Neuro-Oncology

20(6), 743–752, 2018 | doi:10.1093/neuonc/nox191 | Advance Access date 10 October 2017

EGFR heterogeneity and implications for therapeutic intervention in glioblastoma

Eskil Eskilsson, Gro V. Røsland, Gergely Solecki, Qianghu Wang, Patrick N. Harter, Grazia Graziani, Roel G. W. Verhaak, Frank Winkler, Rolf Bjerkvig, and Hrvoje Miletic

Department of Genomic Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA (E.E., Q.W., R.G.W.V.); Department of Biomedicine, University of Bergen, Norway (G.V.R., R.B., H.M.); Department of Neurooncology, University Hospital Heidelberg, Germany (G.S., F.W.); Department of Bioinformatics and Computational Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA (Q.W., R.G.W.V.); Edinger-Institute, Goethe-University Medical School, Frankfurt am Main, Germany (P.N.H.); Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy (G.G.); The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut, USA (R.G.W.V.); KG Jebsen Brain Tumor Research Center, University of Bergen, Norway (R.B., H.M.); Norlux Neuro-Oncology Laboratory, Department of Oncology, Luxembourg Institute of Health, Luxembourg (R.B.); Department of Pathology, Haukeland University Hospital, Bergen, Norway (H.M.)

Corresponding Author: Hrvoje Miletic, MD, PhD, Department of Pathology, Haukeland University Hospital, Jonas Lies vei 65, 5021 Bergen, Norway (Hrvoje.miletic@uib.no); and Eskil Eskilsson, PhD, Department of Genomic Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77054 (eskileskilsson@gmail.com).

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 1α.[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 betacellulin[28] 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 inhibitors[42] 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-IT[51] 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 overexpressed 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 gliomas[63] 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]

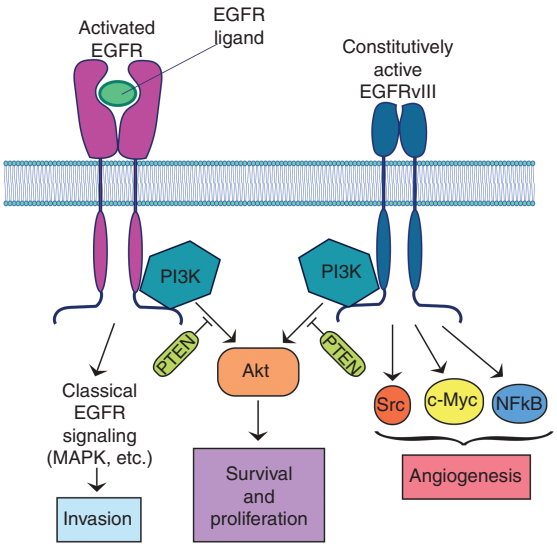

Fig. 1 Schematic of EGFR signaling in GBM. Wild-type EGFR promotes GBM cell invasion through classical EGFR signaling pathways, while constitutive active EGFRvIII fosters angiogenesis through activation of different oncogenic pathways. Both receptors promote GBM cell proliferation and survival through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

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).

