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Mutant Epidermal Growth Factor Receptor Up-Regulates Molecular Effectors of Tumor Invasion[1]

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Abstract

The gene most commonly altered in human glioblastomas is the epidermal growth factor receptor (*EGFR*). We profiled transcripts induced by mutant EGFR to better understand its role in tumor progression. The pattern found suggested enhanced tumor invasion. The highly induced genes included extracellular matrix components, metalloproteases, and a serine protease. We confirmed that mutant EGFR did make glioblastoma cells both more motile and invasive using *in vitro* assays. Furthermore, inhibitors of *EGFR* (OSI-774 and Tyrphostin AG1478) selectively down-regulated these molecular effectors in glioblastoma cells, eliminating enhanced invasion.

Introduction

The *EGFR*[3] is genomically amplified in 40–50% of human glioblastoma tumors (1) and often followed by gene rearrangement. The most common rearrangement is EGFRvIII, an in-frame deletion of amino acids 6–273. The resulting mutant protein is ligand-independent, constitutively phosphorylated, and localizes to the cell surface (2–4). EGFRvIII expression enhances the tumorigenicity of glioma cells *in vivo* by increasing cell proliferation and decreasing cell death (5, 6). EGFRvIII-positive tumors have also been associated with poorer prognosis (7) and shorter life expectancies (8). Unfortunately, little is yet known about the molecular mechanisms that EGFRvIII uses to produce a malignant phenotype.

To understand how EGFRvIII might exert its pathologic effects, we looked for downstream transcriptional targets using SAGE and other expression profiling methods. We have analyzed and compared the transcriptomes of a control and an EGFRvIII-expressing glioblastoma cell line and have identified genes of which the transcript levels were enhanced by EGFRvIII. These targets included ECM proteins, metalloproteases, and a serine protease, which point toward a role in tumor invasion. The effects of EGFR-inhibiting drugs were also evaluated in how they change gene expression. Our data suggests that not only does EGFRvIII expression enhance invasion but points to a small set of extracellular proteins eventually responsible for the malignant behavior of glioblastomas with mutant EGFR.

Materials and Methods

Cell Lines. The retroviral vector, pMFG, containing either the β-galactosidase gene or the EGFRvIII gene was used to generate replication-defective viruses from the CRIP packaging cell line. The viral supernatants were used to transfect the glioblastoma cell line, D54-MG. Stable clones expressing β-galactosidase were designated D54-LacZ. Single cell clones expressing EGFRvIII were isolated, and a clone expressing high levels of the mutant protein was designated D54-EGFRvIII. The glioblastoma cell line U251-MG was also transfected with EGFRvIII as described above (U251-EGFRvIII) and was grown as athymic mice xenografts. The xenografts were removed, dissociated into single cells, and sorted for EGFRvIII expression using the Becton Dickinson FACsort (Franklin Lakes, NJ). These sorted U251-EGFRvIII cells were grown *in vitro* and compared with the parental U251 cell line. U118-MG glioblastoma cell line transfected with EGFRvIII or a vector control were the kind gift of Dr. David James (Mayo Clinic, Rochester, MN). The U87 parental glioblastoma cell line and the U87 cell line transfected with EGFRvIII have been described earlier (5), and were grown as either cell lines or xenografts.

SAGE. Independent SAGE libraries were constructed from the stable clones D54-lacZ and D54-EGFRvIII as described earlier (9). Approximately 2000 plasmid clones were sequenced from each library as part of the CGAP SAGE project. SAGE tags were extracted and their tag frequencies compared using the SAGE software v 4.0. Unique transcript tags were identified as described earlier and mapped to predicted SAGE tags from primate cDNA GenBank entries or UniGene clusters that had a poly(A) signal and/or a poly(A) tail. Gene expression increases were predicted by dividing the fraction of a given SAGE tag in the EGFRvIII-expressing library by the fractional representation in the β-galactosidase-expressing library. The complete SAGE transcript tag counts have been deposited at the National Center for Biotechnology Information SAGEmap website[4] under the library names SAGE_Duke_H54_lacZ and SAGE_Duke_H54_EGFRvIII.

