

Differential Gene Expression Analysis Reveals Generation of an Autocrine Loop by a Mutant Epidermal Growth Factor Receptor in Glioma Cells

Deepti B. Ramnarain,¹ Seongmi Park,^{1,3} Diana Y. Lee,^{5,6} Kimmo J. Hatanpaa,^{2,3} Shane O. Scoggin,⁴ Hasan Otu,⁷ Towia A. Libermann,⁷ Jack M. Raisanen,^{2,3} Raheela Ashfaq,² Eric T. Wong,⁵ Julian Wu,⁶ Robert Elliott,⁵ and Aryn A. Habib^{1,3,4}

Departments of ¹Neurology and ²Pathology, ³Annette G. Strauss Center in Neuro-Oncology, and ⁴Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas and Departments of ⁵Neurology and ⁶Neurosurgery and ⁷Genomics Center, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston Massachusetts

Abstract

The *epidermal growth factor receptor (EGFR)* gene is commonly amplified and rearranged in glioblastoma multiforme leading to overexpression of wild-type and mutant EGFRs. Expression of wild-type EGFR ligands, such as transforming growth factor- α (TGF- α) or heparin-binding EGF (HB-EGF), is also often increased in gliomas resulting in an autocrine loop that contributes to the growth autonomy of glioma cells. Glioblastoma multiforme express a characteristic EGFR mutant (EGFRvIII, de 2-7) that does not bind ligand, signals constitutively, and is more tumorigenic than the wild-type receptor. However, the downstream signals that mediate this increased tumorigenicity are not well understood. We hypothesized that signals induced specifically by EGFRvIII and not the wild-type receptor are more likely to mediate its increased tumorigenic activity and examined the gene expression profiles resulting from inducible expression of comparable levels of either wild-type EGFR or EGFRvIII in a U251-MG glioma cell line. Expression of EGFRvIII resulted in specific up-regulation of a small group of genes. Remarkably, all these genes, which include *TGFA*, *HB-EGF*, *EPHA2*, *IL8*, *MAP4K4*, *FOSL1*, *EMPI1*, and *DUSP6*, influence signaling pathways known to play a key role in oncogenesis and function in interconnected networks. Increased expression of EGFRvIII-induced genes was validated by real-time PCR. The mutant receptor does not bind ligand, and EGFRvIII-induced expression of TGF- α and HB-EGF suggests that EGFRvIII plays a role in generating an autocrine loop using the wild-type EGFR in glioma. It also raises the possibility that EGFRvIII may signal, at least in part, through the wild-type receptor. Indeed, we show that inhibiting the activity of HB-EGF, a potent mitogen, with neutralizing antibodies reduces cell proliferation induced by expression of EGFRvIII. This suggests that the EGFRvIII-HB-EGF-wild-type EGFR autocrine loop plays an important role in signal transduction by EGFRvIII in glioma cells. We also show by immunohistochemistry that HB-EGF expression correlates with the presence of EGFRvIII in glioblastoma multiforme.

Thus, our study provides a new insight into oncogenic signaling by EGFRvIII and improves our understanding of how autocrine loops are generated in glioma. (Cancer Res 2006; 66(2): 867-74)

Introduction

The epidermal growth factor receptor (EGFR; ErbB1) belongs to the ErbB group of receptor tyrosine kinases that also includes ErbB2, ErbB3, and ErbB4 (1). The EGFR is commonly expressed at high levels in various types of human cancer, and EGFR overexpression correlates with a worse prognosis in certain types of cancer (2, 3). *EGFR* gene amplification, rearrangements, and overexpression are a particularly striking feature of glioblastoma multiforme, the most common malignant tumor of the nervous system (4, 5). In glioblastoma multiforme, *EGFR* gene amplification can be detected in 40% to 50% of cases, usually in cases that present without a known preexisting low-grade glioma, called primary or *de novo* glioblastoma multiforme. In ~50% of tumors with EGFR amplification, a specific EGFR mutant (EGFR type III, EGFRvIII, de 2-7, Δ EGFR) can be detected (6). This mutant is generated from a deletion of exons 2 to 7 of the *EGFR* gene, which results in an in-frame deletion of 267 amino acids from the extracellular domain of the receptor. EGFRvIII is unable to bind ligand and signals constitutively. It is important to note that EGFRvIII is usually coexpressed with the wild-type receptor in glioblastoma multiforme (7, 8). In addition, this variant is distinct from the tyrosine kinase domain mutations that have been shown recently to be responsive to specific inhibition of the EGFR (9).

Several studies have shown that EGFRvIII variant is more tumorigenic than the wild-type receptor. Expression of EGFRvIII in fibroblast cells (10) or U87-MG glioma cells (11) resulted in increased growth of the tumor cells in a xenograft model. There is also evidence from transgenic mouse glioma models that EGFRvIII is more tumorigenic than the wild-type receptor (12, 13). Increased EGFRvIII expression may influence multiple aspects of tumor biology, including survival and/or proliferation of cells, motility, and invasiveness (14–16). Expression of EGFRvIII may correlate with a worse prognosis in malignant glioma (17, 18).

Overexpression of EGFRvIII results in a constitutive tyrosine phosphorylation of EGFRvIII. Because this mutant does not bind EGF, its internalization is slowed, promoting a state of low-level, continuous signaling from activated receptors at the cell membrane (19). Increased membrane persistence of activated receptors is known to favor mitogenic signaling (20). The altered kinetics of EGFRvIII could result in a distinct set of downstream signals compared with the wild-type EGFR, and several studies

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Aryn A. Habib, University of Texas Southwestern Medical Center, Mail Code 8813, 6001 Forest Park ND4.136, Dallas, TX 75390-8813. Phone: 214-645-6237; Fax: 214-645-6240; E-mail: Aryn.Habib@UTSouthwestern.edu.

©2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-05-2753

have investigated signal transduction by the mutant EGFR. There are reports of constitutive activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in cells expressing EGFRvIII (21) leading to a down-regulation of p27 (22). In addition, EGFRvIII-mediated activation of Ras (23) and extracellular signal-regulated kinases (ERK; ref. 24) has also been reported. Expression of EGFRvIII in U87-MG cells leads to an increase in Bcl-X_L and resistance to apoptotic cell death in response to chemotherapy (25). Other studies have reported an important role for c-Jun NH₂-terminal kinase activation in EGFRvIII signaling (26). Although the wild-type EGFR also activates these signals in response to ligand, it does not seem to do so constitutively even when overexpressed. It has been proposed that signals generated by the wild-type receptor are terminated more efficiently, because ligand binding is an important mechanism of receptor internalization and signal termination (27). However, several studies have shown that increased expression of the wild-type receptor also overwhelms mechanisms for receptor internalization, dephosphorylation, and degradation and can result in persistent activation of wild-type receptors (28, 29). Thus, questions about how the EGFRvIII is more tumorigenic than the wild-type receptor persist.

