

EGFR heterogeneity and implications for therapeutic intervention in glioblastoma

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Abstract

Patients with glioblastoma (GBM) have a universally poor prognosis and are in urgent need of effective treatment strategies. Recent advances in sequencing techniques unraveled the complete genomic landscape of GBMs and revealed profound heterogeneity of individual tumors even at the single cell level. Genomic profiling has detected epidermal growth factor receptor (EGFR) gene alterations in more than half of GBMs. Major genetic events include amplification and mutation of *EGFR*. Yet, treatment strategies targeting EGFR have thus far failed in clinical trials. In this review, we discuss the clonal and functional heterogeneity of EGFRs in GBM development and critically reassess the potential of EGFRs as therapeutic targets.

Key words

angiogenesis | EGFR | glioblastoma | invasion, targeted therapy | tumor heterogeneity

For more than a decade, standard of care for patients with newly diagnosed glioblastoma (GBM), the most common and most malignant primary brain tumor in adults, has involved surgical tumor resection and temozolomide/radiation therapy.¹ Although the molecular genetics of gliomas have been studied, a void exists in effective targeted treatment. Tumor heterogeneity may contribute broadly to the failure of molecular targeted cancer therapies. Profound heterogeneity has indeed been detected within individual GBMs even at the single cell level.^{2,3}

The majority of GBMs have been identified to harbor genetic events in receptor tyrosine kinase (RTK) signaling pathways.⁴ Among the most relevant pathways, and perhaps also the most cryptic, are those engaged by activation of epidermal growth factor receptor (EGFR).⁵ *EGFR* amplification is common in GBMs,^{4–8} and together with mutation, rearrangement, and/or altered splicing, genetic alteration of

EGFR at large has been observed in 57% of these tumors.^{4,5} A number of studies have assessed targeted intervention of EGFR in GBM using strategies such as antibodies, small-molecule tyrosine kinase inhibitors (TKIs), and vaccines; however, therapeutic benefit has not been achieved. In the present review, we examine (i) the implications of clonal and functional heterogeneity of EGFRs in GBM development and therapy resistance and (ii) the rationale for anti-EGFR targeted therapy in GBM intervention.

Glioblastoma Development and Molecular Characterization

GBM develops predominantly de novo (primary GBM) or via progression from low-grade glioma (secondary GBM).⁹

Both primary and secondary GBMs are composed of infiltrative, less well-differentiated cells than lower-grade astrocytomas, and both show characteristic microvascular proliferation and pseudopalisading necrosis.¹⁰ Mutations in the isocitrate dehydrogenase 1 (*IDH1*) or *IDH2* genes, which are early events in tumor development of low-grade gliomas,¹¹ can thus be used as molecular markers to distinguish between primary and secondary GBMs.¹² GBM is a disease of the entire brain,¹³ and while a number of its features may yield support of this notion, its invasive propensity certainly does. The unknown etiologies and mechanisms underlying GBM invasion make it a therapeutically challenging target.

In addition to the intrinsic invasive capacity of GBMs, mitotic activity and resistance to apoptosis will inevitably increase demand for vascular delivery of adequate oxygen and nutrients. Indeed, the pronounced angiogenesis observed in GBMs suggests that the tumors require it.¹⁰ Yet, tumor angiogenesis is often nonproductive.¹⁴ This is, in part, exemplified by the spontaneous “pseudopalisading” necrosis observed in GBM biopsies, which is speculated to result from a combination of increased cell proliferation/migration in hypoxic areas, along with insufficient vascularization/angiogenesis.¹⁵ Tumor cells surrounding such necrotic areas are known to express angiogenic factors. Vascular endothelial growth factor A (VEGFA) seems to be a key angiogenic factor^{16,17} in this process and is under transcriptional control of hypoxia-inducible transcription factor 1 α .¹⁸

Improved understanding of the genetic and molecular events regulating gliomas has emerged since the turn of the century. A prominent feature of this has been gene expression analysis indicating distinct molecular profiles underlying tumor heterogeneity and malignant progression.¹⁹ In 2006 it was suggested that GBM may be classified into molecular subclasses on the basis of transcriptional profiles.²⁰ More recently, upon being analyzed by The Cancer Genome Atlas (TCGA) Research Network,²¹ Verhaak and colleagues further associated the molecular signatures with alterations in DNA sequence and copy number to produce a refined classification consisting of proneural, classical, and mesenchymal subtypes.^{22,23} Importantly, while widely accepted, the molecular classification is not definite, as several subtypes might be present within different clones of the same patient tumor.²

