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Epidermal growth factor receptor (EGFR) and EGFRvIII in glioblastoma (GBM): signaling pathways and targeted therapies

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Abstract

Amplification of *EGFR* and its active mutant *EGFRvIII* occur frequently in GBM. While EGFR and EGFRvIII play critical roles in pathogenesis, targeted therapy with EGFR-tyrosine kinase inhibitors (TKIs) or antibodies has only shown limited efficacy in patients. Here, we discuss signaling pathways mediated by EGFR/EGFRvIII, current therapeutics, and novel strategies to target *EGFR/EGFRvIII*-amplified GBM.

Keywords

GBM; glioblastoma; epidermal growth factor; EGFR; EGFRvIII; tyrosine kinase inhibitor; TKI

Introduction

GBM is the most common malignant brain tumor in adults, and among the most lethal of all cancers.¹ Current treatment: surgery, radiotherapy and chemotherapy, results in a median survival of only 12–15 months.¹ Understanding the molecular principles and signaling pathways involved in GBM is critical for development of more effective and targeted therapies for this devastating disease.

GBM, WHO grade IV gliomas,² are classified into primary tumors that arise de novo (>90% of all cases) and secondary tumors that progress from low-grade gliomas.³ According to differences in expression patterns, GBMs are divided into four subtypes: classical, proneural, mesenchymal and neural, with intratumoral heterogeneity observed in single-cell RNA-sequencing studies.^{4, 5} *EGFR* amplification is enriched in the classical subtype.⁴ Amplification of the *EGFR* gene occurs in 57.4% of primary GBM patients compared to 8% of secondary GBM patients and is associated with high levels of EGFR protein.^{6, 7}

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Conflict of interest

The authors declare no conflict of interest.

EGFR, also known as HER1 or ERBB1, is a transmembrane receptor tyrosine kinase of the ERBB family.⁸ This is a family of four receptors (ERBB1-4 or HER1-4) with EGFR the best characterized.⁸ Upon binding to ligands which include epidermal growth factor (EGF), Transforming growth factor alpha (TGF- α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), epigen or epiregulin, EGFR forms homodimers or heterodimers with other ERBB family members.⁹ Dimerization of EGFR leads to transphosphorylation (autophosphorylation) of its C-terminal tail, which serves as the docking site for SRC homology 2 (SH2) domain-containing signaling proteins including growth factor receptor-bound protein 2 (GRB2), phosphoinositide 3-kinase (PI3K), SRC homology 2 domain-containing transforming protein 1 (SHC1) and signal transducer and activator of transcription (STAT) proteins.^{10, 11} These signaling proteins regulate downstream physiological and pathological processes.

Activating mutations in the EGFR kinase domain are frequently detected in non-small cell lung cancer. These mutations, L858R in exon 21 and in-frame deletion in exon 19, are rare in GBM.¹² In contrast, a separate group of EGFR deletions and point mutations is found frequently in GBM. EGFR deletions in GBM include *EGFRvI* (N-terminal deletion), *vII* (deletion of exons 14–15), *vIII* (deletion of exons 2–7), *vIV* (deletion of exons 25–27), *vV* (deletion of exons 25–28), among which *vII* and *vIII* are oncogenic.¹² In addition, point mutations in the extracellular region of EGFR such as R108K, A289V/D/T, G598D and other extracellular domain mutations are identified in 24% GBM samples.⁶ These point mutations keep EGFR in an active conformation.¹² Among *EGFR* mutants found in GBM, *EGFRvIII* occurs most commonly;⁶ and is felt to represent a late event, occurring after amplification of *EGFR*^{WT}.¹³ Compared to *EGFR*^{WT}, *EGFRvIII* lacks amino acids 6–273, and deletion of those 268 amino acids creates a junction site with a new glycine residue between amino acids 5 and 274.¹⁴