Several studies have used gene expression profiling to obtain insights into the biology of glioma (30–32). The gene expression profiles of low-grade astrocytomas are distinct from high-grade gliomas (33, 34). A previous study has suggested that the gene expression profiles of glioma subtypes overexpressing the EGFR are distinct from gliomas that do not overexpress the EGFR (35). Another study has looked at the effect of constitutive overexpression of EGFRvIII in a glioma cell and found that the mutant EGFR up-regulates molecular effectors of tumor invasion (15). However, these studies have not addressed differential gene regulation by EGFRvIII versus wild-type receptor.

To understand the downstream signals that make the EGFRvIII more tumorigenic than the wild-type receptor, we inducibly expressed either the wild-type EGFR or EGFRvIII in a glioma cell line at comparable levels to identify genes induced specifically by the EGFRvIII. Genes induced specifically by EGFRvIII include the wild-type EGFR ligands transforming growth factor- α (TGF- α) and heparin-binding EGF (HB-EGF), suggesting that EGFRvIII plays a causal role in generating the autocrine loop that is well described in glioma. Furthermore, we provide evidence that this autocrine loop is biologically significant and propose that EGFRvIII may generate oncogenic signals, in part, through activation of the coexpressed wild-type receptor. In addition, we find that EGFRvIII specifically induces expression of a small group of other genes known to play an important role in signaling pathways key to the pathogenesis of cancer.

Materials and Methods

Plasmids and generation of cell lines. The glioblastoma cell line U251-MG was used to generate cell lines inducibly expressing either wild-type or mutant EGFR. We used the T-Rex Tet-On System from Invitrogen (Carlsbad, CA). U251-MG cells were transfected with a regulatory plasmid (pcDNA6/TR), which encodes the tetracycline repressor. Individual clones were expanded using blasticidin selection and tested for tetracycline induction by transient transfection with a *lacZ* gene in a control inducible plasmid. One clone, TR45, was isolated, which showed very low background expression of *lacZ* combined with strong induction in the presence of tetracycline. Next, a wild-type human *EGFR* gene or a mutant *EGFRvIII* gene was cloned into the inducible expression plasmid (pcDNA4/TO) followed by transfection into U251-TR45 cells and zeocin selection. Individual clones were expanded and

tested for EGFR expression in response to tetracycline. Clones E6 (for wild-type EGFR) and E18 (for EGFRvIII) were selected because they exhibited the strongest induction of EGFR with the least background expression in the absence of tetracycline. An additional cell line was generated expressing an empty vector to serve as an additional control.

Antibodies and Western blotting. EGFR (06-129) and EPH receptor A2 (EphA2; 05-480) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The EGFR antibodies recognize both wild-type and mutant forms of the receptor and were used for Western blotting. Western blotting was done according to standard protocols and as we have described previously (36). Other antibodies used in this study include HB-EGF goat polyclonal neutralizing antibodies (PC319L) from Calbiochem (San Diego, CA), HB-EGF (sc-1413) for immunohistochemistry and goat polyclonal antibodies to betacellulin (sc-5800), Akt (sc-8312), and normal goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA).

RNA isolation. U251-E6 cells (overexpressing the wild-type EGFR) were incubated for 24 hours in serum-free DMEM with or without tetracycline (1 μ g/mL). Cells were then exposed to EGF (100 ng/mL) for 45 minutes or left untreated. E18 cells (expressing EGFRvIII) were incubated in serum-free medium and treated with tetracycline for 3, 6, and 24 hours. RNA was also extracted from U251 cells expressing empty vector following exposure to tetracycline. RNA extraction was done using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Only RNA samples with an A_{260}/A_{280} ratio ≥ 1.8 and with no visible degradation by agarose gel electrophoresis were used for microarray hybridization and cDNA synthesis for real-time PCR experiments.

Microarray probe preparation and Affymetrix GeneChip hybridization. Transcriptional profiling was done on HG-U133A Affymetrix (Santa Clara, CA) GeneChip containing 22,283 transcripts. cDNA was prepared according to the manufacturer's protocol. Total RNA (8 μ g) was used in the first-strand cDNA synthesis with T7-d(T)₂₄ primer and SuperScript II (Life Technologies, Rockville, MD). The second-strand cDNA synthesis was carried out according to the manufacturer's protocol. cRNA (20 μ g) was fragmented and hybridized with a pre-equilibrated HG-U133A Affymetrix GeneChip at 45°C for 16 hours. After the hybridization cocktails were removed, the chips were washed, stained, and scanned according to the manufacturer's protocols as described previously (37).

Data analysis. The chips were scanned in a HP ChipScanner (Affymetrix) to detect hybridization signals. Scanned image output files were visually examined for major chip defects and hybridization artifacts and then analyzed with Affymetrix GeneChip Microarray Analysis Suite version 5.0 software. This was then imported to Gene-Traffic (Iobion, La Jolla, CA) and normalized using Robust Multichip Analysis (38). The normalized data were analyzed using Microsoft Excel. For experimental comparisons, genes showing at least a 2-fold change were identified.

cDNA synthesis and real-time PCR. For validation of array results, real-time PCR was done. cDNA was prepared in duplicate from total RNA of two independent experiments using a combination of oligo(dT), random primers, deoxynucleotide triphosphates, SuperScript II (Invitrogen), and Superase.in (RNA inhibitor from Ambion, Austin, TX). PCR primers for each gene were obtained from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/links.html>) or were designed using Primer3 software, with a melting temperature at 58°C to 60°C and a resulting product of ~100 bp. Each PCR was carried out in triplicate in a 20 μ L volume using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) for 15 minutes at 95°C for initial denaturing followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in the ABI Prism 7700 Sequence Detection System. cDNA prepared from Universal RNA (Stratagene, La Jolla, CA) was used to construct a standard curve for each gene. Values for each gene were normalized to expression levels of 18S RNA. Primer sequences are available on request. Despite repeated attempts, we were unable to design a suitable primer for *FOSL1* and this gene was not tested.

Primary tumors/immunohistochemistry. Frozen tissue specimens of human glioblastomas were received from the Research Repository of Human Brain Tumors and Brain Tissue, University of Texas Southwestern Medical Center. Paraffin-embedded glioblastoma specimens were obtained from the archives of the Division of Neuropathology at the University of

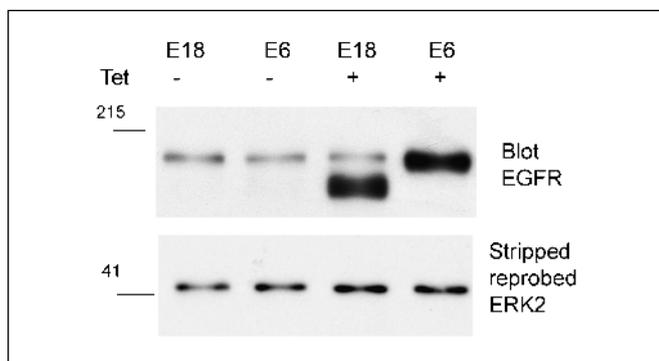


Figure 1. Generation of a U251-MG clone (E6) inducibly overexpressing the wild-type EGFR in response to tetracycline (*Tet*) and a U251 clone (E18) inducibly expressing a high level of mutant EGFR (EGFRvIII de 2-7) in response to tetracycline. *Top*, wild-type EGFR; *bottom*, mutant EGFR (faster migrating band). The endogenous EGFR levels can also be seen in this blot. This experiment shows that the relative amounts of wild-type (E6) and mutant (E18) induced by tetracycline are similar. This was confirmed by densitometry (data not shown). The blot was stripped and reprobbed with ERK2 to show loading (bottom).