Chromosome 10q loss of heterozygosity is the most frequently occurring gross genomic alteration in GBM, and many GBMs have lost an entire copy of chromosome 10.⁹ Meanwhile, the most prominent focal aberrations in protein coding sequences include *EGFR* amplification, *CDKN2A* deletion, *TP53* mutation, and *PTEN* mutation.⁹ The significance of these genetic aberrations in the context of GBM pathogenesis has yet to be fully elucidated; however, a convergence of a highly interconnected network of genetic aberrations on 3 fundamental signaling pathways—the RTK/RAS/phosphatidylinositol-3 kinase (PI3K), tumor protein (TP)53, and retinoblastoma pathways—has been identified.²¹ Aimed partially at facilitating the discovery of viable therapeutic targets, an expanded TCGA study effectively produced a comprehensive catalogue of somatic alterations in GBM.⁴

Receptor Tyrosine Kinases in Glioblastoma Development

Tyrosine phosphorylation is recognized to be important for signal transduction in multicellular organisms. Among a number of known functions, tyrosine phosphorylation is implicated in cellular processes, including differentiation, proliferation, migration, and survival.²⁴ RTKs are membrane-spanning proteins with N-terminal extracellular ligand-binding domains and C-terminal intracellular catalytic domains.²⁵ The majority of RTKs are activated via binding of their extracellular domain to ligands.²⁵ Ligand binding of the extracellular domain elicits RTK oligomerization and activation of the intracellular catalytic domain.²⁵ It is also proposed that active dimers can exist in the absence of ligands by mere RTK overexpression.²⁴ Activation facilitates recruitment of proteins that initiate a signaling cascade, and integration of the numerous signaling pathways subsequently results in specific cellular responses.^{24,25}

RTK encoding genes such as *EGFR*, platelet derived growth factor receptor (*PDGFRA*), and *MET* have been implicated in GBM development. These genes are often overexpressed or even amplified or coamplified in GBM.²⁶ Amplified genes are often located on extrachromosomal DNA known as double minutes.²⁷ *EGFR* is the most frequently amplified RTK (~40%–45% of GBMs). *EGFR* monomers can homodimerize or form heterodimers with other RTK family members.²⁸ This process can either (i) be dependent on ligand binding of one of the ligands EGF, transforming growth factor alpha, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, epigen, or betacellulin²⁸ with activation of canonical signaling pathways such as extracellular signal-regulated kinase (ERK) and Akt, or (ii) be independent of ligand binding, which leads to activation of interferon regulatory transcription factor 3.²⁹ In addition to amplification, *EGFR* can harbor point mutations or deletions that lead to constitutive activation of the receptor and is independent of ligand binding.³⁰ The most frequently occurring deletion is of exon 2–7 in the extracellular domain of *EGFR*, which results in the truncated mutant variant III (*EGFRvIII*).^{31–33} The cellular processes activated by *EGFR* or mutants of the receptor might be dependent on the specific cell type. Activated *EGFR* may engage a number of signaling pathways, including PI3K/Akt, Ras/Raf/Mek/ERK, signal transducer and activator of transcription 3 (STAT3), and phospholipase C gamma,³⁴ which translates to different cellular functions, including proliferation, invasion, angiogenesis, and resistance to apoptosis.

Anti-EGFR Cancer Therapy and Pharmacology

The prevalence of *EGFR* across a number of prominent cancers makes RTK an appealing target for therapeutic intervention, and a number of strategies have been pursued to achieve targeted inhibition of *EGFR* signaling. Importantly, expression of *EGFR* or other molecular targets

in all tumor cells might not be necessary to achieve a therapeutic effect, as clinical indications for targeted therapies are warranted even when the respective molecular target is expressed in a small fraction of tumor cells.³⁵

The anti-EGFR monoclonal antibody (mAb) cetuximab is thought to occupy the ligand binding domain of EGFR to prevent dimerization at the cell surface and subsequent cross-activation that initiates downstream signal transduction. Cetuximab is approved for the treatment of a subset of colorectal cancers and head and neck cancers.³⁶ While the mAb has been proposed for the treatment of non-small cell lung cancer (NSCLC), benefit has not been established. Cetuximab has also failed to demonstrate benefit in the treatment of GBM.

