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


Departments of Neurology and Pathology, Annette G. Strauss Center in Neuro-Oncology, and Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas and Departments of Neurology and Neurosurgery and Genomics 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 multiformes 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 TGFα, HB-EGF, EPHA2, IL8, MAP4K4, FOSL1, EMP1, 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 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, ΔEGFR) 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: Amyn A. Habib, University of Texas Southwestern Medical Center, Mail Code 8813, 6001 Forest Park ND14.136, Dallas, TX 75390-8813. Phone: 214-645-6257; Fax: 214-645-6240; E-mail: Amyn.Habib@UTSouthwestern.edu.

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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, 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 inductively 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 pathogenesis of cancer.

Materials and Methods

Plasmids and generation of cell lines. The glioblastoma cell line U251-MG was used to generate cell lines inductively expressing either wild-type or mutant EGFR. We used the T-Rex Tet-Off 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 plasticidin selection and tested for tetracycline induction by transient transfection with a lacZ gene in a control inducible plasmid. One clone, TB45, 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 (66–129) and EPH receptor A2 (EphA2, 64–580) 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-MG cells (overexpressing the wild-type EGFR) were incubated for 24 hours in serum-free DMEM with or without tetracycline (1 μg/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 RNaseasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Only RNA samples with an A260/A280 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. Transcriptomic 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 μg) was used in the first-strand cDNA synthesis with 74-dT24 primer and SuperScript II (Life Technologies, Rockville, MD). The second-strand cDNA synthesis was carried out according to the manufacturer's protocol. cRNA (20 μg) 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 (RMA). 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 PrimerBankhttp://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 μL 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 Texas Southwestern Medical Center.
Texas Southwestern Medical Center. Sections (4 μm) 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 1:500 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 radioimmunoprecipitation 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.

Western blotting. E18 cells were plated in a six-well tissue culture plate in the presence and absence of tetracycline (1 μg/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 μg/mL). After −24 hours of plating, control antibodies (betacellulin) and HB-EGF-neutralizing antibodies (GF10; Calbiochem) were added at a concentration of 5 μg/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 control. The cells were trypsinized and counted after 72 hours of antibody exposure. At least three independent experiments were done in triplicate.

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 + EGFRvIII.
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 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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Accession no.</th>
<th>Affymetrix annotation</th>
<th>Unigene</th>
<th>Fold increase by EGFRvIII (RT-PCR)</th>
<th>Fold increase by EGFRvIII (Affymetrix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB-EGF–like growth factor (diphtheria toxin receptor)</td>
<td>HB-EGF</td>
<td>NM_001945</td>
<td>203821_at</td>
<td>Hs.799</td>
<td>3.93</td>
<td>2.14</td>
</tr>
<tr>
<td>Dual-specificity phosphatase 6</td>
<td>DUSP6</td>
<td>BC003143</td>
<td>208892_s_at</td>
<td>Hs.298654</td>
<td>11.48</td>
<td>3.03</td>
</tr>
<tr>
<td>EphA2</td>
<td>EPHA2</td>
<td>NM_004431</td>
<td>203499_at</td>
<td>Hs.171596</td>
<td>11.04</td>
<td>2.11</td>
</tr>
<tr>
<td>Epithelial membrane protein 1</td>
<td>EMP1</td>
<td>NM_001423</td>
<td>203125_s_at</td>
<td>Hs.306692</td>
<td>13.12</td>
<td>2.53</td>
</tr>
<tr>
<td>IL-8</td>
<td>IL8</td>
<td>NM_000584</td>
<td>202859_x_at</td>
<td>Hs.624</td>
<td>8.01</td>
<td>2.72</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase kinase 4</td>
<td>MAPK4</td>
<td>NM_004834</td>
<td>206571_s_at</td>
<td>Hs.6631</td>
<td>1.76</td>
<td>2.1</td>
</tr>
<tr>
<td>TGF-α</td>
<td>TGFA</td>
<td>NM_003236</td>
<td>205016_at</td>
<td>Hs.170009</td>
<td>11.67</td>
<td>2.32</td>
</tr>
</tbody>
</table>

**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.
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, we found that neutralizing antibodies to HB-EGF 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 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 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 TGFα, HB-EGF, EPHA2, IL8, MAP4K4, FOSLI, EMPI, 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 TGFα, HB-EGF, EPHA2, IL8, MAP4K4, DUSP6, and EMPI. 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 STATI (data not shown). For all the genes tested, we were able to confirm the microarray results with real-time PCR analysis.

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

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

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.
increased expression of EGFRvIII correlates with expression of HB-EGF in human glioma. Five of the 11 glioblastoma multiforme tumors we tested showed expression of EGFRvIII. Four of the EGFRvIII-expressing tumors also showed 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 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-$\alpha$ expression did not reach statistical significance. Whereas expression of TGF-$\alpha$ can be detected in all the tumors that express EGFRvIII, TGF-$\alpha$ is also expressed in tumors that do not express EGFRvIII (Table 2). This suggests the presence of alternative pathways that lead to TGF-$\alpha$ expression in glioblastoma multiforme.

In addition to HB-EGF and TGF-$\alpha$, 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 of 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-$\alpha$ 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-$\alpha$ and HB-EGF. TGF-$\alpha$ 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-$\alpha$ and HB-EGF by EGFRvIII expression suggests that EGFRvIII ($\alpha$) plays a role in generating an autocrine/paracrine loop in gliomas and in promoting continuous activation of wild-type EGFR and ($\beta$) raises
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 α5β1. 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 MAPK/ERK, also known as hepatocyte growth factor-related/mitogen-activated protein 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). EMP1 has been reported to be increased in response to ErbB2 overexpression in human mammary cells (52) and in uterine leiomyoma (53). FOGL1 (Fra1) is a heterodimeric partner of activator protein-1 and is induced by 12-O-tetradecanoylphorbol-13-acetate (54). FOGL1 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 EGFR11 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.

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