Functional domains of EGFR and *EGFRvIII* are illustrated in Figure 1. *EGFRvIII* does not contain a ligand-binding domain, and is constitutively active.^{15, 16} Our data suggests that *EGFRvIII* is a substrate for EGFR,¹⁷ and that the kinase activity of *EGFRvIII* is dispensable for some of the augmented signaling observed, when *EGFR*^{WT} and *EGFRvIII* are co-expressed. The kinase activity of *EGFRvIII* is much weaker than that for ligand-activated full-length EGFR, and this weak constitutive kinase activity has been reported as sufficient to confer growth advantage to tumors.^{18, 19} However, most mouse and human cells express some *EGFR*^{WT}. It is therefore possible that some of the growth advantage observed in *EGFRvIII* transduced cells results from *EGFR*^{WT}-directed phosphorylation of *EGFRvIII*, rather than from *EGFRvIII* alone.

EGFRvIII forms molecular clusters on the cell membrane, which may be important for its pro-tumorigenic function,²⁰ although intracellular localization of *EGFRvIII* has also been proposed to contribute to activity.²¹ Expression of *EGFRvIII* promotes cell proliferation, angiogenesis, and invasion in different model systems.^{22–25} *EGFRvIII* expression has been found only in tumors and not in normal tissue, suggesting it as a good candidate for targeted therapy.²⁶ However, recent observations that *EGFRvIII* amplicons can hide during therapy, only to re-emerge; combined with the recent failure of a phase III clinical trial (ACT IV) testing an anti-*EGFRvIII* vaccine on newly diagnosed *EGFRvIII*-positive patients, indicates

that targeting EGFRvIII will be challenging.^{27, 28} In this review, we discuss signaling pathways mediated by EGFR and EGFRvIII, current therapeutics targeting EGFR and/or EGFRvIII, and future directions in treating *EGFR/EGFRvIII*-amplified GBM.

Signaling mediated by EGFR

Signaling pathways mediated by EGFR are summarized in Figure 2. EGFR signaling can be divided based on EGFR localization. The first group of pathways are mediated by EGFR signaling from the plasma membrane, including the RAS/mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, PI3K/protein kinase B (PKB/AKT) pathway, Janus Kinase (JAK)/STAT pathway, and protein kinase C (PKC) pathway. The second group includes signaling driven by localization of EGFR in non-plasma membrane compartments, including nuclear- (signaling molecules include DNA-PK, PCNA, Histone H4 and STAT proteins) and mitochondrial (signaling molecules include COXII)-localized EGFR. How EGFR signals to these targets is more poorly characterized but is attracting increasing attention. EGFRvIII shares signaling pathways with EGFR but also displays unique features, as presented below. Some signaling molecules downstream of EGFR/EGFRvIII are mutated frequently in GBM, emphasizing the importance of EGFR/EGFRvIII signaling in GBM. The most frequent mutations of EGFR/EGFRvIII-regulated molecules are summarized in Table 1.

Signaling mediated by the membrane-bound RTK function of EGFR

The RAS/MAPK/ERK pathway—RAS proteins are small GTPases.²⁹ The GTP to GDP switch is regulated by guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs), and controls activation/inactivation of RAS proteins.²⁹ RAS-GEFs include son of sevenless (SOS), while RAS-GAPs include the tumor suppressor neurofibromin 1 (NF1).²⁹

Upon activation, EGFR recruits the SH2 domain-containing protein GRB2 in a preformed complex with RAS GEF SOS, facilitating activation of RAS.³⁰ Activated RAS drives the RAF-MAPK/ERK kinase (MEK)-ERK1/2 signaling cascade. Activated ERK1/2 in turn phosphorylate a large number of downstream substrates, and translocate to the nucleus to modulate the activity of a range of proteins and transcription factors.³¹ ERK1/2 play critical roles in regulating proliferation, survival and metabolism. ERK1/2 can also function in tumor suppressing pathways and this function is dependent on activation strength.³¹