DNA Array Analysis. Poly(A) RNA isolated from D54-lacZ and D54-EGFRvIII cell lines were used to probe the Atlas Human Cancer 1.2 cDNA Array as described by the manufacturers (Clontech Laboratories, Palo Alto, CA). Briefly, the message RNA was reverse transcribed in the presence of [α-^{32}P]dATP to generate the probe, the membranes were prehybridized at 68°C for 30 min, hybridized overnight at 68°C, washed twice with 2× SSC/1% SDS and twice with 0.1× SSC/0.5% SDS for 30 min each at 68°C each, and then visualized by exposure to X-ray film.

Inhibitor Studies. Stock solutions of the tyrosine kinase inhibitors Tyrphostin AG1478 (Sigma-Aldrich, St. Louis, MO) and OSI-774 (Tarceva; courtesy of Ken Iwata, OSI Pharmaceuticals, Melville, NY) were prepared in DMSO. Glioblastoma cell lines were treated with either 25 μM Tyrphostin or 20 μM OSI-774 for the indicated time points. Control cells were treated with an equal volume of DMSO. Total RNA was isolated, and cDNA was synthesized using standard techniques. Transcript levels were assayed by quantitative PCR.

Quantitative PCR. Quantitative PCR was performed on cDNA templates using a thermocycler with continuous fluorescent monitoring capabilities (LightCycler; Roche Diagnostics) and SYBR Green I (Molecular Probes, Eugene, OR) using PCR conditions and data analysis as described earlier (10). The integrity of the cDNA and normalization of the cDNA yields were performed using primers specific for β-actin. Primers specific for genes induced by EGFRvIII were designed to generate 140–240-bp products, and their sequences are available on request.

Immunohistochemistry. Immunohistochemical staining was performed on 5 μm formalin-fixed, paraffin-embedded glioblastoma tissue sections for EGFRvIII using the mouse monoclonal antibody L84A at a concentration of 3

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; ECM, extracellular matrix; SAGE, Serial Analysis of Gene Expression; GBM, glioblastoma multiforme; CGAP, Cancer Genome Anatomy Project; poly(A), polyadenylic acid; MMP, matrix metalloproteinase; FAP, fibroblast activation protein; IHC, immunohistochemistry.

[4] Internet address: http://www.ncbi.nlm.nih.gov/SAGE/.

μg/ml, for Fibrillin-1 using the mouse monoclonal clone 12A5.18 (Neomarkers, Fremont, CA) at a concentration of 3 μg/ml and for carbonic anhydrase 9 using the mouse monoclonal M75 antibody (generous gift of E. Oosterwijk, University Medical Center, Nijmegen, the Netherlands) at a dilution of 2.8 μg/ml. The slides were immersed in solvent to remove paraffin, rehydrated, and blocked for endogenous peroxidase activity. The sections were then blocked with horse serum and sequentially incubated with primary antibody (1 h, 37°C), biotinylated secondary antibody, and streptavidin-conjugated horseradish peroxidase (Super sensitive detection system; Biogenex, San Ramon, CA). Bound antibody was detected using 3,3′-diaminobenzidine and hydrogen peroxide, counterstained with 1% hematoxylin, and permanently mounted.

Scratch Assay. D54-lacZ and D54-EGFRvIII cells were seeded in triplicates in six-well fibronectin or collagen IV coated plates (Becton Dickinson Labware, Bedford, MA). A scratch through the central axis of the plate was gently made using a pipette tip when the cells were 80% confluent. Migration of the cells into the scratch was observed at six preselected points at 0, 4, 8, 16, and 24 h, and one D54-lacZ and one D54-EGFRvIII plate were stained using Diff Quik (Dade Behring, Newark, DE) after 8, 16, and 24 h, and photographed at ×10 magnification.

***In Vitro* Invasion Assays.** The BD Biocoat Matrigel Invasion Chamber assay was performed as described by the manufacturers (Becton Dickinson Labware). Briefly, the Matrigel inserts were rehydrated, and 2.5×10^4 D54-lacZ or D54-EGFRvIII cells were added to the invasion or control chamber wells. Twenty-four h later, the cells on the upper side of the chamber were scraped, and the ones on the lower side of the chamber were stained with Diff Quik (Dade Behring) and counted using light microscopy with a standardized grid. The cells were treated with either 0, 10, or 20 μM OSI-774 for 24 h prior and while in the invasion chambers, for a total inhibitor exposure time of 48 h. All of the experimental and control groups were done in triplicates. The percentage of invasion was calculated by dividing the mean number of cells invading through the Matrigel with those that migrate through the control. The invasion index was calculated by taking the ratio of the percentage of invasion in D54-EGFRvIII with that in D54-lacZ.

