two-tailed χ² test), as seen in Table 1.

EGF expression, measured by quantitative reverse transcription-PCR, was significantly less in tumors with the homozygous A/A +61 EGF genotype when compared with homozygous G (P = 0.011) or heterozygous G/A genotypes (P = 0.004), as seen in Fig. 1A. The mean EGF:GAPDH mRNA ratio was 2.88 [95% confidence interval (CI), 1.68–4.07], 9.75 (95% CI, 5.74–13.76), and 5.77 (95% CI, 4.12–7.41) for the A/A, G/A, and G/G genotypes, respectively. There was no significant difference in EGF expression between tumors homozygous or heterozygous for the 61G allele (P = 0.187). In addition, there was no significant relationship between EGF levels and EGF +61 genotype (Fig. 1B). The mean EGF:GAPDH mRNA ratio was 4.45 (95% CI, 2.37–6.53), 3.72 (95% CI, 2.44–5.04), and 3.68 (95% CI, 2.58–4.78) for A/A, G/A, and G/G genotypes, respectively. Reverse transcription-PCR quantitation values were validated by ELISA, showing a high concordance rate between tumoral EGF protein levels and quantified EGF mRNA levels (R² = 0.83), as seen in Fig. 2. Finally, there was no relationship between EGF expression as measured by reverse transcription-PCR or ELISA, and whether the tumor was a primary or secondary GBM or whether there was amplification of the EGFR (data not shown).

Patients homozygous for the 61A allele were not significantly dissimilar to subjects with the 61G allele in age, sex, extent of resection, or postoperative adjuvant therapy. Fig. 3A demonstrates 61A homozygotes have an average of 3.5 months longer progression-
free survival than 61G allele carriers ($P = 0.036$). Furthermore, 61A homozygotes had a statistically significantly longer overall survival when compared with subjects with the 61G allele (Fig. 3B).

**Discussion**

GBM is the most common primary brain tumor found in adults (1–3). It is uniformly lethal, with a mean survival time of ~12 months after diagnosis (1–3). Known predictors for survival include clinical factors such as age, level of function, radiological and histopathological evidence of tumor necrosis, tumor size, and extent of resection (1–3, 10–12). Evidence showing the effectiveness of specific genetic mutations in predicting GBM prognosis has been mixed or, at best, revealed a complex relationship between multiple clinical and genetic factors (1–3, 11). However, the effect of common genetic variances, manifested as polymorphisms in the general population, have not been investigated as factors that may have functional significance in predicting clinical outcome in GBM patient populations.

EGFR amplification/overexpression or constitutive activation through mutation have been well established as common pathological genetic alterations in >40% of all glioblastomas (4–5). When combined with other known genetic alterations found in GBMs, animal models of the EGFR mutation or overexpression show new glial cell malignant transformation or acceleration of the onset of the malignant phenotype (3, 13, 14). Amplification or activating mutations of EGFR have also been identified in other neoplasms, including breast adenocarcinoma, malignant melanoma, prostate adenocarcinoma, and lung carcinomas (15–17). Interestingly, targeted overexpression of an EGFR ligand, transforming growth factor α, has been shown both in vitro and in vivo to cause malignant transformation of mammary duct epithelia, but only in the presence of EGFR (18). This implicates the levels of common EGFR ligands, EGF, transforming growth factor α, heregulin, and amphiregulin, among others, as important factors in tumorigenesis, especially in EGFR overexpressing tumors.

A single nucleotide polymorphism found in the 5' UTR region of the EGF gene was found recently to have functional significance in patients with melanoma (9). The polymorphism, an A-to-G variant found 61 bp downstream of the EGF promoter, is found in 43.9% of all of the EGF alleles in the European Caucasian population but was present in nearly 66% of patients with malignant melanoma (9). Increased EGF expression in activated peripheral blood mononuclear cells collected from normal individuals that were homozygous and heterozygous for the 61G allele was also demonstrated by Shahbazi et al. (9), which suggests a possible functional mechanism for the polymorphism. These findings led us to investigate the relevance of this single nucleotide polymorphism in patients with glioblastoma multiforme, in which alterations of growth factor signaling through the EGFR are common.

In this study, we find that a single nucleotide polymorphism in the 5' UTR of EGF is significantly correlated with increased expression of EGF in resected GBM tumor samples, whether from primary or secondary GBMs. These data are supported by previous in vitro findings of increased EGF production found in stimulated peripheral blood mononuclear cells from normal subjects homozygous or heterozygous for the EGF 61G allele (9). Importantly, these findings suggest that the mechanism by which the EGF expression is increased continues to be active after malignant transformation of glial cells. We also show that the association of the 61G allele with increased EGF is independent of EGFR overexpression or mutation. This would indicate that this common genetic variant does not promote genetic alteration of EGFR, nor is it affected by increased activation of the receptor that is commonly altered in glioblastoma.

We also find that the EGF 61G allele is found in significantly higher numbers in GBM patients compared with a normal, tumor-free population and, thus, may serve to promote glial tumor development.
in susceptible patients. Because the 61G allele is associated with increased EGF expression, increased EGF expression may promote GBM tumor development and progression. This is supported by in vitro data showing increased proliferation and invasiveness of GBM primary cell cultures treated with EGF (19). Furthermore, aberrant activation of EGFR has long been established as an important genetic alteration found in sporadic, de novo GBM development (1–8). Increased EGF expression provides a mechanism of EGFR activation in those patients with GBMs that do not have EGFR overexpression or an activating mutation of the receptor. This may also be an early event promoting tumor growth before EGFR mutation, as previous data indicate that more than one form of activating mutation can occur in subsequent amplification events after early amplification of wild-type EGFR (7).

The clinical finding that EGF 61G homozygotes and heterozygotes had significantly shorter progression-free survival than 61A homozygous cohorts indicates that increased EGF expression is not only active during growth and progression of GBMs but likely has a significant biological effect that promotes tumor growth. Due to the rare incidence of GBMs in the general population of 1 in 100,000 individuals and the high prevalence of the 61G allele, this polymorphism cannot be considered a significant risk factor for GBM development. However, this is a unique observation demonstrating a common genetic variance that is usually phenotypically silent but in the setting of glial malignancy, becomes clinically relevant for tumor progression.

The mechanism by which increased EGF expression is associated with the EGF 5′UTR polymorphism is unknown. It may be possible that the 61G allele is linked to a locus involved in EGF regulation. The polymorphic site otherwise could be functional, causing differential EGF mRNA degradation or providing a binding site for transcription factors. As noted in Fig. 1, expression of EGF was higher in GA than in GG, although there was no statistical difference; this may represent a function of the numbers studied or other technical features unrelated to the polymorphism itself. Functional studies to dissect the nature of the polymorphism are ongoing. In this study, due to the lack of normal tissue from the subjects, we were unable to determine whether the 61G allele was inherited or if it had been acquired in these tumors through a single nucleotide mutation. The possibility that such a unique mutation could occur repeatedly in multiple different tumors is small. However, such an occurrence would provide evidence that the polymorphic site is a cis-acting locus, directly affecting transcription of EGF. Nevertheless, future studies of EGF physiology could benefit from the use of the EGF 5′UTR polymorphism as a marker of differential EGF expression, which is the aim of an ongoing, prospective clinical research study at the NIH (03-N-0160). Furthermore, it also may be a useful stratification tool in glioblastoma patients for future studies investigating treatments targeting EGF and its receptor.

Because time to tumor recurrence after primary resection is a strong determinant of mortality in GBM patients, the EGF 5′UTR polymorphism has the potential to be applied as a prognostic indicator for clinical outcome.

References

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