The three human *RAS* genes (*K-RAS*, *H-RAS* and *N-RAS*) are mutated in 20–30% of all human cancers.³² Although mutation of *RAS* is rare in GBM (only 2%), high RAS activity in the tumor is frequently observed.^{33, 34} Additionally, the RAS-GAP *NF1* is mutated or deleted in 18% of GBM patients. Tumors with *NF1* mutation/deletion show activation of RAS, measured by p-ERK and p-MEK.^{6, 33} These results indicate that the EGFR/RAS/MEK/ERK pathway plays an important role in pathogenesis. A recent study indicates that oncogenic *KRAS* and *EGFR* mutation in lung adenocarcinoma leads to synthetic lethality.³⁵ It will be interesting to know if oncogenic *RAS* coupled with *EGFR/EGFRvIII* amplification similarly promotes synthetic lethality in GBM, which may provide insight into the low frequency of *RAS* mutations in GBM.

The PI3K/AKT pathway—The PI3Ks are kinases that phosphorylate cellular lipids.³⁶ Based on differences in structure and substrate specificity, PI3Ks can be classified into three different classes, among which Class IA PI3Ks play major roles in cancers. The functions and signaling pathways of PI3Ks have been reviewed recently.^{11, 36}

Class IA PI3Ks contain catalytic p110 and regulatory p85 subunits.¹¹ Active EGFR associates with p85 through dimerization with human epidermal growth factor receptor 3 (HER3), or through the docking protein GRB2-associated binder 1 (GAB1), and relieves the inhibitory effect of p85.³⁷ P110 is able to phosphorylate phosphatidylinositol 4,5-Bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serves as a membrane-docking site for AKT.³⁶ Phosphoinositide-dependent kinase 1 (PDK1) then partially activates AKT by phosphorylating it at T308, and mammalian target of rapamycin complex 2 (mTORC2) fully activates it by phosphorylating S473.³⁶ Phosphatase and Tensin Homolog (*PTEN*), a tumor suppressor frequently mutated in GBM, dephosphorylates PIP3 to PIP2 and negatively regulates the PI3K/AKT signaling.³⁶ A recent study indicates that loss of chromosome 10q (including *PTEN*) is an early event that precedes *EGFR* amplification.³⁸

AKT substrates, proteins critical in regulating cell proliferation and survival, include tuberous sclerosis complex (TSC),³⁹ BCL2 associated death protein (BAD),⁴⁰ Beclin 1,⁴¹ Caspase-9,⁴² inhibitor of nuclear factor kappa-B (NFκB) kinase subunit alpha (IKKα),⁴³ transcription factors cAMP response element-binding protein (CREB)⁴⁴ and forkhead homeobox type O (FOXO).⁴⁵ AKT also promotes metabolism by facilitating membrane localization and expression of glucose transporters,⁴⁶ phosphorylating critical enzymes in metabolism such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase,⁴⁷ and phosphorylating ATP-citrate lyase (ACLY), enabling production of acetyl Co-A production.⁴⁸ ACLY regulates histone acetylation, which correlates with levels of p-AKT.⁴⁸

The JAK/STAT pathway—JAKs are receptor tyrosine kinases that interact with cytokine receptors. The interaction of JAK2 with EGFR confers resistance to EGFR inhibitors.⁴⁹ Upon cytokine binding, JAK is activated and phosphorylates STAT proteins, which dimerize and translocate to the nucleus.⁵⁰ Nuclear STAT proteins can activate or repress transcription of target genes to facilitate transformation, inflammation-associated tumorigenesis, stemness and migration. STAT3 can also be directly phosphorylated and activated by EGFR.⁵¹ Phosphorylation at Y705 by EGFR induces dimerization of STAT3.^{51, 52} AKT phosphorylation of EZH2, which mediates DNA methylation, could further activate STAT3.⁵³ As AKT is also downstream of EGFR, EGFR can activate STAT3 through multiple downstream effectors.