Results and Discussion

Model System. Our experimental model was to constitutively overexpress EGFRvIII in glioblastoma cells using retroviral-mediated gene transfer. Stable cell populations expressing either the β-galactosidase gene (D54-lacZ) or the EGFR deletion mutant, EGFRvIII (D54-EGFRvIII), were generated in the human glioblastoma (GBM) cell line, D54-MG. *EGFRvIII* transcript levels were 42-fold higher in D54-EGFRvIII when compared with D54-lacZ using real-time PCR (data not shown). D54-MG was chosen because it had one of the lowest *EGFR* transcript levels for a cultured glioblastoma cell line (11). Flow cytometry using an EGFRvIII-specific antibody revealed that the mutant protein was expressed and detected on the cell surface of D54-EGFRvIII (data not shown).

Expression Analysis. To find downstream targets of EGFRvIII, SAGE libraries were constructed from D54-EGFRvIII and D54-lacZ. A total of 124,177 SAGE tags representing 22,945 unique transcript tags derived from the two SAGE libraries were compared. There were 70 transcripts with 5-fold different expression below a 0.01 *P* chance. Of these 70 transcripts, 38 were induced by the stable expression of EGFRvIII and the remainder repressed. The complete transcript counts can be viewed or downloaded from the SAGEmap website. We concentrated our efforts on a subset of induced genes shown in Table 1, which included ECM proteins, some metalloproteases, a collagen, and a serine protease. Using a DNA filter array, *MMP13* was additionally identified as up-regulated by EGFRvIII (data not shown). The transcript tag levels for this gene were too low to yield a significant difference for the amount of sequencing performed and, therefore, missed by SAGE alone.

Verifying Expression Differences. Real-time PCR quantification was used to confirm the differences observed by SAGE in the D54-MG-derived cell lines and to test gene expression differences in other glioblastoma model systems that either express EGFRvIII or a control gene (Table 1). Transcript levels of EGFRvIII in these cell lines or xenografts compared with the corresponding parental or control cell line were 13–60-fold higher. The expression of the EGFRvIII downstream genes found by SAGE were also induced in other cell lines with experimental induction of the mutant protein. This indicated that the up-regulation of these genes was independent of the genetic backgrounds of different cell lines. However, the GBM cell line U87 grown instead in nude mice as a xenograft showed reduced expression of these same EGFRvIII targets (Table 1). These s.c. xenografts of U87 are not invasive but grow as well-circumscribed tumors. The host tissue environment may be influencing the expression pattern reflected by lack of invasion in this artificial tumor model. Because our one "*in vivo*" system tested thus far did not have invasion properties that corresponded to human GBM, additional evaluation of the EGFRvIII target genes and system was needed to determine whether these genes were relevant to glioblastoma pathology.

A comparison of the expression levels of the EGFRvIII target genes in normal tissues using the public gene expression database, SAGEmap, revealed that most of these genes were either absent or

Table 1 *Genes induced by EGFRvIII*

SAGE Tag sequence	Gene symbol (name)[a]	Accession no.[b]	Fold Inc SAGE D54[c]	Fold Inc PCR D54[d]	Fold Inc PCR U87c[e]	Fold Inc PCR U87x[e]	Fold Inc PCR U118[e]	Fold Inc PCR U251[e]
TAAAAACAAA	*COL8A1* (Collagen, type VIII, α 1)	BC013581	22x	154x	4.3x	(−16x)	4.6x	2.4x
ACAGATTTGA	*COL8A1* (Alternative Transcript of *COL8A1*)	AL359062	22x	30x	11.5x	(−3x)	6.6x	2.2x
GCCAAATGTT	*IL13RA2* (Interleukin 13 receptor, α 2)	NM_000640	16x	4.0x	3.8x	(−183x)	2.5x	1x
CATTTTGCTT	*FAP* (fibroblast activation protein α)	NM_004460	8x	7.3x	9.8x	5.6x	2.9x	1x
TGCAGTCACT	*MMP1* (matrix metalloproteinase 1)	NM_002421	8x	12x	5.2x	(−9.4x)	(−3.1x)	2.2x
AATCTGTCTC	*MME* (Membrane metalloendopeptidase)	NM_007289	6x	4x	7.5x	46.3x	1.5x	1x
TGCAATATGC	*FBN1* (fibrillin 1)	NM_000138	6x	4.3x	6x	(−2x)	2.4x	1x
	MMP13[f] (matrix metalloproteinase 13)	NM_002427	–	29x	2.5x	ND[g]	ND[g]	1x
	EGFRvIII[h]		–	42x	60x	18x	36x	13x