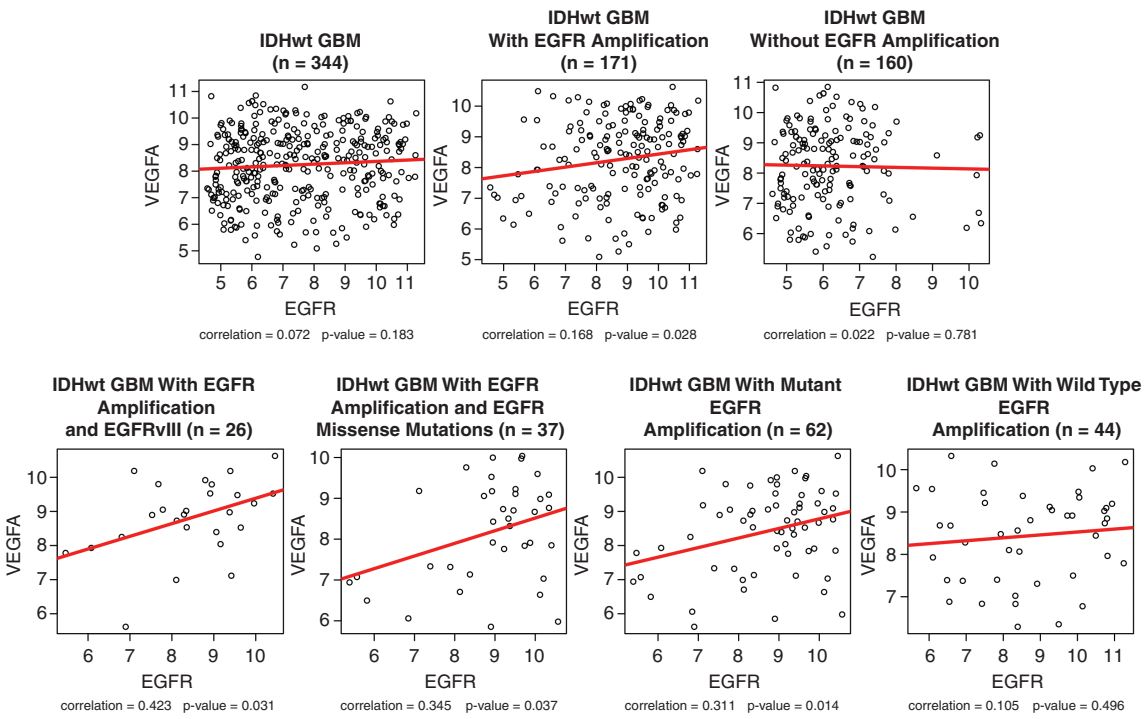

Fig. 2 Expression analysis of EGFR vs VEGFA in TCGA GBMs. TCGA GBM expression array (platform: AffyU133a, version: 2016-08-16), somatic mutation (platform: IlluminaGA, assembly: hg19, method = RADIA, version: 2016-08-16), and copy number (type: gene-level GISTIC2 thresholded, version: 2016-08-16)[94] 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 GBMs[4] 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 proposed[83] 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 orthothopic xenograft models harboring *EGFR* amplification.[67,68] Clinical studies using CED or other methods of overcoming the BBB[46] 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

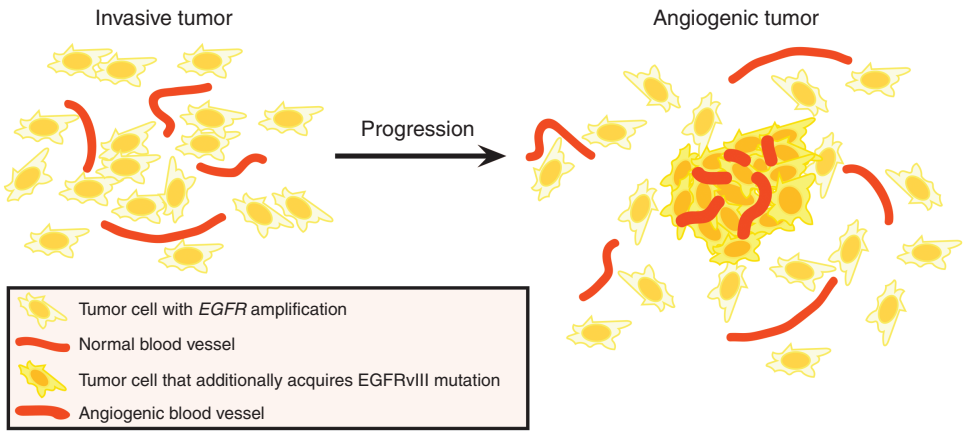

Fig. 3 Functional heterogeneity of EGFRs in GBM development. EGFR amplification is acquired by GBM cells early in tumorigenesis and substantially contributes to the invasive process. Upon tumor progression, GBM cells acquire EGFRvIII mutations which contribute to the angiogenic switch and more aggressive 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–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 CAR T cells are an emerging technology in the treatment of GBM, so it is worth noting that the FDA recently approved the first CAR T 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]

Funding

This work was supported by the Research Council of Norway, the Norwegian Cancer Society, Helse Vest, Haukeland University Hospital, University of Bergen, the K. G. Jebsen Research Foundation, and a grant to G.G. from the Italian Association for Cancer Research (AIRC 2013 IG 14042).

Conflict of interest statement. The authors declare that they have no conflicts of interest.