The role of STAT3 in GBM tumorigenesis depends on the mutational status of other genes.⁵⁴ For example, STAT3 suppresses *PTEN* loss-induced malignant transformation of astrocytes.⁵⁴ However, when EGFRvIII is expressed, STAT3 forms a complex with EGFRvIII and drives malignant transformation.^{17, 54} STAT3 also initiates mesenchymal transformation in high-grade gliomas.⁵⁵ Activation of STAT3 promotes tumor growth and survival by suppressing the antitumor response of the immune system, and also by inducing tumor stemness and angiogenesis.^{56–58} Inhibition of STAT using small molecule inhibitors of

JAK2 sensitizes U87.EGFR and U87.EGFRvIII cells to the EGFR inhibitor gefitinib in vitro.⁵⁹

The phospholipase C (PLC)/PKC pathway—Active EGFR is able to recruit and activate PLC, which catalyzes the cleavage of PIP₂ to inositol 1,4,5-trisphosphate (IP₃) and diacyl glycerol (DAG).⁶⁰ Activated PLC activates PKC. PKCs are a large family of phospholipid-dependent serine/threonine kinases classified into three families based on structure and activation mechanisms:⁶¹ classic PKCs (PKC α , β I, β II, and γ), nonclassic PKCs (δ , ϵ , η , and θ) and atypical PKCs [PKC ζ and ι (λ)]. PKCs may be tumor suppressors or oncogenes depending on context.^{61–63} PKCs are able to activate a large group of effector molecules to regulate tumor proliferation, angiogenesis, infiltration, and survival. Downstream effectors of PKC isoenzymes include cell cycle regulators p53 and p21, cell growth and proliferation regulators RAS-RAF1 and glycogen synthase kinase 3 (GSK3), cell motility regulators integrins, cell survival regulators BCL2 and BAD, and the inflammation regulator NF κ B.⁶¹

Different types of tissues usually show expression of different PKC isoenzymes. In GBM, PKC isoenzymes including PKC α , PKC η and PKC δ showed pro-tumorigenic functions.^{64–67} High levels of PLC γ are correlated inversely with survival.⁶⁸ EGFR transduces signaling to mTOR in a PKC-dependent manner.⁶⁴ Inhibition of PKC decreased viability of GBM cells, indicating that PKC is a critical signaling node.⁶⁴

The function of non-plasma-membrane-bound EGFR

EGFR also plays important roles in intracellular compartments in the mitochondria and the nucleus (topology shown in Fig. 2). Phosphorylation of Y845 of EGFR by SRC stimulates the mitochondrial trafficking of EGFR.⁶⁹ Mitochondrial EGFR associates with and phosphorylates a subunit of Complex IV, cytochrome oxidase subunit II (COXII) at the inner mitochondrial membrane, leading to decrease in COXII activity and respiratory inhibition.⁶⁹

EGFR also localizes to the nucleus,⁷⁰ where it functions as a cofactor alongside transcription factors such as STAT3/5 to enhance transcription of inducible nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*COX2*), *MYC*, Aurora-A kinase (*AURKA*) and other genes critical in cell growth and survival.⁷⁰ Nuclear EGFR (nEGFR) can also phosphorylate DNA-dependent protein kinase (DNA-PK), proliferating cell nuclear antigen (PCNA) and histone H4 to function in DNA repair and replication.^{71–73} Nuclear translocation of EGFR is regulated by SRC Family Kinases (SFKs), AKT, Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), AXL and PKC ϵ .^{74–76} EGFR can phosphorylate EGFRvIII to activate STAT3/5 in the nucleus, and facilitate tumor progression.¹⁷ nEGFR/nEGFRvIII also play critical roles in GBM growth and survival.^{77, 78}

Signaling mediated by EGFRvIII

EGFRvIII confers a growth advantage to GBM. Patients with EGFRvIII have significantly shortened survival.⁷⁹ The pro-tumorigenic function of EGFRvIII results from its ability to promote tumor growth, survival, invasion, stemness, metabolism and angiogenesis.^{23, 24, 80–82} EGFRvIII signaling is different from EGFR signaling both quantitatively