[a] HUGO/GDB nomenclature committee approved symbols for individual genes when available. Genes with no approved symbols are marked with an asterisk (*). Each gene sequence had a poly(A)signal and/or poly(A)tail and was matched to the SAGE tag.

[b] The GenBank accession number was used to identify the gene and contains the differentially expressed tag.

[c] The fold increase in gene expression predicted by SAGE was calculated by normalizing the total number of tags in the two SAGE libraries and taking the ratio of the tags in D54-EGFRvIII to D54-lacZ.

[d] The fold increase in gene expression by real-time PCR was calculated by comparing the rate of amplification of a gene-specific product in D54-EGFRvIII and D54-lacZ-derived cDNA relative to a set of standards.

[e] The fold increase in gene expression by real-time PCR in different glioblastoma cell lines (U87c, U118, and U251) and xenografts (U87x) expressing EGFRvIII compared to either the parental cell line or the same cell line expressing β-galacatosidase. Values in brackets with a negative sign indicate a fold decrease in gene expression.

[f] MMP13 was identified as induced by EGFRvIII using DNA filter arrays.

[g] There was no detectable PCR product.

[h] The fold induction of EGFRvIII was calculated in the different glioblastoma systems using real-time PCR.

expressed at very low levels in most normal tissues (Fig. 1). The exception was cultured fetal astrocytes. However, these astrocytes were cultured with supplemental epidermal growth factor in the medium, perhaps inducing some of these genes. The specific induction of the genes listed in Table 1 using mutant EGFR protein in cultured cells, in conjunction with a lack of expression in normal adult brain, suggested that additional investigation was warranted.

EGFR Inhibitor Effects. To help implicate the genes in Table 1 as EGFRvIII downstream genes, we next studied the effect of known EGFR inhibitors on the transcripts. Transcript levels of the EGFRvIII targets were inhibited in a concentration and a time-dependent manner in the presence of tyrosine kinase inhibitors (Fig. 2). Tyrphostin AG1478 is a highly specific inhibitor of EGFR (12) and inhibits EGFRvIII with a slight preference over EGFR in glioma cells (13). Inhibition by 25 μM Tyrphostin AG1478 occurred as early as 6 h after treatment and increased progressively through 24 h (Fig. 2*A*).

OSI-774 is a quinazoline derivative that inhibits the kinase activity of EGFR. It has shown efficacy in Phase I trials for epidermoid malignancies (14) and is currently being evaluated in Phase II trials (15). Inhibition by OSI-774 was tested at a concentration of 20 μM and an exposure time of 48 h. In seven of the eight selected genes, inhibition by OSI-774 resulted in at least a 50% reduction of gene expression (Fig. 2*B*). There was no effect on control genes, suggesting specificity in the transcriptional alteration. Although these inhibitors likely have transcriptional effects beyond down-regulation of EGFRvIII target genes, this observation indicates that the initial induction of ECM genes seen with SAGE was not likely an artifact of the retroviral model system. Therefore, the EGFRvIII target genes might be useful biomarkers for optimizing the efficacy of OSI-774, or other drugs targeting EGFR or EGFRvIII.