Gefitinib was the first EGFR-targeted small-molecule TKI to be approved. Initial clinical studies showed that gefitinib was safe, but tumor responses were observed in only a subset of patients with chemotherapy-refractory advanced NSCLC, and the addition of gefitinib to traditional chemotherapy did not provide benefit.³⁷ But the response was profound in those patients who responded, and it was identified that a subgroup of patients with NSCLC had specific mutations in the *EGFR* gene which correlate with clinical responsiveness to gefitinib.³⁷ The mutations cluster near the ATP cleft of the tyrosine kinase domain, and it was suggested that the mutations stabilized the interaction of both ATP and gefitinib with EGFR. Another first-generation EGFR-targeted small-molecule TKI, erlotinib, was shown to prolong survival in patients with NSCLC upon chemotherapy.³⁸ Similar to gefitinib, the presence of *EGFR* mutations was associated with increased responsiveness to erlotinib, although initial studies suggested that *EGFR* gene mutation was not indicative of a survival benefit from this agent.³⁹

It must be recognized that colorectal, head and neck, and lung cancers are entirely different diseases than GBMs. Aside from the differences in tissues and associated therapeutic accessibility, EGFR is also molecularly heterogeneous among these cancers. *EGFR* mutations in GBMs occur within the extracellular domain while *EGFR* mutations in lung cancers typically occur in the kinase domain.⁴⁰ GBMs, therefore, are not sensitized to first-generation EGFR inhibitors such as gefitinib and erlotinib in the same way as NSCLCs. Of course, this is thought to contribute to the limited success of these drugs in the therapeutic intervention of GBM where initial studies indicated that gefitinib and erlotinib are not generally effective.⁴¹ However, next-generation inhibitors⁴² may not produce substantially greater promise in combating GBM. Including tumor heterogeneity, a number of mechanisms have been proposed to underlie GBM resistance to EGFR-targeted therapies.⁴³ Compensatory activation of other RTKs^{44,45} and an intact blood-brain barrier (BBB) are also thought to contribute to anti-EGFR therapy failure.⁴⁶

Several clinical studies are being carried out either in newly diagnosed GBM with anti-EGFR agents in combination with standard radiochemotherapy or in recurrent/refractory tumors as monotherapy. Clinical trials with agents that did not produce satisfactory results in previous studies are aimed at achieving higher drug concentrations in the central nervous system. An overview of clinical trials

assessing anti-EGFR therapeutic strategies for GBM is provided in Table 1.

The spectrum of EGFR-targeted small molecules currently under investigation in clinical trials includes first- and second-generation TKIs. A number of the TKIs target multiple kinases, and importantly, preclinical studies suggest that some are capable of effectively crossing the BBB. One such TKI, tesevatinib,⁴⁷ is being evaluated in patients with recurrent GBM. The study will enable comparison of drug activity in GBMs with and without EGFRvIII as well as those with and without *EGFR* amplification.

A wide range of biologics are also under investigation in clinical trials, and a number of initiatives incorporate strategies to cross the BBB. A phase I clinical trial recently established that superselective intra-arterial cerebral infusion of cetuximab upon osmotic disruption of the BBB with mannitol is safe,⁴⁸ and studies are currently evaluating efficacy. Other efforts to cross the BBB and target EGFR with biologics involve utilizing convection-enhanced delivery (CED). CED of the EGFR-targeted toxin TP-38 showed some encouraging results in a phase I clinical trial,⁴⁹ and CED of the immunotoxin D2C7-IT is currently being studied.⁵⁰ D2C7-IT⁵¹ is based on the mAb D2C7, which has been shown to bind both wild-type (wt)EGFR and EGFRvIII,⁵² and preclinical studies suggest its therapeutic potential is promising.⁵³ Efficacy of ABT-414, an anti-EGFR mAb–drug conjugate reportedly capable of crossing the BBB, is also currently being evaluated.⁵⁴ ABBV-221 is a mAb–drug conjugate based on ABT-414 with higher affinity for over-expressed EGFR.⁵⁵ Particularly with respect to targeting EGFRvIII, a number of initiatives are under way to exploit anti-EGFR chimeric antigen receptor (CAR) T cells in the treatment of GBM.

Experimental Model Systems to Study EGFR Function in Glioblastoma

In order to develop more effective anti-EGFR targeted strategies, experimental model systems that reflect the genetics and behavior of patient GBMs are urgently needed. In the past decades, cell lines based on monolayer cultures in serum-containing media, such as U87, U251, and U373, have been standards for maintaining and expanding GBM cells. However, these cultures do not preserve the geno- and phenotypes of patient biopsies. In particular, *EGFR* amplification seems to disappear in monolayer cultures, while it is preserved in xenografts established from patient biopsies that are directly transplanted to animals without subculturing.⁵⁶

The inability to preserve EGFR aberrations in culture systems has necessitated alternative strategies to study EGFR functions, including overexpression of the receptors.⁵⁷ However, new culture systems have been developed that can retain *EGFR* amplification and possibly also EGFRvIII mutation. One such system was introduced decades ago by Bjerkvig and colleagues, who cultured biopsy spheroids in flasks covered with agar in an effort to prevent cell attachment.⁵⁸ This approach preserves the pheno- and genotypes of patient biopsies.⁵⁹ We recently showed that this