Texas Southwestern Medical Center. Sections (4 μ m) were cut and deparaffinized. All subsequent incubations were at room temperature. For HB-EGF immunohistochemistry, the antigen retrieval method was pretreatment with proteinase K (DAKO, Carpinteria, CA) for 15 minutes. The sections were exposed to the goat polyclonal HB-EGF antibody (Santa Cruz Biotechnology) at a dilution of 1:100 for 25 minutes. The signal was detected by using the 4 PLUS detection kit (Biocare Medical, Walnut Creek, CA) according to the manufacturer's instructions. The sections were counterstained lightly with hematoxylin. For TGF- α immunohistochemistry, the antigen retrieval method was trypsin (BioGenex, San Ramon, CA) at a dilution of 1:10 for 15 minutes. The sections were exposed to the mouse monoclonal TGF- α antibody (Calbiochem) at a dilution of 1:400 for 60 minutes. The detection system was Envision Dual Link (DAKO). The sections were counterstained lightly with hematoxylin.

For protein extraction, resected tumors were initially frozen at -80°C . Protein was extracted for Western blotting using a modified radio-immunoprecipitation buffer (150 mmol/L NaCl, 1% NP40, 0.25% deoxycholate, 1 mmol/L EGTA, 1 mmol/L NaF, 50 mmol/L Tris-HCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 mmol/L sodium orthovanadate) and quantitated.

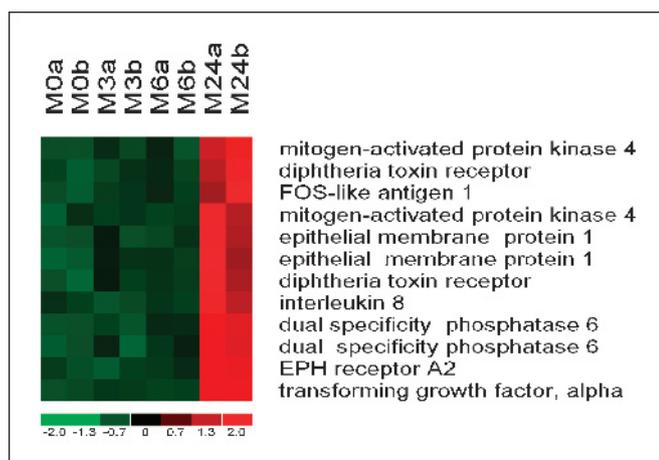


Figure 2. Dendrogram of EGFRvIII-specific gene expression. *M*, mutant EGFRvIII. RNA was extracted in two independent experiments at time 0 (no tetracycline), 3 hours after tetracycline addition, 6 hours after tetracycline addition, and 24 hours after tetracycline addition. The color code shows differences in the expression of the eight EGFRvIII-specific genes up-regulated by >2 -fold in the range of +2 (red) to -2 (green).

Western blotting. E18 cells were plated in a six-well tissue culture plate in the presence and absence of tetracycline (1 $\mu\text{g}/\text{mL}$). Lysates were prepared as described previously (36) followed by electrophoresis and immunoblotting.

Cell proliferation assays. E18 cells were plated at 20,000 per well in a 12-well tissue culture plate in the presence and absence of tetracycline (1 $\mu\text{g}/\text{mL}$). After ~ 24 hours of plating, control antibodies (betacellulin) and HB-EGF-neutralizing antibodies (GF10; Calbiochem) were added at a concentration of 5 $\mu\text{g}/\text{mL}$. Normal goat IgG was used as an additional control. The cells were trypsinized and counted after 72 hours of antibody exposure. At least three independent experiments were done in triplicate.

Results

Generation of cell lines expressing either wild-type or mutant EGFR. EGFRvIII is more tumorigenic than the wild-type receptor. To identify downstream signals that drive EGFRvIII-mediated gliomagenesis, we expressed either wild-type EGFR or EGFRvIII in U251-MG glioma cells using a tetracycline-inducible system. U251 cells express a moderate level of endogenous EGFR and have been widely used as an *in vitro* and xenograft model of glioma. Because EGFRvIII does not bind ligand and signals constitutively, an inducible system is particularly useful for studying this mutant. We identified one clone for the wild-type receptor (E6) and one clone for the mutant EGFRvIII (E18) that show a strong induction with tetracycline with a low background. Both wild-type EGFR and EGFRvIII are expressed at a similar level in clones E6 and E18 (Fig. 1). By gene expression analysis, we detect ~ 7 -fold increase in EGFR receptor mRNA levels in response to tetracycline in both E6 and E18 cells (Supplementary Table S1). We find a similar 7-fold increase in EGFR protein level by densitometry of Western blots (Fig. 1; data not shown), with $<10\%$ difference between levels of wild-type EGFR and EGFRvIII in tetracycline-treated E6 and E18 cells. In E6 cells, we could detect a small increase in wild-type EGFR expression compared with parental cells even in the absence of tetracycline, suggesting some leakiness in tetracycline regulation. We have, therefore, also used U251 cells transfected with an empty vector as an additional

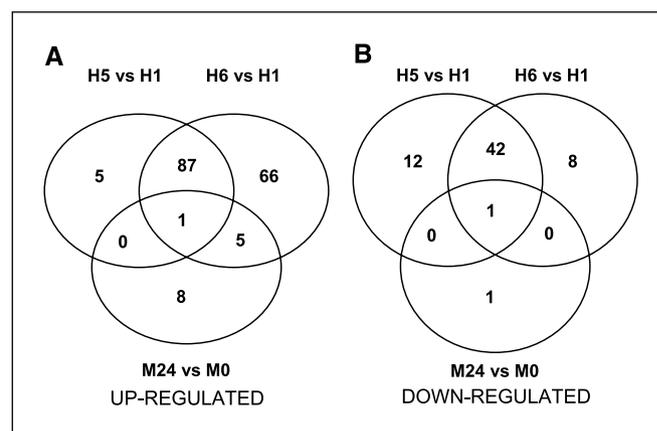


Figure 3. Comparisons of cell lines overexpressing wild-type or mutant EGFR were analyzed for genes that were differentially expressed. Known genes in which the fold expression ratios were >2 -fold were organized into Venn distributions, so that the independent effects of mutant and varying levels of wild-type EGFR can be displayed. *Circles*, all genes of pairwise combinations; *intersecting areas*, genes common to another pairwise combination. M24, E18 cells treated with tetracycline for 24 hours (and thus expressing EGFRvIII); M0, untreated E18 cells; H1, vector-transfected cells expressing a low level of endogenous wild-type EGFR; H5, cells express a high level of wild-type EGFR (E6 cells exposed to tetracycline for 24 hours); H6, H5 cells + EGFR.

control in our studies, giving us a low (vector-transfected U251 cells), intermediate (E6 cells without tetracycline), and high (E6 cells plus tetracycline) levels of wild-type EGFR. In E18 cells, we do not detect leaky expression of EGFRvIII in the absence of tetracycline. We found that maximal induction of both wild-type and mutant EGFR following tetracycline exposure takes 16 to 24 hours (data not shown). For microarray analysis, RNA was extracted from E18 cells after 3, 6, and 24 hours of tetracycline exposure to identify genes expressed at early time points. However, for E18 cells, as noted below, the only gene expressed at the 3- and 6-hour time points was the *EGFRvIII* itself. For wild-type EGFR-expressing E6 cells, RNA was extracted after tetracycline exposure for 24 hours, with or without EGF for 45 minutes.