***In Vivo* Expression.** The next question was if any of these genes were induced in human tumors in addition to our cell culture models. Primary glioblastoma tissue sections were stained with an EGFRvIII-specific antibody that allowed us to classify GBMs as either EGFRvIII-positive or EGFRvIII-negative (Fig. 3). Two cases of each type were immunostained for Fibrillin-1, a component of the ECM. The two EGFRvIII-positive tumors were strongly positive for Fibrillin-1 (Fig. 3, *B* and *E*). In the case of one of them, strong staining around vessel structures was seen (Fig. 3*B*) in addition to other regions of the tumor. The EGFRvIII-negative tumors did not express Fibrillin-1 (Fig. 3, *H* and *K*). However, restricted staining around some vessel structures was observed in one of them (Fig. 3*K*). To rule out any differences in the tissue preparation that might produce the staining observed, control staining was performed with carbonic anhydrase IX antibody, a hypoxia marker (10), and mouse IgG. There was an independent pattern of hypoxia staining in all four of the sections (Fig. 3, *C*, *F*, *I*, and *L*), and staining with mouse IgG was negative (data not shown). In summary, Fibrillin-1 and EGFRvIII protein levels colocalized to the same region in all five of the glioblastomas tested using immunohistochemistry.

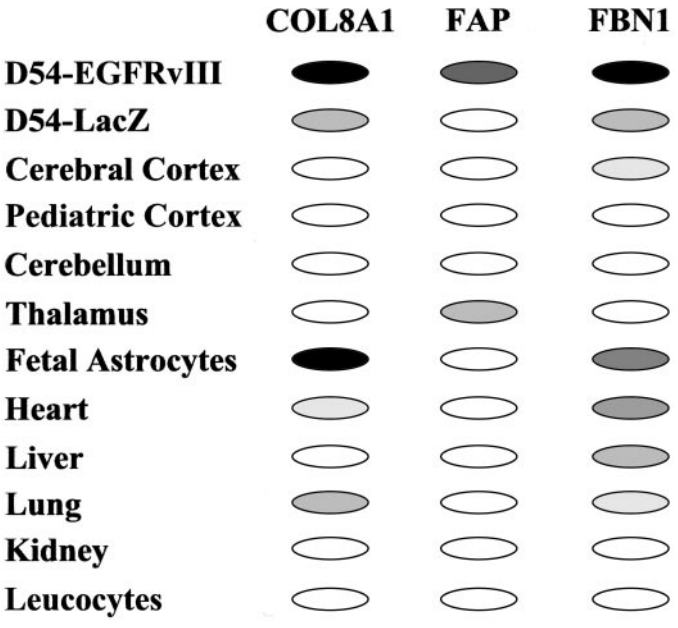

Fig. 1. Virtual Northern of three EGFRvIII target genes. SAGE libraries from a variety of normal and cancer tissues deposited in the public gene expression database SAGEmap were used to display expression levels of the EGFRvIII-induced genes, Collagen VIII A1 (*COL8A1*, alternative transcript), *FAP*, and Fibrillin-1 (*FBN1*). The SAGE libraries shown are the GBM cell lines expressing either EGFRvIII (*D54-EGFRvIII*) or β-galactosidase (*D54-LacZ*) and various normal tissues. Fetal astrocytes are untransformed human astrocytes grown in a short-term cell culture. *Blank ovals* indicate no detectable expression. *Progressive shading* indicates increasing levels of expression to a maximum of 128/100,000 SAGE tags for the *black oval*.