References

1. Stupp R, Mason WP, van den Bent MJ, et al; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352(10):987–996.
2. Sottoriva A, Spiteri I, Piccirillo SG, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A*. 2013; 110(10):4009–4014.
3. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science*. 2014;344(6190):1396–1401.
4. Brennan CW, Verhaak RG, McKenna A, et al; TCGA Research Network. The somatic genomic landscape of glioblastoma. *Cell*. 2013;155(2):462–477.
5. Furnari FB, Cloughesy TF, Cavenee WK, Mischel PS. Heterogeneity of epidermal growth factor receptor signalling networks in glioblastoma. *Nat Rev Cancer*. 2015;15(5):302–310.
6. Chaffanet M, Chauvin C, Lainé M, et al. EGF receptor amplification and expression in human brain tumours. *Eur J Cancer*. 1992;28(1):11–17.
7. Libermann TA, Nusbaum HR, Razon N, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature*. 1985;313(5998):144–147.
8. Libermann TA, Razon N, Bartal AD, Yarden Y, Schlessinger J, Soreq H. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Res*. 1984;44(2):753–760.
9. Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. *Am J Pathol*. 2007;170(5):1445–1453.
10. Maher EA, Furnari FB, Bachoo RM, et al. Malignant glioma: genetics and biology of a grave matter. *Genes Dev*. 2001;15(11):1311–1333.
11. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med*. 2009;360(8):765–773.
12. Lai A, Kharbanda S, Pope WB, et al. Evidence for sequenced molecular evolution of IDH1 mutant glioblastoma from a distinct cell of origin. *J Clin Oncol*. 2011;29(34):4482–4490.
13. Agarwal S, Sane R, Oberoi R, Ohlfest JR, Elmquist WF. Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. *Expert Rev Mol Med*. 2011;13:e17.
14. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov*. 2011;10(6):417–427.
15. Brat DJ, Van Meir EG. Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis, and accelerated growth in glioblastoma. *Lab Invest*. 2004;84(4):397–405.
16. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. 1992;359(6398):845–848.
17. Miletic H, Niclou SP, Johansson M, Bjerkvig R. Anti-VEGF therapies for malignant glioma: treatment effects and escape mechanisms. *Expert Opin Ther Targets*. 2009;13(4):455–468.
18. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 1992;359(6398):843–845.
19. Nigro JM, Misra A, Zhang L, et al. Integrated array-comparative genomic hybridization and expression array profiles identify clinically relevant molecular subtypes of glioblastoma. *Cancer Res*. 2005;65(5):1678–1686.
20. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell*. 2006;9(3):157–173.
21. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008; 455(7216):1061–1068.
22. Verhaak RG, Hoadley KA, Purdom E, et al; Cancer Genome Atlas Research Network. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17(1):98–110.