Gene expression induced by constitutive EGFR expression. EGFRvIII does not bind ligand, and previous studies have shown that expression of EGFRvIII results in constitutive signaling. For example, previous studies have shown that EGFRvIII induces the activation of ERKs and the PI3K/Akt pathways. We detect an increase in ERK activation when EGFRvIII is expressed in E18 cells following exposure to tetracycline (data not shown). Consistent with previous studies, we do not detect constitutive activation of ERK when the wild-type EGFR is overexpressed in E6 cells.

Microarray analysis with Affymetrix HG-U133A chip revealed up-regulation of 14 genes and down-regulation of 1 gene in response to expression of EGFRvIII in E18 cells (Figs. 2 and 3; Table 1). A gene was considered to be regulated if the fold change was >2-fold. In the Venn diagram shown in Fig. 3, we have compared gene expression induced by the mutant and wild-type EGFR (with or without EGF). The mutant EGFR is compared with the wild-type EGFR expressed at a similar level with or without EGF exposure. The Venn diagram (Fig. 3A) shows that eight genes are specifically up-regulated by EGFRvIII but not by the wild-type EGFR either with or without EGF stimulation.

The Venn diagram (Fig. 3A) also shows that increased expression of the wild-type receptor without EGF stimulation results in up-regulation of a total of 93 genes and down-regulation of 55 genes (Fig. 3A and B), suggesting that the wild-type receptor also signals constitutively. If EGF is added to cells expressing high levels of wild-type EGFR, there is a further increase in the number of genes

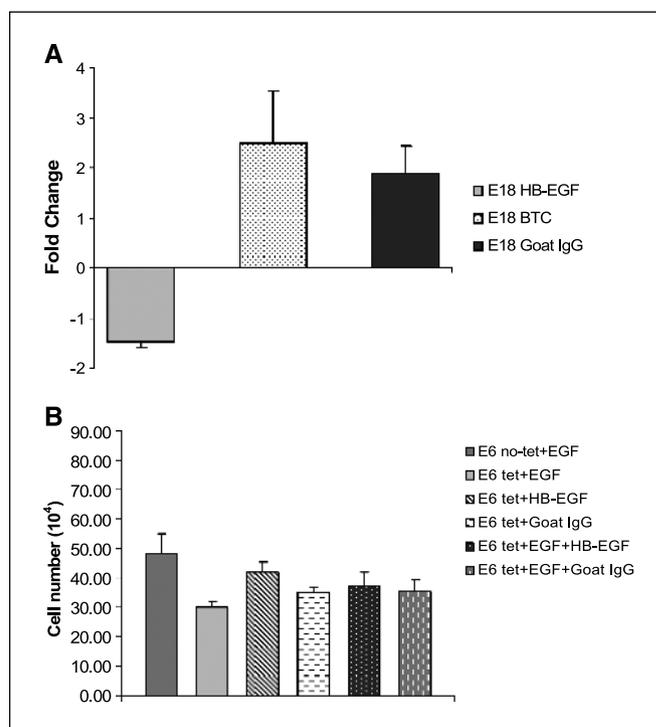


Figure 4. A, HB-EGF-neutralizing antibodies inhibit EGFRvIII-induced proliferation in E18 cells. E18 cells with or without tetracycline exposure were also exposed to either HB-EGF-neutralizing antibodies or betacellulin (*BTC*) antibodies or normal goat IgG as negative controls. The concentration of antibodies used was 5 μ g/mL. Cells were trypsinized and counted in a hemocytometer 72 hours after exposure to antibodies. *Columns*, fold increase in the number of cells on exposure to tetracycline. Exposure to tetracycline increases cell number to ~2-fold in E18 cells treated with control antibodies (betacellulin or normal goat IgG), whereas exposure to HB-EGF-neutralizing antibodies blocks the tetracycline-induced increase in cell number in E18 cells. B, E6 cells overexpressing the EGFR (*E6 Tet*) are not growth inhibited by treatment with HB-EGF antibodies with or without EGF under the same conditions as in (A). Cell numbers are similar whether cells are exposed to HB-EGF antibodies or normal goat IgG. *Bars*, SE.

expressed to 159. As previous studies have suggested, signals generated by the wild-type receptor seem more robust in terms of the number of genes altered and the magnitude of the change (Fig. 3A; Supplementary Table S1). However, at least a part of the

Table 1. Genes induced by EGFRvIII

| Gene name | Gene symbol | Accession no. | Affymetrix annotation | Unigene | Fold increase by EGFRvIII (RT-PCR) | Fold increase by EGFRvIII (Affymetrix) |
|---|---------------|---------------|-----------------------|-----------|------------------------------------|--|
| HB-EGF-like growth factor (diphtheria toxin receptor) | <i>HB-EGF</i> | NM_001945 | 203821_at | Hs.799 | 3.93 | 2.14 |
| Dual-specificity phosphatase 6 | <i>DUSP6</i> | BC003143 | 208892_s_at | Hs.298654 | 11.48 | 3.03 |
| EphA2 | <i>EPHA2</i> | NM_004431 | 203499_at | Hs.171596 | 11.04 | 2.11 |
| Epithelial membrane protein 1 | <i>EMPI</i> | NM_001423 | 201325_s_at | Hs.306692 | 13.12 | 2.53 |
| IL-8 | <i>IL8</i> | NM_000584 | 202859_x_at | Hs.624 | 8.01 | 2.72 |
| Mitogen-activated protein kinase kinase kinase kinase 4 | <i>MAP4K4</i> | NM_004834 | 206571_s_at | Hs.6631 | 1.76 | 2.1 |
| TGF- α | <i>TGFA</i> | NM_003236 | 205016_at | Hs.170009 | 11.67 | 2.32 |

NOTE: Expression of EGFRvIII-induced genes by real-time PCR and Affymetrix microarray analysis. Real-time PCR was conducted as described in Materials and Methods. The fold increase in gene expression for each gene is shown. Values for each gene were normalized to 18S RNA. The fold increase in gene expression by microarray analysis is also shown in column 7.