(difference in signal strength) and qualitatively (difference in signaling molecules). EGFRvIII has lower-level constitutive kinase activity, which is partially due to impaired endocytosis and degradation.⁸³ As a constitutively active mutant of EGFR, EGFRvIII is able to transduce signals via traditional EGFR pathways controlled by RAS/MAPK, PI3K/AKT and JAK/STAT. The pro-tumorigenic function of EGFRvIII is dependent on its kinase activity, as kinase dead EGFRvIII in some contexts fails to promote tumor formation and growth.¹⁶ Epigenomic and transcriptomic analyses indicate that EGFRvIII specifically activates more than 2000 enhancers and regulates GBM sensitivity to the small-molecule bromodomain ligand JQ1 by modulating transcription.⁸⁴ Below we discuss some examples of EGFRvIII-regulated pathways.

EGFRvIII phosphorylates other kinases and receptors—EGFRvIII interacts with and phosphorylates both kinases and receptor molecules. For example, EGFRvIII phosphorylates SFKs, which further activate dedicator of cytokinesis 1 (DOCK1).⁸⁵ DOCK1 plays critical roles in mediating cell growth and migration, contributing to the pro-tumorigenic function of EGFRvIII.^{23, 85} EGFRvIII can also increase phosphorylation of DOCK1 through PKA.²³ EGFRvIII forms a complex with the cytokine receptor OSMR, which regulates the EGFRvIII-STAT3 signaling axis.⁸⁶ Additionally, SFK activation promotes mitochondrial localization of EGFRvIII, and increases cell survival under low glucose conditions.⁸⁷ Moreover, EGFRvIII activates hepatocyte growth factor receptor (MET), which in-turn drives STAT3.^{88, 89} EGFRvIII also activates c-SRC, which promotes secretion of vascular endothelial growth factor (VEGF) and angiogenesis.¹³ Phosphorylation of these kinases further activates downstream signaling pathways and contributes to tumor progression.

EGFRvIII modulates gene and protein expression—EGFRvIII is able to remodel global transcription.⁸⁴ EGFRvIII increases expression of proteins critical in apoptosis, invasion, stemness, metabolism and angiogenesis including BCL-xL, extracellular matrix (ECM) proteins, abnormal spindle-like microcephaly-associated protein (ASPM), glucose transporter protein type 1 (GLUT1), leukemia inhibitory factor (LIF) and heterogeneous nuclear ribonucleoprotein A1 splicing factor (hnRNPA1).^{24, 78, 82, 90–92} These molecules contribute to proliferation and survival.

EGFR/EGFRvIII-related mouse models of glioma

Mice carrying targeted deletions in the *Ink4a/arf* locus displayed glioma-like lesions in the setting of an active mutant of EGFR.⁹³ The EGFR homologue v-erbB expressed under the S100 β promoter led to low-grade gliomas, which were higher grade and more aggressive after crossing to mice with *p53* or *Ink4a/arf* mutations.⁹⁴ These observations suggested that other mutations cooperated with v-erbB to promote high-grade glioma. Neither EGFR nor EGFRvIII expressed under the GFAP promoter generated tumors.⁹⁵ However, EGFRvIII was able to accelerate tumors driven by *HRas*^{G12V}, changing the histopathology of these gliomas from astrocytoma to oligodendroglioma or mixed oligoastrocytoma.⁹⁵

Crosstalk among EGFR, EGFRvIII and other RTKs

EGFRvIII regulates EGFR activity by inducing expression of EGFR ligands such as TGF- α and HB-EGF.⁹⁶ EGFRvIII can also form transient dimers with EGFR, ERBB family members, and self-dimerize.^{97–99} Forced-dimerization of EGFRvIII increased its capacity to promote tumor growth.¹⁰⁰ We and others have shown that EGFRvIII is a substrate of EGFR, but EGFRvIII is unable to phosphorylate EGFR.^{17, 98} A previous publication indicated that EGFRvIII was able to phosphorylate wild-type EGFR in a murine cell line Ba/F3 that did not express endogenous EGFR.⁹⁹ Whether different experimental systems in these studies led to distinct results awaits further evaluation.