Table 1 Investigational EGFR-targeted therapies for adult high-grade gliomas

Agent Name	Class	Mechanism of Action	References of Clinical Study Results	Ongoing Clinical Trials	Development Status
Erlotinib	Small molecule	1st generation TKI (EGFR selective)	⁴⁶ for review	NCT01257594 NCT02239952	Phase I
Gefitinib	Small molecule	1st generation TKI (EGFR selective)			*
Lapatinib	Small molecule	1st generation TKI (dual EGFR and HER2/neu)		NCT01591577 NCT02101905	Phase II
Afatinib	Small molecule	2nd generation TKI (pan-erbB)		NCT02423525	Phase I
Vandetanib	Small molecule	1st generation TKI (EGFR, VEGFR, and RET multitarget)	⁹⁶	NCT02239952	Phase I
Dacomitinib	Small molecule	2nd generation TKI (pan-erbB)	⁹⁷	NCT01112527 NCT01520870	Phase II
Tesevatinib	Small molecule	2nd generation TKI (EGFR, HER2/neu and Src multitarget)		NCT02844439	Phase II
Cetuximab	Biologic	Chimeric mAb	⁴⁸	NCT02800486 NCT02861898	Phase II
Nimotuzumab	Biologic	Humanized mAb	⁹⁸		*
Sym004	Biologic	mAb mixture		NCT02540161	Phase II
(125)I-mAb 425	Biologic	Radiolabeled murine mAb	⁹⁹		*
EGFR(V)-EDV-Dox	Biologic	Toxin-loaded minicell-mAb conjugate	¹⁰⁰	NCT02766699	Phase I
ABT-414	Biologic	mAb-drug conjugate	⁵⁴	NCT02573324 NCT02343406 NCT02590263	Phase II
ABBV-221	Biologic	mAb-drug conjugate		NCT02365662	Phase I
D2C7-IT	Biologic	Recombinant mAb immunotoxin		NCT02303678	Phase I
TP-38	Biologic	Recombinant ligand toxin	⁴⁹		*
Rindopepimut	Biologic	Vaccine	⁹⁰		*
Unknown	Biologic	CART cells		NCT02331693	Phase I
Unknown	Biologic	CART cells		NCT02844062	Phase I
Unknown	Biologic	CART cells		NCT02209376	Phase I
Unknown	Biologic	CART cells		NCT01454596	Phase I
Unknown	Biologic	CART cells		NCT02664363	Phase I

*Indicates that development has been discontinued or status is not available.

culture system additionally maintains *EGFR* amplification, particularly in tumor cells with high levels of *EGFR* amplification.⁶⁰ We further demonstrated that *EGFR* amplification is not lost in other culture systems but GBM cells are rather outgrown by other cell populations that do not harbor *EGFR* amplification.⁶⁰ An additional culture condition, which is now established as a standard for GBM research, is based on the formation of neurospheres in neural basal medium supplemented with basic fibroblast growth factor and EGF. This culture system also better preserves patient genotypes compared with traditional monolayer cultures.⁶¹ Meanwhile, Schulte et al observed that the addition of EGF to the cultures has a negative effect on the

expansion of *EGFR*-amplified cells and, accordingly, may be omitted when culturing these cells.⁶² The establishment of new culture methods provides an important platform to study the impact of endogenous *EGFR* alterations.

Wild-type EGFR Function and Signaling in Glioblastoma

Tumor cell invasion is a hallmark of diffuse gliomas⁶³ and is regarded as a major escape mechanism of targeted therapies. Anti-angiogenic therapy for GBM has largely failed

in clinical trials,⁶⁴ most likely due to enhanced invasive properties of tumor cells by different mechanisms, including RTK signaling.^{65,66} We recently showed in clinically relevant animal models that wtEGFR is an important mediator of tumor cell invasion independent of angiogenesis *in vivo*.⁶⁷ The tumor cells were derived from *EGFR*-amplified patient specimens and, upon implantation into nude rat brains, developed diffusely invasive tumors without inducing angiogenesis even at late stages. While these tumors responded to treatment with cetuximab,⁶⁷ treatment with the anti-angiogenic agent bevacizumab did not affect tumor growth (unpublished data). Similar results have been reported using cetuximab and the antibody DC101 against VEGFR-2.⁶⁸ While a few reports have indicated that wtEGFR activation in GBM cells can lead to increased secretion of angiogenic factors, in particular VEGFA, these studies were based on *in vitro* experiments with serum-cultured GBM cell lines that overexpress the receptor.^{69,70} The majority of *in vivo* studies indicate that wtEGFR is an important mediator of tumor cell invasion. As illustrated in a schematic of EGFR signaling in GBM (Fig. 1), classical downstream signaling pathways such as Ras/Raf/Mek/ERK may be involved in the invasive process downstream of wtEGFR,^{71,72} and we have effectively inhibited GBM cell invasion *in vitro* using ERK inhibitors (unpublished data).