Table 2. Correlation between EGFRvIII, HB-EGF, and TGF- α expression in glioblastoma multiforme

| Tumor samples | EGFRvIII | EGFRvIII expression (intensity/mm ²) | HB-EGF expression (immunohistochemistry) | TGF- α expression (immunohistochemistry) | Tumor type |
|---------------|----------|--|--|---|-------------------------|
| A | – | 0.1 | 1 | 3 | Glioblastoma multiforme |
| B | – | 1.3 | 1 | 1 | Normal brain |
| F | – | 0 | 0 | 2 | Glioblastoma multiforme |
| G | – | 0 | 0 | 2 | Glioblastoma multiforme |
| I | – | 0 | 0 | 0 | Glioblastoma multiforme |
| J | – | 0 | 1 | 0 | Glioblastoma multiforme |
| K | – | 0 | 1 | 0 | Glioblastoma multiforme |
| C | + | 11,870 | 3 | 2 | Glioblastoma multiforme |
| D | + | 7,455 | 3 | 2 | Glioblastoma multiforme |
| E | + | 41,133 | 1 | 2 | Glioblastoma multiforme |
| H | + | 42,667 | 3 | 2 | Glioblastoma multiforme |
| L | + | 14,735 | 2 | 2 | Glioblastoma multiforme |

NOTE: Glioblastoma multiforme samples were studied to understand the relation between expression levels of EGFRvIII, HB-EGF, and TGF- α by Western blot (WB) analysis and immunohistochemistry. EGFRvIII expression was detected by Western blotting and quantitated by densitometry. For detection of HB-EGF and TGF- α , immunohistochemistry was used and signal intensity was graded as 0, + or 1, ++ or 2, and +++ or 3. The result shows a significant correlation between EGFRvIII and HB-EGF by Student's *t* test ANOVA ($P = 0.0006$). Analysis for EGFRvIII and TGF- α expression did not show a statistically significant correlation ($P = 0.075$). $P < 0.05$ was considered statistically significant.

signals generated by constitutive activation of the EGFRvIII are distinct and may provide clues as to what make EGFRvIII more tumorigenic than the wild-type receptor. Figure 2 shows genes induced specifically by EGFRvIII.

Genes up-regulated by EGFRvIII include *TGFA*, *HB-EGF*, *EPHA2*, *IL8*, *MAP4K4*, *FOSL1*, *EMPI1*, and *DUSP6*. By microarray analysis, the gene expression changes induced by EGFRvIII are quantitatively modest (Table 1) and delayed. For example, whereas activation of wild-type EGFR is well known to increase expression of immediate early genes, EGFRvIII-mediated gene expression takes longer, and although the mutant EGFR starts to express by 3 hours of tetracycline exposure, we did not detect any other changes in gene expression at 3 or 6 hours after addition of tetracycline to E18 cells (data not shown). It is important to note that almost all of the EGFRvIII up-regulated genes are involved in some aspect of growth control/oncogenesis and may function in an interconnected network.

Validation of microarray results. In this study, we have focused on genes expressed by EGFRvIII. We used quantitative reverse transcription-PCR (QRT-PCR) to validate the results of our microarray analysis. Among the genes induced specifically by EGFRvIII, we measured changes in transcript levels for *TGFA*, *HB-EGF*, *EPHA2*, *IL8*, *MAP4K4*, *DUSP6*, and *EMPI1*. The results of real-time PCR are shown in Table 1. In addition, we measured levels of transcripts of three genes induced by the wild-type EGFR. These were *GADD45B*, *IRF1*, and *STAT1* (data not shown). For all the genes tested, we were able to confirm the microarray results with real-time PCR analysis.

Biological effect of EGFRvIII expression. Previous studies have shown that expression of EGFRvIII results in increased proliferation of cells and decreased apoptosis in animal models (14). EGFRvIII expression may also result in increased proliferation of cells in tissue culture (22). We have found that tetracycline-induced expression of EGFRvIII in U251 cells (E18 cells) results in an increase in cell proliferation compared with untreated cells (Fig. 4A). As noted, expression of EGFRvIII results in a specific

increase in expression of TGF- α and HB-EGF, which are known EGFR ligands. This suggests that EGFRvIII (which is usually coexpressed with the wild-type EGFR) generates an autocrine loop in glioma cells. Moreover, it raises the possibility that EGFRvIII may signal, at least in part, through the wild-type receptor. Indeed, we find that neutralizing antibodies to HB-EGF block EGFRvIII-induced proliferation in E18 cells as shown in Fig. 4A. As a control for HB-EGF goat polyclonal antibodies, we used two negative controls, normal goat IgG and goat polyclonal betacellulin antibodies. Betacellulin, like HB-EGF, is a ligand for the wild-type receptor, but its expression is not increased by EGFRvIII. The control antibodies failed to inhibit EGFRvIII-induced cell proliferation (Fig. 4A).

We also examined whether neutralizing antibodies to HB-EGF would influence cell proliferation in E6 cells overexpressing the wild-type EGFR. Firstly, we find that increased wild-type EGFR expression in (tetracycline-exposed) E6 cells does not result in an increase in proliferation of cells with or without EGF (Fig. 4B; data not shown). Instead, as has been noted previously for other wild-type EGFR-overexpressing cells (36), EGF has a slight growth-suppressive effect. Secondly, HB-EGF antibodies fail to inhibit the proliferation of tetracycline-induced E6 cells under either EGF-treated or untreated conditions (Fig. 4B). This shows that the effect of HB-EGF-neutralizing antibodies is specific to cells expressing EGFRvIII.

In vivo expression of EGFRvIII-induced genes. Several studies have shown the coexpression of TGF- α and/or HB-EGF with the amplified EGFR in human malignant glioma (39, 40). EGFRvIII is commonly coexpressed with the wild-type EGFR in malignant glioma and we are particularly intrigued by the finding that EGFRvIII increases the expression of TGF- α and HB-EGF, ligands for the wild-type EGFR. This suggests a causal role for EGFRvIII in generating the HB-EGF and TGF- α -EGFR autocrine loop in human glioma. We studied the expression of EGFRvIII, HB-EGF, and TGF- α in glioblastoma samples by Western blotting and immunohistochemistry. Consistent with previous studies, we also find that

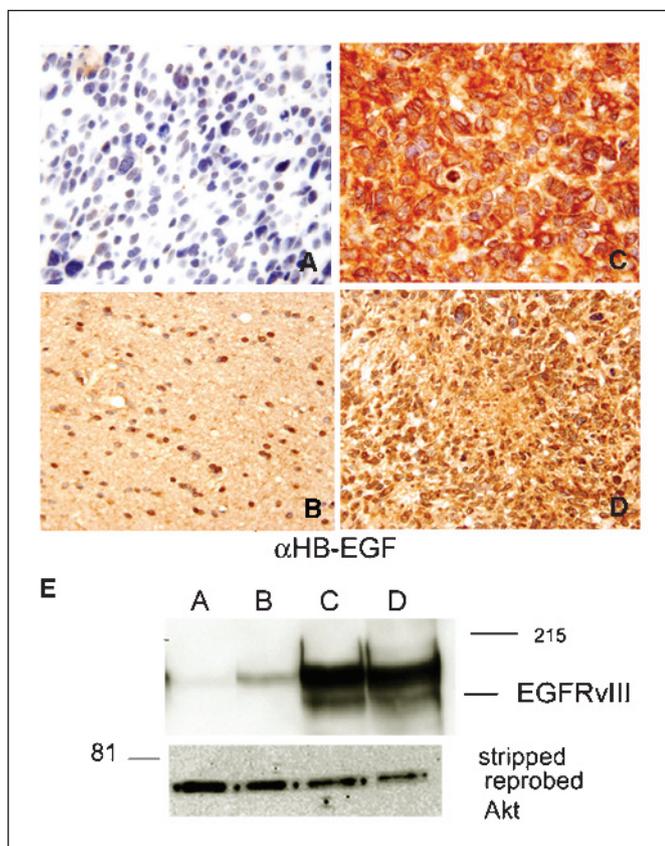


Figure 5. Expression of HB-EGF in two glioblastoma multiforme tumors that express EGFRvIII (C and D) and absence or weak expression of HB-EGF in a glioblastoma negative for EGFR amplification (A) or normal brain (B). Immunohistochemistry was done as described in Materials and Methods. Magnification, $\times 20$. Expression of wild-type and mutant EGFR in these tumors (and the normal brain) was detected by Western blotting (E). The faster migrating band represents EGFRvIII.