23. Wang Q, Hu B, Hu X, et al. Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. *Cancer Cell*. 2017;32(1):42–56.e6.
24. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000;103(2):211–225.
25. Hunter T. Tyrosine phosphorylation: thirty years and counting. *Curr Opin Cell Biol*. 2009;21(2):140–146.
26. Snuderl M, Fazlollahi L, Le LP, et al. Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. *Cancer Cell*. 2011;20(6):810–817.
27. Bigner SH, Wong AJ, Mark J, et al. Relationship between gene amplification and chromosomal deviations in malignant human gliomas. *Cancer Genet Cytogenet*. 1987;29(1):165–170.
28. Schneider MR, Wolf E. The epidermal growth factor receptor ligands at a glance. *J Cell Physiol*. 2009;218(3):460–466.
29. Guo G, Gong K, Wohlfeld B, Hatanpaa KJ, Zhao D, Habib AA. Ligand-independent EGFR signaling. *Cancer Res*. 2015;75(17):3436–3441.
30. Lee JC, Vivanco I, Beroukhim R, et al. Epidermal growth factor receptor activation in glioblastoma through novel missense mutations in the extracellular domain. *PLoS Med*. 2006;3(12):e485.
31. Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res*. 2000;60(5):1383–1387.
32. Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc Natl Acad Sci U S A*. 1990; 87(21):8602–8606.
33. Humphrey PA, Wong AJ, Vogelstein B, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc Natl Acad Sci U S A*. 1990; 87(11):4207–4211.
34. Han W, Lo HW. Landscape of EGFR signaling network in human cancers: biology and therapeutic response in relation to receptor subcellular locations. *Cancer Lett*. 2012;318(2):124–134.
35. Früh M, Pless M. EGFR IHC score for selection of cetuximab treatment: ready for clinical practice? *Transl Lung Cancer Res*. 2012;1(2):145–146.
36. Wee P, Wang Z. Epidermal growth factor receptor cell proliferation signaling pathways. *Cancers*. 2017; 9(5).
37. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350(21):2129–2139.
38. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al; National Cancer Institute of Canada Clinical Trials Group. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med*. 2005;353(2):123–132.
39. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. *N Engl J Med*. 2005;353(2):133–144.
40. Vivanco I, Robins HI, Rohle D, et al. Differential sensitivity of glioma- versus lung cancer-specific EGFR mutations to EGFR kinase inhibitors. *Cancer Discov*. 2012;2(5):458–471.
41. Brandes AA, Franceschi E, Tosoni A, Hegi ME, Stupp R. Epidermal growth factor receptor inhibitors in neuro-oncology: hopes and disappointments. *Clin Cancer Res*. 2008;14(4):957–960.
42. Reardon DA, Wen PY, Mellinghoff IK. Targeted molecular therapies against epidermal growth factor receptor: past experiences and challenges. *Neuro-oncology*. 2014; 16(Suppl 8):viii7–13.
43. Thorne AH, Zanca C, Furnari F. Epidermal growth factor receptor targeting and challenges in glioblastoma. *Neuro Oncol*. 2016;18(7):914–918.
44. Stommel JM, Kimmelman AC, Ying H, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science*. 2007;318(5848):287–290.
45. Clark PA, Iida M, Treisman DM, et al. Activation of multiple ERBB family receptors mediates glioblastoma cancer stem-like cell resistance to EGFR-targeted inhibition. *Neoplasia*. 2012;14(5):420–428.
46. Westphal M, Maire CL, Lamszus K. EGFR as a target for glioblastoma treatment: an unfulfilled promise. *CNS drugs*. 2017.
47. Tonra JR, Poyurovsky M, Liu KG, et al. Abstract 2590: KD019: blood brain barrier penetrant HER2/neu, Src, and EGFR inhibitor. *Cancer Res*. 2015; 75(15 Supplement):2590-2590.
48. Chakraborty S, Filippi CG, Wong T, et al. Superselective intraarterial cerebral infusion of cetuximab after osmotic blood/brain barrier disruption for recurrent malignant glioma: phase I study. *J Neurooncol*. 2016;128(3):405–415.
49. Sampson JH, Akabani G, Archer GE, et al. Intracerebral infusion of an EGFR-targeted toxin in recurrent malignant brain tumors. *Neuro Oncol*. 2008;10(3):320–329.
50. Randazzo D, Desjardins A, Chandramohan V, et al. Phase 1 single-center, dose escalation study of D2C7-IT administered intratumorally via convection-enhanced delivery for adult patients with recurrent malignant glioma. *J Clin Oncol*. 2017; 35(15_suppl):e13532–e13532.
51. Chandramohan V, Bao X, Keir ST, et al. Construction of an immunotoxin, D2C7-(scdsFv)-PE38KDEL, targeting EGFRwt and EGFRvIII for brain tumor therapy. *Clin Cancer Res*. 2013;19(17):4717–4727.
52. Zalutsky MR, Boskovitz A, Kuan CT, et al. Radioimmunotargeting of malignant glioma by monoclonal antibody D2C7 reactive against both wild-type and variant III mutant epidermal growth factor receptors. *Nucl Med Biol*. 2012;39(1):23–34.
53. Bao X, Pastan I, Bigner DD, Chandramohan V. EGFR/EGFRvIII-targeted immunotoxin therapy for the treatment of glioblastomas via convection-enhanced delivery. *Receptors Clin Investig*. 2016; 3(4).
54. Reardon DA, Lassman AB, van den Bent M, et al. Efficacy and safety results of ABT-414 in combination with radiation and temozolomide in newly diagnosed glioblastoma. *Neuro Oncol*. 2017;19(7):965–975.
55. Calvo E, Cleary JM, Moreno V, et al. Preliminary results from a phase 1 study of the antibody-drug conjugate ABBV-221 in patients with solid tumors likely to express EGFR. *J Clin Oncol*. 2017; 35(15_suppl):2510-2510.
56. Pandita A, Aldape KD, Zadeh G, Guha A, James CD. Contrasting in vivo and in vitro fates of glioblastoma cell subpopulations with amplified EGFR. *Genes Chromosomes Cancer*. 2004;39(1):29–36.
57. Mishima K, Johns TG, Luwor RB, et al. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of monoclonal antibody (mAb) 806, a novel monoclonal antibody directed to the receptor. *Cancer Res*. 2001;61(14):5349–5354.
58. Bjerkvig R, Tønnesen A, Laerum OD, Backlund EO. Multicellular tumor spheroids from human gliomas maintained in organ culture. *J Neurosurg*. 1990;72(3):463–475.
59. Sakariassen PO, Prestegarden L, Wang J, et al. Angiogenesis-independent tumor growth mediated by stem-like cancer cells. *Proc Natl Acad Sci U S A*. 2006; 103(44):16466–16471.
60. Talasila KM, Brekka N, Mangseth K, et al. Tumor versus stromal cells in culture—survival of the fittest? *PLoS One*. 2013;8(12):e81183.
61. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell*. 2006;9(5):391–403.
62. Schulte A, Günther HS, Martens T, et al. Glioblastoma stem-like cell lines with either maintenance or loss of high-level EGFR amplification, generated via modulation of ligand concentration. *Clin Cancer Res*. 2012;18(7):1901–1913.
63. Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. *J Neurooncol*. 2004;70(2):217–228.