Although a major hallmark of GBM, angiogenesis is evidently not required for growth of some GBM cell subpopulations. Tumors can escape angiogenesis inhibition by enhancing invasive tumor growth. And what about the reverse scenario? By inactivating wtEGFR signaling we demonstrated that tumors can switch from highly invasive, angiogenesis-independent growth to profoundly

angiogenic and less invasive growth.⁶⁷ The switch to angiogenesis was associated with upregulation of the transcription factors STAT3, CCAAT-enhancer binding homologous protein beta, and basic helix-loop-helix family member e40, which are key regulators of the mesenchymal GBM subtype.⁷³ This suggests that therapeutic targeting of wtEGFR may drive a switch to a more angiogenic and mesenchymal tumor phenotype. Interestingly, it has been shown that the switch to the mesenchymal subtype is a common escape mechanism of GBM cells after therapy.⁷⁴

EGFRvIII Function and Signaling in Glioblastoma

While EGFRvIII alone lacks the ability to transform cells, in the context of other mutations, it can contribute to transformation of normal cells.⁷⁵ Nagane et al showed that by overexpressing EGFRvIII in GBM cell lines, constitutive phosphorylation of the receptor confers enhanced tumorigenicity by increasing proliferation and reducing apoptosis.⁷⁶ In GBM stemlike cells, we recently demonstrated that EGFRvIII promotes angiogenic tumor growth, while activation of wtEGFR enhances tumor cell invasion. We deduced that wtEGFR and EGFRvIII elicit differential signaling cascades to drive different growth modalities—perhaps a scenario in which pathway engagement is shifted differentially between the 2 receptors. Experiments indeed revealed differential signaling orchestrated by wtEGFR and EGFRvIII.⁷⁷ By analyzing the role of Src family kinases as signaling partners for EGFRvIII, we observed that c-Src was specifically upregulated and activated downstream of EGFRvIII and responsible for the angiogenic tumor growth mediated by EGFRvIII.⁷⁷ Others have shown that nuclear factor-kappaB/interleukin-8, c-Myc/angiopoietin-like 4, and tissue factor might be additional important targets downstream of EGFRvIII that promote angiogenesis in GBM models^{78–80} (Fig. 1). EGFRvIII might also be involved in the invasive process of GBMs,⁸¹ and because wtEGFR and EGFRvIII are usually co-expressed, signaling pathways might converge to stimulate both invasion and angiogenesis.⁸²

In addition to EGFRvIII, several EGFR-activating point mutations, which are frequently located in the extracellular domain of EGFR, have been detected in GBM samples.⁴ These mutations have oncogenic activity which seems to be similar to that of EGFRvIII.³⁰ To analyze whether or not other *EGFR* mutations might be important for angiogenesis in GBM, we performed correlation analysis across samples registered with TCGA (Fig. 2). We detected a slight correlation between EGFR and VEGFA expression when considering only GBM samples with *EGFR* amplification ($n = 171$, correlation = 0.168, $P = 0.028$), but no correlation was identified in those without *EGFR* amplification ($n = 160$, correlation = -0.022 , $P = 0.781$) or when considering all GBM samples together ($n = 344$, correlation = 0.072, $P = 0.183$). Interestingly, strong correlation between EGFR and VEGFA expression in GBM samples with *EGFR* amplification was limited to those that additionally harbored EGFRvIII ($n = 26$, correlation = 0.423, $P = 0.031$) and/or *EGFR* missense mutations ($n = 37$, correlation = 0.345, $P = 0.037$).