increased expression of EGFRvIII correlates with expression of HB-EGF in human glioma. Five of the 11 glioblastoma multiforme tumors we tested show expression of EGFRvIII. Four of the EGFRvIII-expressing tumors also show significant expression of HB-EGF, whereas none of the EGFRvIII-negative tumors have a significant expression of HB-EGF (Table 2). The correlation between EGFRvIII and HB-EGF expression is statistically significant ($P = 0.0006$). Figure 5 shows expression of EGFRvIII and immunohistochemistry for HB-EGF for some of the tumors studied. EGFRvIII is truncated and can be distinguished from the slower migrating wild-type EGFR by Western blotting. The correlation between EGFRvIII and TGF- α expression did not reach statistical significance. Whereas expression of TGF- α can be detected in all the tumors that express EGFRvIII, TGF- α is also expressed in tumors that do not express EGFRvIII (Table 2). This suggests the presence of alternative pathways that lead to TGF- α expression in glioblastoma multiforme.

In addition to HB-EGF and TGF- α , we also studied the expression of another EGFRvIII-induced gene, *EPHA2*, in human glioma. As can be seen in Fig. 6, the four glioblastoma multiformes showing a significant expression of EGFRvIII also show expression of EphA2 by Western blotting. In contrast, only one the four glioblastoma multiformes that lack EGFRvIII expression show a significant expression of EphA2. We find a statistically significant correlation between EGFRvIII and EphA2

expression in glioblastoma multiforme ($P = 0.027$). This finding further supports the probability that EGFRvIII drives expression of the genes we have identified (in E18 cells) in glioblastoma multiforme.

Discussion

EGFRvIII is more tumorigenic than the wild-type receptor. We show that inducible expression of EGFRvIII results in a gene expression profile that is distinct from the wild-type receptor. We find that increased expression of the wild-type receptor also results in constitutive signaling and gene regulation in the absence of exogenous ligand. However, overexpression of wild-type EGFR results in increased expression of a wide spectrum of genes, including genes involved not only in proliferation but also in growth suppression, immune modulation, metabolism, and transcription (Supplementary Table S1). The mutant EGFRvIII, in contrast to the wild-type receptor, induces specific expression of a narrow range of genes (shown in Fig. 2), all of which could play key roles in oncogenesis and could mediate increased tumorigenicity. For example, both TGF- α and HB-EGF are potent mitogens (41), and HB-EGF also promotes angiogenesis (42). Interleukin-8 (IL-8) is a chemotactic factor that has been shown recently to have a key role in Ras-induced angiogenesis and tumor development (43). Not unexpectedly, genes induced by EGFRvIII are closely linked to receptor tyrosine kinase and Ras signaling pathways.

A key finding of our study is that expression of EGFRvIII (but not the wild-type EGFR) in glioma cells results in increased expression of two known EGFR ligands, TGF- α and HB-EGF. TGF- α and HB-EGF are mitogens with established roles in cancer. Although EGFRvIII does not bind ligand, it is usually coexpressed with the wild-type EGFR in glioma. A recent study has identified populations within the same glioma of cells that express either wild-type EGFR or EGFRvIII (44). The induction of TGF- α and HB-EGF by EGFRvIII expression suggests that EGFRvIII (a) plays a role in generating an autocrine/paracrine loop in gliomas and in promoting continuous activation of wild-type EGFR and (b) raises

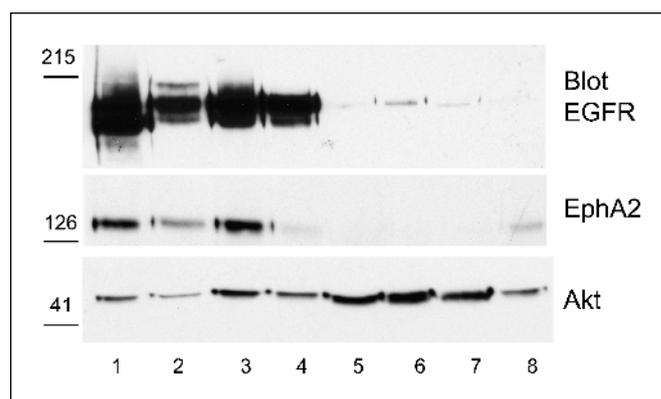


Figure 6. Expression of EGFRvIII correlates with expression of EphA2 in glioblastoma multiforme. Lysates from eight glioblastoma tumors were examined for the presence of EphA2. The blot was stripped and reprobed with EGFR antibodies to detect expression of EGFRvIII (faster migrating band). The same Western blot was stripped and reprobed with Akt antibodies to detect loading. EphA2 can be detected in all four EGFRvIII-expressing tumors. EphA2 can be detected in only one tumor that does not express EGFRvIII, suggesting that, although there is a strong correlation between EGFRvIII and EphA2 expression by densitometry ($P = 0.027$), alternative pathways may also lead to EphA2 expression in glioblastoma multiforme.

the possibility that EGFRvIII may signal and promote growth, in part, through the wild-type EGFR.

Autocrine loops, in which both the receptor and the ligand are produced by the same tumor cells, may be an important contributor to the growth autonomy of cancer cells (45). Coexpression of EGFR and TGF- α is well documented in EGFR amplification-positive glioma as is the coexpression of EGFR and HB-EGF (39, 40). We find that expression of HB-EGF correlates well with the presence of EGFRvIII in glioma (Fig. 5; Table 2), whereas the correlation between EGFRvIII and TGF- α did not reach statistical significance, suggesting that pathways other than EGFRvIII may also regulate expression of TGF- α . The major experimental support for the significance of autocrine loops is derived from studies that show that, whereas expression of the receptor alone (EGFR) has a weak transforming effect on cells, coexpression of ligand (TGF- α) results in a robust increase in transformation. In addition, strategies aimed at neutralizing ligands, such as TGF- α , have been shown to decrease growth of cells harboring such loops (39, 46). Thus, one mechanism of oncogenic signaling by EGFRvIII may be the production of autocrine loops in glioma cells by increasing expression of ligands for the wild-type EGFR. Our experimental findings support this view. We have found that inhibiting the activity of HB-EGF using neutralizing antibodies inhibits EGFRvIII-induced cell proliferation in glioma cells. However, this is unlikely to be the only mechanism of EGFRvIII-induced cell growth because the mutant also activates other oncogenic signals. Furthermore, EGFRvIII can transform fibroblast cells that do not express the wild-type receptor (10).