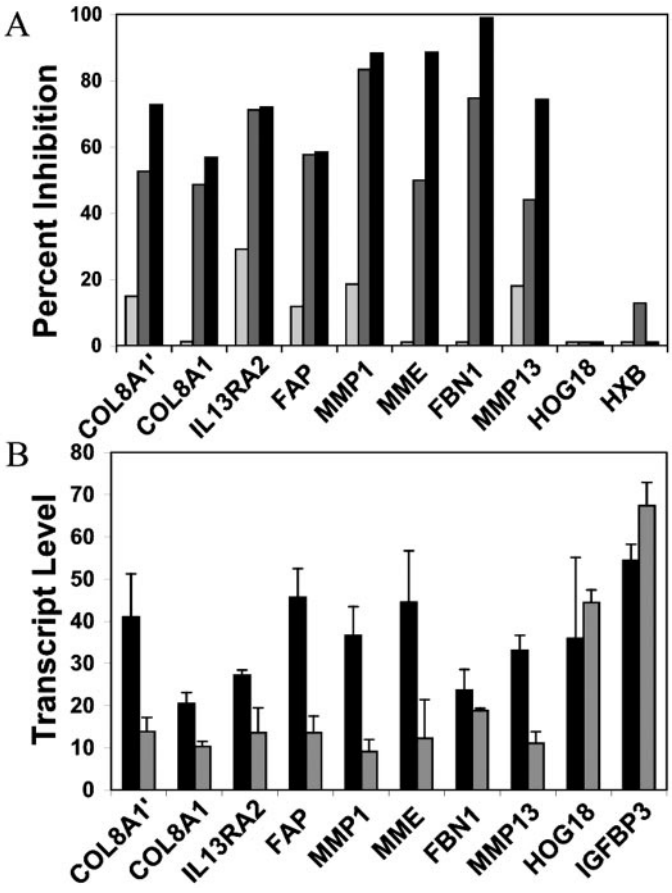

Fig. 2. EGFRvIII transcriptional targets are inhibited by the EGFR-targeting drugs Tyrphostin AG1478 and OSI-774. *A*, in a short time course, D54-EGFRvIII cells were treated with DMSO solvent or 25 μM Tyrphostin AG1478 for 6 h (*light gray columns*), 12 h (*dark gray columns*), or 24 h (*black columns*). Transcript levels were analyzed by quantitative PCR and expressed as a percentage of the total transcript levels in the control (DMSO-treated) cells. *COL8A1′* refers to a longer alternative transcript of COL8A1. *B*, OSI-774 reproducibly inhibited EGFRvIII targets at 48 h. D54-EGFRvIII cells were treated with DMSO or 20 μM OSI-774, and the transcript levels of the EGFRvIII targets were analyzed by quantitative PCR. Relative transcript levels of the EGFRvIII-regulated genes in the controls (*black columns*) and the OSI-774-treated cells (*gray columns*) are plotted. Transcript levels of *HOG18*, *HXB*, and *IGFBP3* hypoxia-regulated genes not influenced by EGFRvIII were used as controls and were unaffected by Tyrphostin AG1478 (*A*) and OSI-774 (*B*); *bars*, ±SD.

Invasion Potential. Because our data thus far supports the fact that ECM proteins and protease genes are induced by EGFRvIII, and repressed by its inhibitors, we hypothesized that EGFRvIII enhances tumor invasion in glioblastomas. Human GBMs have a particularly invasive