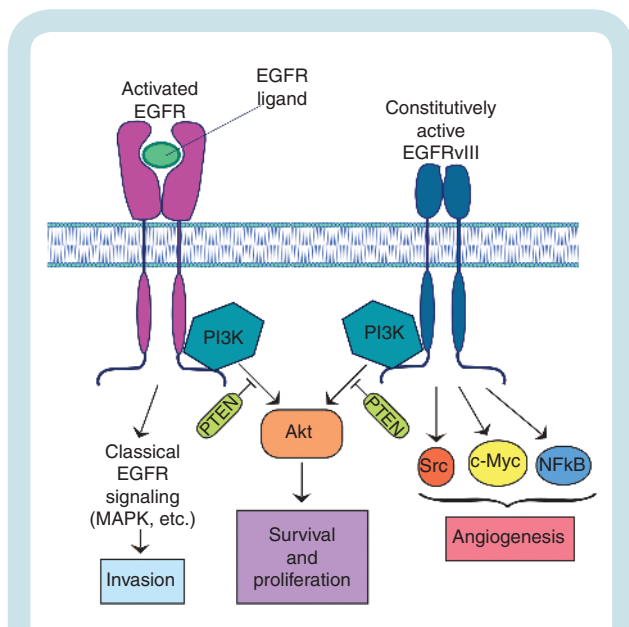


Fig. 1 Schematic of EGFR signaling in GBM. Wild-type EGFR promotes GBM cell invasion through classical EGFR signaling pathways, while constitutive active EGFRvIII fosters angiogenesis through activation of different oncogenic pathways. Both receptors promote GBM cell proliferation and survival through PI3K/Akt activation.

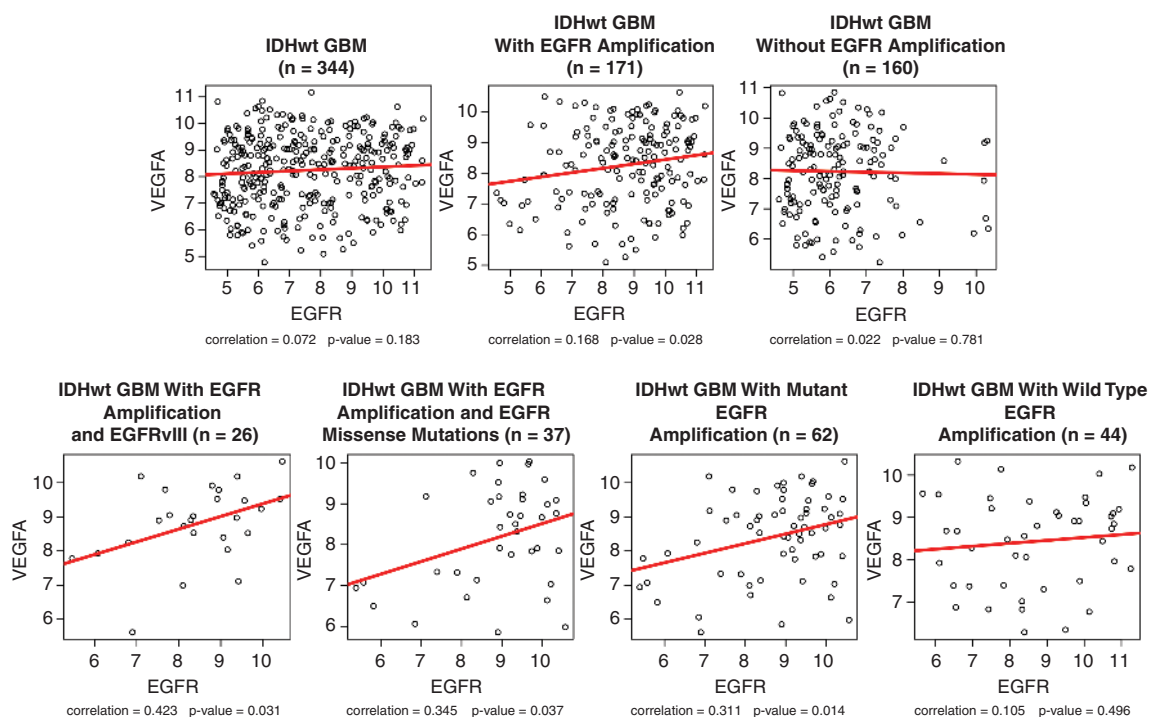


Fig. 2 Expression analysis of EGFR vs VEGFA in TCGA GBMs. TCGA GBM expression array (platform: AffyU133a, version: 2016-08-16), somatic mutation (platform: IlluminaGA, assembly: hg19, method = RADIA, version: 2016-08-16), and copy number (type: gene-level GISTIC2 thresholded, version: 2016-08-16)⁹⁴ data were downloaded from the University of California–Santa Cruz Xena Public Data Hubs website at <http://xena.ucsc.edu/>. Previously identified IDHwt, non–glioma cytosine-phosphate-guanine island methylator phenotype primary GBMs⁴ are succinctly labeled as IDHwt GBM throughout the figure. GBMs with amplified EGFR reflect those with high *EGFR* amplification (ie, thresholded values of Genomic Identification of Significant Targets in Cancer [GISTIC] = 2), and GBMs without EGFR amplification reflect those with low or no *EGFR* amplification (ie, GISTIC2 thresholded values <2). Where indicated, EGFRVIII status was assigned as previously identified.⁹⁵ GBMs which are EGFRVIII-positive and/or have *EGFR* missense mutations are labeled as harboring mutant EGFR. GBMs which do not have *EGFR* missense mutations and are EGFRVIII-negative are labeled as harboring wild-type EGFR. Pearson correlation coefficients and their *P*-values were calculated using R v3.3.2 in conjunction with RStudio. Trend lines were determined by linear regression model.