Although both TGF- α and HB-EGF engage the wild-type EGFR, there are differences in the delivery, processing, and action of the ligands (41). Both HB-EGF and TGF- α are produced as transmembrane proteins, cleaved and released as soluble proteins. TGF- α undergoes a rapid cleavage and is quickly consumed by EGFR in the vicinity, whereas HB-EGF has a longer residency time, and cleavage is delayed resulting in the majority of HB-EGF remaining on the plasma membrane as pro-HB-EGF. This membrane-associated pro-HB-EGF can engage the EGFR on adjacent cells (juxtacrine stimulation). Pro-HB-EGF is not merely a precursor of the soluble form. Membrane-associated HB-EGF is biologically active and may also interact with other proteins, such as CD9 and integrin $\alpha_3\beta_1$. These interactions may also help to stabilize the interaction of HB-EGF with the EGFR. HB-EGF induces the expression of the matrix metalloproteinases (MMP), MMP-9 and MMP-3, leading to enhanced cell migration (42). It is of particular interest that active MMP-9 expression is associated with a primary glioblastoma subtype and correlates highly with EGFRvIII expression (47). This raises the possibility that EGFRvIII may induce up-regulation of MMP-9 via expression of HB-EGF. HB-EGF expression is also reported to increase activation of cyclin D1 promoter as well as induction of vascular endothelial growth factor resulting in increased angiogenesis and enhanced tumorigenicity in mice (42). Thus, HB-EGF is a potent inducer of tumor growth and angiogenesis and may play an important role in growth induced by EGFRvIII. It is likely that the different EGFR ligands produce different patterns of EGFR stimulation *in vivo*, and the presence of multiple ligands may generate a more powerful autocrine loop in cancer.

The EphA2 is a receptor tyrosine kinase that is frequently overexpressed in cancer, and EphA2 overexpression confers tumorigenic and metastatic characteristics to cells (48, 49).

EphA2 expression is associated with increased invasiveness and decreased apoptosis of cancer cells (49). In normal cells, EphA2 is stimulated by ligands anchored on the surface of surrounding cells and tends to inhibit growth. In the absence of ligand stimulation because of unstable cell-cell contacts in cancer, EphA2 promotes oncogenesis. We have found that EphA2 expression correlates with the presence of mutant EGFR in human tumor samples, strongly suggesting that it is a EGFRvIII-induced gene in glioblastoma multiforme.

Increased expression of the chemokine IL-8/CXCL-8 has been described in several human cancers and is generally associated with tumor vascularization and a metastatic phenotype (43). In a recent study, expression of constitutively active Ras up-regulated CXCL-8 expression and inhibition of CXCL8 resulted in a decreased growth of Ras-induced tumors. CXCL8 is thought to recruit inflammatory cells to the tumor, leading to increased vascularization of the tumor and tumor progression.

Relatively less is known about the other genes up-regulated specifically by EGFRvIII in glioma cells. However, these genes are linked to known signaling pathways activated by receptor tyrosine kinases. The MAP4K4, also known as hepatocyte progenitor kinase-like/germinal center kinase-like kinase, is reported to be broadly expressed in human tumor cells, can modulate cellular transformation and invasion, and may be required for Ras-mediated transformation (50). Another gene, *DUSP6* or *MKP3*, seems to be up-regulated by Ras signaling (51). *EMPI* has been reported to be increased in response to ErbB2 overexpression in human mammary cells (52) and in uterine leiomyoma (53). *FOSL1* (Fra1) is a heterodimeric partner of activator protein-1 and is induced by 12-*O*-tetradecanoylphorbol-13-acetate (54). *FOSL1* is induced in response to activation of Ras or β -catenin pathways.

Our study suggests that expression of the mutant EGFRvIII in glioma cells results in the selective up-regulation of a few oncogenic genes that are not induced by the wild-type receptor. We suggest that the EGFRvIII may play a causal role in generating the autocrine loop in glioma and may signal cell proliferation, at least in part, through this loop and the wild-type EGFR. A previous study comparing expression of EGFRvIII to *lacZ* expression in D54-MG glioma cells has shown that EGFRvIII expression results in increased expression of genes involved in tumor invasion (15). Our study also finds that the genes induced selectively by expression of EGFRvIII, seem to function in pathways closely linked to oncogenic signaling, such as the Ras signaling pathway, and influence key aspects of cancer progression, such as cell proliferation, invasiveness, and angiogenesis. The differences between the genes identified in the two studies may reflect the differences in the study design (wild-type versus mutant receptor in our study, whereas in the previous study EGFRvIII expression was compared with *lacZ* expression), cell type used (D54-MG versus U251), mode of EGFRvIII expression (constitutive versus induced), and differences in GeneChip design (Clontech, Mountain View, CA versus Affymetrix). Future studies will address the *in vivo* significance of the EGFRvIII up-regulated genes.

Acknowledgments

Received 8/4/2005; revised 10/13/2005; accepted 11/7/2005.

Grant support: NIH grant CA 78741 (A.A. Habib), American Cancer Society Institutional Research Grant, Annette G. Strauss Center in Neuro-Oncology, and Children's Brain Tumor Foundation of the Southwest. The Research Repository of Human Brain Tumors and Brain Tissue is supported by the Division of

Neuropathology and the Annette G. Strauss Center in Neuro-Oncology at the University of Texas Southwestern Medical Center.

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

We thank the members of the Department of Neurosurgery at the University of Texas Southwestern Medical Center, Drs. Bruce Mickey, Christopher Madden, Tony

Whitworth, and Kevin Morrill for providing samples of resected tumor tissue and generous support; Dr. Frank Furnari (University of California at San Diego) for EGFRvIII plasmid; Dr. Charles L. White III for support; Drs. Benjamin Neel, John Minna, and Ramasamy Paulmurugan for helpful discussions and advice; and Dr. Adi Gazdar for critically reading this article. The immunohistochemical stains were done at the University of Texas Southwestern Pathology Immunohistochemistry Laboratory.