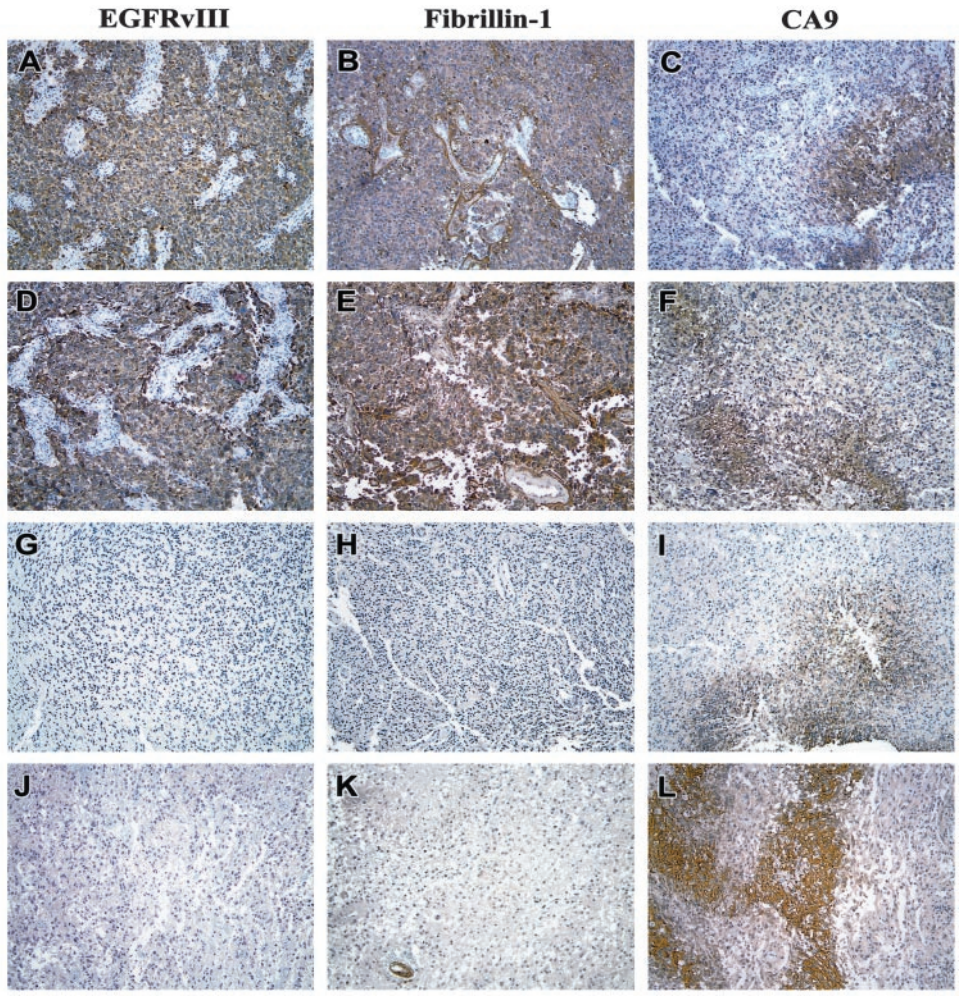

Fig. 3. *In vivo* expression of Fibrillin-1 in primary glioblastoma tumors. IHC with a monoclonal antibody specific for EGFRvIII was used to classify primary glioblastomas as EGFRvIII-positive (*A* and *D*) or EGFRvIII-negative (*G* and *J*). *In vivo* expression of Fibrillin-1 by IHC (*B*, *E*, *H*, and *K*) correlated with EGFRvIII IHC (*first column*). Sections were stained with carbonic anhydrase 9 (*C*, *F*, *I*, and *L*), a hypoxia marker, as a control showing an independent pattern of staining. Original magnification is ×10 for all panels.

phenotype. They virulently invade the surrounding normal brain, making complete surgical resection difficult. We tested the EGFRvIII-invasion hypothesis using *in vitro* scratch and membrane assays to compare the migratory and invasive potential of D54-EGFRvIII *versus* D54-lacZ cells. A scratch-wound assay provides a qualitative picture of how rapidly cells migrate back into a disrupted region from a monolayer. As anticipated, we found that D54-EGFRvIII showed greater migration than D54-lacZ, and this difference was more pronounced on fibronectin-coated plates when compared with collagen IV-coated plates (Fig. 4*A*). By 16 h, the wound was barely visible in the D54-EGFRvIII plates but was clear in the D54-lacZ plates.

A more quantitative assay, using Matrigel invasion chambers, measures the number of invading cells through a simulated ECM. In addition to having an increased migratory potential, this assay also showed that D54-EGFRvIII cells were more invasive compared with D54-lacZ (Fig. 4*B*). The invasion index was 4.3, indicating that D54-EGFRvIII cells were several-fold more invasive than D54-lacZ control cells. OSI-774 showed specific inhibition of EGFRvIII transcriptional targets at a concentration of 20 μM over a 48-h exposure time and was also tested in the invasion assays. After the application of OSI-774 to the invasion assay, there was a decrease in the invasion index from 4.3 to almost 1, removing the entire difference between the invasive potential of D54-EGFRvIII and D54-lacZ. OSI-774 decreased the invasive ability of D54-EGFRvIII, and there was negligible invasion inhibition of the D54-lacZ control. EGFRvIII appears to enhance tumor invasion, likely in part using the ECM genes identified, and EGFR inhibitors have potential to block invasive properties.

Genes Promoting Invasion. EGFR and, more recently, EGFRvIII have been implicated in invasion and a more malignant-acting tumor. In ovarian cancer, antisense EGFR and EGFR inhibitors repressed the invasive phenotype (16). Also, in small cell lung cancer, EGFRvIII enhanced *in vitro* invasion to levels comparable with those found in our study on GBMs (17). It is known that EGFRvIII enhances the tumorigenicity of GBMs. Insight is provided into the molecular mechanism of this action by the EGFRvIII-induced genes (Table 1).