One sample was identified in both datasets, and upon examining the 2 datasets together, correlation between EGFR and VEGFA expression remained high as the significance increased ($n = 62$, correlation = 0.311, $P = 0.014$). Meanwhile, EGFR expression did not correlate with VEGFA expression in *EGFR*-amplified GBMs with wtEGFR ($n = 44$, correlation = 0.105, $P = 0.496$). Combined, our results suggest that *EGFR* mutations, but not *EGFR* amplification, are important for VEGFA upregulation. Contrary to wtEGFR, *EGFR* missense mutations may have oncogenic/angiogenic functions similar to EGFRVIII. Further evaluation in larger datasets such as those proposed⁸³ may yield additional insight.

Heterogeneity of EGFRs in Glioblastoma Development

Tumor heterogeneity has been shown for many different tumor types, even on the single cell level. By analyzing patient samples acquired through a unique surgical

multisampling technique, we recently described how *EGFR* amplification and EGFRVIII mutations evolve during tumor evolution.⁷⁷ While *EGFR* amplification was observed in all samples of individual patients, EGFRVIII mutations were only detectable in subclones of the tumor, which suggests they are late events in tumor development.⁷⁷ Heterogeneity of EGFRVIII has also been observed on the protein level.⁸⁴ In contrast, wtEGFR expression is much more abundant and lacks the profound heterogeneity observed of EGFRVIII. However, EGFRVIII might control wtEGFR function by inducing a cytokine circuit which activates wtEGFR.⁸⁵ Heterogeneity of another mutation, EGFRVII, which is less frequent in GBM, has been detected using single cell sequencing.⁸⁶ Accordingly, certain selection pressures within the tumor microenvironment seem to favor the occurrence of EGFRVIII or other mutations in *EGFR*-amplified tumor cells at later stages of tumor development. *EGFR* mutations, which are present in subclones at diagnosis, might get lost at tumor recurrence as other dominant clones that do not harbor *EGFR* mutations take over. This hypothesis is supported by a recent study showing loss of EGFRVIII in a fraction of recurrent tumors.⁸⁷ Another scenario, termed mutational

switching, refers to replacement of one *EGFR* mutation by another upon tumor recurrence and was recently described for GBM.⁸⁸ This indicates that both occurrence and disappearance of *EGFR* mutations are frequent processes which significantly contribute to tumor heterogeneity.

Apart from the clonal heterogeneity of EGFRs, there might be a profound functional heterogeneity between amplified wtEGFR and mutated EGFRs as described and analyzed herein. Sequential *EGFR* amplification and *EGFR* mutation are aligned with GBM pathophysiology, as tumors in the early phase of development may not depend on angiogenesis due to the rich vasculature present in the normal brain. This is also demonstrated in secondary GBMs, which develop through progression from invasive, lower-grade tumors. *EGFR* amplification and subsequent activation of a classical EGFR signaling cascade will lead to enhanced invasion of tumor cells able to co-opt host vasculature. Angiogenesis is required later in tumor development to survive in hypoxic environments. Environmental pressures are regional and can explain the focal emergence of EGFRvIII and other *EGFR* mutations which promote angiogenesis through oncogenic signaling (Fig. 3).

Impact of Tumor Heterogeneity on EGFRs as Therapeutic Targets

Due to recently accumulated knowledge related to heterogeneity of *EGFR* mutations, it is highly debated whether or not they are still important targets for treatment of GBMs. In this regard, it was recently shown that EGFRvIII can be eliminated from extrachromosomal DNA of tumor cells as a resistance mechanism when tumor cells are treated with EGFR TKIs. However, upon drug removal, EGFRvIII reappears.⁸⁹ Thus, while the exact mechanisms remain poorly understood, the elimination of *EGFR* mutations and their reappearance under certain conditions highlight