References

- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *Br Med Bull* 1991;47:87–98.
- Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001;37 Suppl 4:S9–15.
- Liebermann TA, Razon N, Bartal AD, Yarden Y, Schlesinger J, Soreq H. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Res* 1984;44:753–60.
- Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res* 2000;60:1383–7.
- Wong AJ, Ruppert JM, Bigner SH, et al. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 1992;89:2965–9.
- Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor α , and epidermal growth factor and their expression in human gliomas *in vivo*. *Cancer Res* 1991;51:2164–72.
- Biernat W, Huang H, Yokoo H, Kleihues P, Ohgaki H. Predominant expression of mutant EGFR (EGFRvIII) is rare in primary glioblastomas. *Brain Pathol* 2004;14:131–6.
- Minna JD, Gazdar AF, Sprang SR, Herz J. Cancer. A bull's eye for targeted lung cancer therapy. *Science* 2004;304:1458–61.
- Batra SK, Castelino-Prabhu S, Wikstrand CJ, et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ* 1995;6:1251–9.
- Nishikawa R, Ji XD, Harmon RC, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A* 1994;91:7727–31.
- Holland EC, Hively WP, DePinho RA, Varmus HE. A constitutively active epidermal growth factor receptor cooperates with disruption of G_1 cell-cycle arrest pathways to induce glioma-like lesions in mice. *Genes Dev* 1998;12:3675–85.
- Bachoo RM, Maher EA, Ligon KL, et al. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. *Cancer Cell* 2002;1:269–77.
- Nagane M, Coufal F, Lin H, Bogler O, Cavenee WK, Huang HJ. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res* 1996;56:5079–86.
- Lal A, Glazer CA, Martinson HM, et al. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* 2002;62:3335–9.
- Boockvar JA, Kapitonov D, Kapoor G, et al. Constitutive EGFR signaling confers a motile phenotype to neural stem cells. *Mol Cell Neurosci* 2003;24:1116–30.
- Shinojima N, Tada K, Shiraishi S, et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res* 2003;63:6962–70.
- 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;63:700–7.
- Huang HS, Nagane M, Klingbeil CK, et al. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* 1997;272:2927–35.
- Di Fiore PP, Gill GN. Endocytosis and mitogenic signaling. *Curr Opin Cell Biol* 1999;11:483–8.
- Choe G, Horvath S, Cloughesy TF, et al. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients *in vivo*. *Cancer Res* 2003;63:2742–6.
- Narita Y, Nagane M, Mishima K, Huang HJ, Furnari FB, Cavenee WK. Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. *Cancer Res* 2002;62:6764–9.
- Prigent SA, Nagane M, Lin H, et al. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. *J Biol Chem* 1996;271:25639–45.
- Lorimer IA, Lavictore SJ. Activation of extracellular-regulated kinases by normal and mutant EGF receptors. *Biochim Biophys Acta* 2001;1538:1–9.
- Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. *Proc Natl Acad Sci U S A* 1998;95:5724–9.
- Antonyak MA, Kenyon LC, Godwin AK, et al. Elevated JNK activation contributes to the pathogenesis of human brain tumors. *Oncogene* 2002;21:5038–46.
- Waterman H, Yarden Y. Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. *FEBS Lett* 2001;490:142–52.
- Wiley HS. Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. *J Cell Biol* 1988;107:801–10.
- Lund KA, Oprekso LK, Starbuck C, Walsh BJ, Wiley HS. Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J Biol Chem* 1990;265:15713–23.
- Nutt CL, Mani DR, Betensky RA, et al. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Cancer Res* 2003;63:1602–7.
- Mischel PS, Cloughesy TF, Nelson SF. DNA-microarray analysis of brain cancer: molecular classification for therapy. *Nat Rev Neurosci* 2004;5:782–92.
- Freije WA, Castro-Vargas FE, Fang Z, et al. Gene expression profiling of gliomas strongly predicts survival. *Cancer Res* 2004;64:6503–10.
- Rickman DS, Bobek MP, Misk DE, et al. Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. *Cancer Res* 2001;61:6885–91.
- Sallinen SL, Sallinen PK, Haapasalo HK, et al. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. *Cancer Res* 2000;60:6617–22.
- Mischel PS, Shai R, Shi T, et al. Identification of molecular subtypes of glioblastoma by gene expression profiling. *Oncogene* 2003;22:2361–73.
- Habib AA, Chun SJ, Neel BG, Vartanian T. Increased expression of epidermal growth factor receptor induces sequestration of extracellular signal-related kinases and selective attenuation of specific epidermal growth factor-mediated signal transduction pathways. *Mol Cancer Res* 2003;1:219–33.
- Voisine P, Ruel M, Khan TA, et al. Differences in gene expression profiles of diabetic and nondiabetic patients undergoing cardiopulmonary bypass and cardioplegic arrest. *Circulation* 2004;110:H280–6.
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003;31:e15.
- Tang P, Steck PA, Yung WK. The autocrine loop of TGF- α /EGFR and brain tumors. *J Neurooncol* 1997;35:303–14.
- Mishima K, Higashiyama S, Asai A, et al. Heparin-binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas. *Acta Neuropathol (Berl)* 1998;96:322–8.
- Harris RC, Chung E, Coffey RJ. EGF receptor ligands. *Exp Cell Res* 2003;284:2–13.
- Onghsaha PP, Kwak JC, Zwible AJ, et al. HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 2004;64:5283–90.
- Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 2004;6:447–58.
- Nishikawa R, Sugiyama T, Narita Y, Furnari F, Cavenee WK, Matsutani M. Immunohistochemical analysis of the mutant epidermal growth factor, Δ EGFR, in glioblastoma. *Brain Tumor Pathol* 2004;21:53–6.
- Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. *N Engl J Med* 1980;303:878–80.
- Filmus J, Shi W, Spencer T. Role of transforming growth factor α (TGF- α) in the transformation of ras-transfected rat intestinal epithelial cells. *Oncogene* 1993;8:1017–22.
- Choe G, Park JK, Jouben-Steele L, et al. Active matrix metalloproteinase 9 expression is associated with primary glioblastoma subtype. *Clin Cancer Res* 2002;8:2894–901.
- Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res* 2001;61:2301–6.
- Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. *Oncogene* 2004;23:1448–56.
- Wright JH, Wang X, Manning G, et al. The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion. *Mol Cell Biol* 2003;23:2068–82.
- Bloethner S, Chen B, Hemminki K, et al. Effect of common B-RAF and N-RAS mutations on global gene expression in melanoma cell lines. *Carcinogenesis* 2005;26:1224–32.
- Mackay A, Jones C, Dexter T, et al. cDNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. *Oncogene* 2003;22:2680–8.
- Arslan AA, Gold LI, Mittal K, et al. Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: new evidence and a systematic review. *Hum Reprod* 2005;20:852–63.
- Adisheshaiah P, Papaiahgari SR, Vuong H, Kalvakolanu DV, Reddy SP. Multiple *cis*-elements mediate the transcriptional activation of human fra-1 by 12-O-tetradecanoylphorbol-13-acetate in bronchial epithelial cells. *J Biol Chem* 2003;278:47423–33.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Differential Gene Expression Analysis Reveals Generation of an Autocrine Loop by a Mutant Epidermal Growth Factor Receptor in Glioma Cells

Deepti B. Ramnarain, Seongmi Park, Diana Y. Lee, et al.

Cancer Res 2006;66:867-874.

Updated version

Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/66/2/867>

Supplementary Material

Access the most recent supplemental material at:
<http://cancerres.aacrjournals.org/content/suppl/2006/01/24/66.2.867.DC1>

Cited articles

This article cites 53 articles, 33 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/66/2/867.full.html#ref-list-1>

Citing articles

This article has been cited by 24 HighWire-hosted articles. Access the articles at:
</content/66/2/867.full.html#related-urls>

E-mail alerts

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

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.