Tumor cell invasion is a complex and multistep process involving interaction of the tumor cell with the extracellular barrier, proteolytic digestion of this ECM, and, finally, cell migration through the space created. MMPs aid in invasion by degrading a broad range of ECM components in gliomas and in other tumors. Both MMP1 and MMP13 are collagenases that have been implicated in tumor cell invasion. MMP13 is primarily expressed by malignant tumor cells and has been shown to markedly increase the invasion of fibrosarcoma cells through type I collagen and Matrigel (18). MMP1 expression has been related to the invasive property of a variety of cancers including melanoma (19) and ovarian tumors (20). FAP α, also known as *Seprase*, is an integral membrane serine protease that has been associated with the invasive behavior of melanoma (21) and breast carcinoma cells (22). ECM components like COL8A1 and Fibrillin-1 were also up-regulated in our model. Fibrillin-1 protein expression was correlated in this study with EGFRvIII in human glioblastomas. Our study suggests that the accumulation of a number of the above protein products assist in glioblastoma invasion.

In addition to genes with a potential role in tumor invasion,

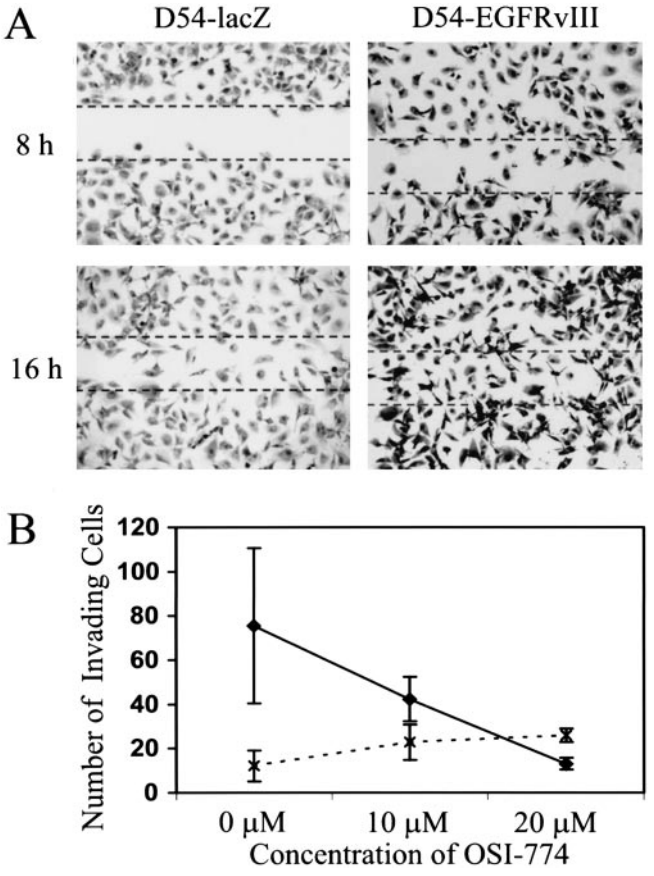

Fig. 4. Comparison of the *in vitro* migration and invasion abilities of D54-lacZ and D54-EGFRvIII. *A*, EGFRvIII-expressing cells show higher motility in a standard scratch assay. The migration of D54-lacZ and D54-EGFRvIII cells was qualitatively assessed 8 h and 16 h after the introduction of a scratch in monolayer cultures of these cells grown on fibronectin. The *gray dotted lines* represent the position of the initial scratch. *B*, the number of D54-EGFRvIII cells that invade through the Matrigel decreases as the concentration of OSI-774 increases (*solid line*), but the D54-lacZ (*dotted line*) cells show no effect or a slight increase. The Y-axis represents the number of cells invading per mm^2; *bars*, ±SD.

IL13RA1 was interesting because it had been identified previously as a marker for GBM (23). IL13R expressed in GBM tissue is both quantitatively and qualitatively different from IL13R expressed in normal tissues, and is being exploited currently for therapeutic purposes (23). Our studies indicate that expression of *IL13R* is at least partially influenced by EGFRvIII. Additional downstream targets of EGFRvIII might be exploited as therapeutic targets or as biomarkers for the pathologic effects of the mutant gene.

In conclusion, EGFRvIII induces a specific pattern of genes, and many of these genes are likely effectors of tumor invasion. The two drugs tested that target EGFR both selectively inhibit the expression of the invasive genes. The targets of EGFRvIII identified by our study provide insight into the molecular mechanism of EGFRvIII-enhanced invasion and are potential tumor markers or biomarkers for drug screens of EGFR inhibition.

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