the flexibility of GBM cells to shape their mutational repertoire. A recent study additionally showed that EGFRvIII, although present in the primary tumor, was not detected in approximately half of recurrent tumors after standard therapy, suggesting that tumor growth may not be dependent on EGFRvIII.⁸⁷ Clonal subpopulations of the tumor might benefit from *EGFR* mutations, but oncogene “addiction” is likely not at play. Results from a phase III clinical trial in which GBM patients were treated with the EGFRvIII vaccine rindopepimut support this hypothesis, as it failed to significantly impact patient overall survival.⁹⁰ Additional support comes from the observation that EGFRvIII was not detectable in recurrent tumors after rindopepimut vaccination therapy in a phase II trial.⁹¹ However, what about amplification of wtEGFR, which is present in the majority of tumor cells and seems to occur much earlier in tumorigenesis compared with *EGFR* mutations? In this case, systemic delivery of many anti-EGFR therapies might not be effective, as *EGFR*-amplified cells are highly invasive and detected in areas with an intact BBB. Clinical trials have indeed revealed disappointing results. Yet, we and others have shown in experimental models that local delivery of cetuximab produces a significant therapeutic effect in orthotopic xenograft models harboring *EGFR* amplification.^{67,68} Clinical studies using CED or other methods of overcoming the BBB⁴⁶ are necessary to investigate whether or not EGFR-targeted therapies are effective.

Conclusions and Perspectives

The EGFR signaling landscape is exceedingly influential in GBM development. We and others have shown that aberrant expression of EGFR is a major driver of GBM invasion and angiogenesis.^{67,77} Yet, therapeutic targeting of EGFR has failed to produce efficacy in the clinic. Based on recent results from clinical trials and observations, the role of

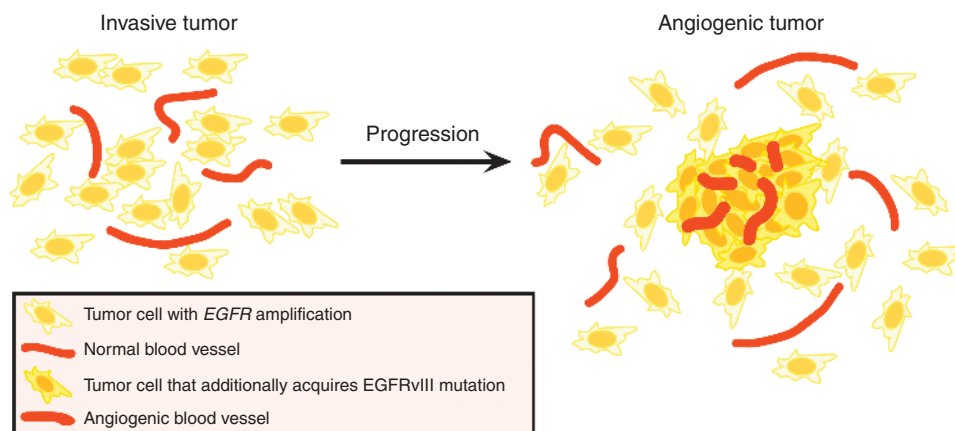


Fig. 3 Functional heterogeneity of EGFRs in GBM development. EGFR amplification is acquired by GBM cells early in tumorigenesis and substantially contributes to the invasive process. Upon tumor progression, GBM cells acquire EGFRvIII mutations which contribute to the angiogenic switch and more aggressive tumor growth.

EGFRvIII as a target for future therapies should be critically revised due to its subclonal presence and elimination from recurrent tumors upon therapy. This heterogeneity also pertains to other *EGFR* mutations. Targeting wtEGFR, meanwhile, might still be a valid possibility. Tumor cells in *EGFR*-amplified GBMs often express wtEGFR, and it promotes invasion, which is a major cause of tumor recurrence.

An important reason for failure of anti-EGFR therapies might be compensatory upregulation of other RTKs, including PDGFRA and MET or other pathways such as the recently identified tumor necrosis factor- α -c-Jun N-terminal kinase-Axl-ERK signaling axis.^{44,45,92} These receptors/pathways might also be drivers in other subclones of the same tumor and, accordingly, might mediate important escape mechanisms. In the future, this phenomenon may implicate the application of combinatorial treatments upon carefully analyzing the subclonal distribution of RTKs in individual patient tumors.

Inefficient drug penetration and distribution in the CNS might be another major reason for failure of anti-EGFR therapies in clinical trials. The BBB, which is intact in invasive tumor areas, limits effective drug delivery and likely undermines strategies to exploit systemic administration of otherwise effective targeted therapies. Although small-molecule TKIs should overcome the BBB, data from experimental studies show that these drugs may reach low concentrations at the target site upon systemic delivery due to elimination by drug transporters in endothelial cells.⁹³

As highlighted in this review, a number of initiatives are under way to effectively target EGFRs in GBM. Some initiatives involve overcoming the BBB with small-molecule TKIs or biologics. Anti-EGFR CART cells are an emerging technology in the treatment of GBM, so it is worth noting that the FDA recently approved the first CART cell therapy for a subset of patients with B-cell precursor acute lymphoblastic leukemia. Additional strategies for targeting EGFRs in GBM are in development.⁴⁶

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