64. Niyazi M, Harter PN, Hattingen E, et al. Bevacizumab and radiotherapy for the treatment of glioblastoma: brothers in arms or unholy alliance? *Oncotarget*. 2016;7(3):2313–2328.
65. Jahangiri A, De Lay M, Miller LM, et al. Gene expression profile identifies tyrosine kinase c-Met as a targetable mediator of antiangiogenic therapy resistance. *Clin Cancer Res*. 2013;19(7):1773–1783.
66. Keunen O, Johansson M, Oudin A, et al. Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proc Natl Acad Sci U S A*. 2011; 108(9):3749–3754.
67. Talasila KM, Soentgerath A, Euskirchen P, et al. EGFR wild-type amplification and activation promote invasion and development of glioblastoma independent of angiogenesis. *Acta Neuropathol*. 2013;125(5):683–698.
68. Martens T, Laabs Y, Günther HS, et al. Inhibition of glioblastoma growth in a highly invasive nude mouse model can be achieved by targeting epidermal growth factor receptor but not vascular endothelial growth factor receptor-2. *Clin Cancer Res*. 2008;14(17):5447–5458.
69. Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell*. 1993;4(1):121–133.
70. Maity A, Pore N, Lee J, Solomon D, O'Rourke DM. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3'-kinase and distinct from that induced by hypoxia. *Cancer Res*. 2000;60(20):5879–5886.
71. Ji H, Wang J, Nika H, et al. EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-catenin from beta-catenin and transactivation of beta-catenin. *Mol Cell*. 2009;36(4):547–559.
72. Glading A, Chang P, Lauffenburger DA, Wells A. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem*. 2000;275(4):2390–2398.
73. Carro MS, Lim WK, Alvarez MJ, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature*. 2010;463(7279):318–325.
74. Halliday J, Helmy K, Pattwell SS, et al. In vivo radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. *Proc Natl Acad Sci U S A*. 2014; 111(14):5248–5253.
75. Li L, Dutra A, Pak E, et al. EGFRvIII expression and PTEN loss synergistically induce chromosomal instability and glial tumors. *Neuro Oncol*. 2009;11(1):9–21.
76. Nagane M, Coufal F, Lin H, Bögler O, Cavenee WK, Huang HJ. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res*. 1996;56(21):5079–5086.
77. Eskilsson E, Rosland GV, Talasila KM, et al. EGFRvIII mutations can emerge as late and heterogenous events in glioblastoma development and promote angiogenesis through Src activation. *Neuro Oncol*. 2016;18(12):1644–1655.
78. Bonavia R, Inda MM, Vandenberg S, et al. EGFRvIII promotes glioma angiogenesis and growth through the NF-κB, interleukin-8 pathway. *Oncogene*. 2012;31(36):4054–4066.
79. Katanasaka Y, Kodera Y, Kitamura Y, Morimoto T, Tamura T, Koizumi F. Epidermal growth factor receptor variant type III markedly accelerates angiogenesis and tumor growth via inducing c-myc mediated angiopoietin-like 4 expression in malignant glioma. *Mol Cancer*. 2013;12:31.
80. Magnus N, Garnier D, Rak J. Oncogenic epidermal growth factor receptor up-regulates multiple elements of the tissue factor signaling pathway in human glioma cells. *Blood*. 2010;116(5):815–818.
81. Feng H, Hu B, Vuori K, et al. EGFRvIII stimulates glioma growth and invasion through PKA-dependent serine phosphorylation of Dock180. *Oncogene*. 2014;33(19):2504–2512.
82. Fan QW, Cheng CK, Gustafson WC, et al. EGFR phosphorylates tumor-derived EGFRvIII driving STAT3/5 and progression in glioblastoma. *Cancer Cell*. 2013;24(4):438–449.
83. Eskilsson E, Verhaak RG. Longitudinal genomic characterization of brain tumors for identification of therapeutic vulnerabilities. *Neuro Oncol*. 2016;18(8):1037–1039.
84. Biernat W, Huang H, Yokoo H, Kleihues P, Ohgaki H. Predominant expression of mutant EGFR (EGFRvIII) is rare in primary glioblastomas. *Brain Pathol*. 2004;14(2):131–136.
85. Inda MM, Bonavia R, Mukasa A, et al. Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. *Genes Dev*. 2010;24(16):1731–1745.
86. Francis JM, Zhang CZ, Maire CL, et al. EGFR variant heterogeneity in glioblastoma resolved through single-nucleus sequencing. *Cancer Discov*. 2014;4(8):956–971.
87. van den Bent MJ, Gao Y, Kerkhof M, et al. Changes in the EGFR amplification and EGFRvIII expression between paired primary and recurrent glioblastomas. *Neuro Oncol*. 2015;17(7):935–941.
88. Wang J, Cazzato E, Ladewig E, et al. Clonal evolution of glioblastoma under therapy. *Nat Genet*. 2016;48(7):768–776.
89. Nathanson DA, Gini B, Mottahedeh J, et al. Targeted therapy resistance mediated by dynamic regulation of extrachromosomal mutant EGFR DNA. *Science*. 2014;343(6166):72–76.
90. Malkki H. Trial Watch: Glioblastoma vaccine therapy disappointment in Phase III trial. *Nat Rev Neurol*. 2016;12(4):190.
91. Sampson JH, Heimberger AB, Archer GE, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol*. 2010;28(31):4722–4729.
92. Guo G, Gong K, Ali S, et al. A TNF-JNK-Axl-ERK signaling axis mediates primary resistance to EGFR inhibition in glioblastoma. *Nat Neurosci*. 2017.
93. Elmeliegy MA, Carcaboso AM, Tagen M, Bai F, Stewart CF. Role of ATP-binding cassette and solute carrier transporters in erlotinib CNS penetration and intracellular accumulation. *Clin Cancer Res*. 2011;17(1):89–99.
94. Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G. GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol*. 2011;12(4):R41.
95. Ozawa T, Riester M, Cheng YK, et al. Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell*. 2014;26(2):288–300.
96. Lee EQ, Kaley TJ, Duda DG, et al. A multicenter, phase II, randomized, noncomparative clinical trial of radiation and temozolomide with or without vandetanib in newly diagnosed glioblastoma patients. *Clin Cancer Res*. 2015;21(16):3610–3618.
97. Sepulveda-Sanchez JM, Vaz MA, Balana C, et al. Phase II trial of dacomitinib, a pan-HER (human epidermal growth factor receptor) tyrosine kinase inhibitor, in recurrent glioblastoma patients with EGFR amplification. *Neuro Oncol*. 2017.
98. Westphal M, Heese O, Steinbach JP, et al. A randomised, open label phase III trial with nimotuzumab, an anti-epidermal growth factor receptor monoclonal antibody in the treatment of newly diagnosed adult glioblastoma. *Eur J Cancer*. 2015;51(4):522–532.
99. Li L, Quang TS, Gracely EJ, et al. A phase II study of anti-epidermal growth factor receptor radioimmunotherapy in the treatment of glioblastoma multiforme. *J Neurosurg*. 2010;113(2):192–198.
100. Whittle JR, Lickliter JD, Gan HK, et al. First in human nanotechnology doxorubicin delivery system to target epidermal growth factor receptors in recurrent glioblastoma. *J Clin Neurosci*. 2015;22(12):1889–1894.