While rare cells in GBM co-express EGFR and EGFRvIII,¹⁷ the majority of tumor cells express predominantly EGFR or EGFRvIII. These observations suggest a hierarchical model, with EGFR and EGFRvIII potentially signaling in cancer stem cell compartment, and segregating separately to daughter cells in the tumor (Figure 3). In cells where EGFR and EGFRvIII are expressed in separate cells, EGFRvIII promotes the growth of cells expressing wild-type EGFR via a paracrine interleukin 6 (IL-6) and/or LIF-dependent manner. These cytokines activate EGFR via glycoprotein 130 (GP130).⁹²

The EGFRvIII RNA and protein can also be transferred to other cells via microvesicles to increase the fitness of the tumor.^{101, 102} Importantly, single-cell sequencing verified that EGFRvIII only exists in a subset of tumor cells. Different cells in the same tumor can have distinct *EGFR* mutations,¹⁰³ and, distinct tumor cell subpopulations can amplify different RTKs.¹⁰⁴ For example, in platelet derived growth factor receptor alpha (*PDGFRA*)-amplified GBM tumors, amplification of *MET* or *EGFR* is also observed frequently.¹⁰⁵ Inhibition of a single RTK can lead to upregulation of other RTKs. For example, inhibition of EGFR upregulated transcription of *PDGFR β* and promoted resistance to EGFR TKI treatment.¹⁰⁶ Tumor cell subpopulations harboring distinct *EGFR* mutations or RTK amplifications add to the complexity of GBM and contribute to resistance to EGFR/EGFRvIII-directed therapies.^{103, 105} As compensatory activation of ERBB family receptors also occurs in the setting of EGFR inhibition,¹⁰⁷ co-inhibition of EGFR and other ERBBs may cooperate to inhibit GBM growth.

Targeting EGFR/EGFRvIII for therapy in GBM

Current anti-EGFR/EGFRvIII therapeutics are summarized in Table 2. They include, but are not limited to small molecule TKIs, antibodies, vaccines, chimeric antigen receptor (CAR) T cells and RNA-based therapies.

Small molecule inhibitors of EGFR/EGFRvIII—First generation EGFR inhibitors were designed to orthosterically block the ATP/substrate-binding pocket of EGFR in the tyrosine kinase domain. FDA-approved first-generation EGFR inhibitors include gefitinib (ZD1839; Iressa), erlotinib (OSI-774; Tarceva) and lapatinib (GW572016; Tykerb/Tyverb). Although these inhibitors showed promising results in inhibiting growth and improving survival in preclinical models, they have had limited activity in patients.^{108, 109} Erlotinib and gefitinib are known to affect EGFR activity in patients with lung cancer, whose activating

mutations typically lie in exons 19 and 21 of the tyrosine kinase domain.¹¹⁰ These mutations do not exist in GBM, potentially contributing to the lack of survival benefit in patients treated with erlotinib or gefitinib.

The blood brain barrier represents a real barrier to effective use of EGFR TKIs. Melanoma patients treated with vemurafenib required a dose that blocked over 80% of downstream p-ERK to achieve a clinical response.¹¹¹ If pathway inhibition of >80% is required for clinical responses more generally, then the blood brain barrier represents a clear obstacle. While the blood brain barrier in GBM is compromised, this magnitude of this compromise is likely inadequate to enable such efficient inhibition. Indeed, for patients treated with lapatinib (which binds EGFR better than erlotinib or gefitinib¹¹²), only modest inhibition of p-EGFR was achieved in biopsied post-treatment tumors.¹¹² In addition, erlotinib has lower affinity for EGFRvIII compared to EGFR mutants in non-small-cell lung cancer (NSCLC), and releases more rapidly than from EGFR^{WT}.^{112, 113} The blood-brain barrier clearly limits drug concentration in tumors, and is an important factor in the resistance of GBM to EGFR inhibitors.¹¹⁴

Second generation EGFR-TKIs are designed to bind irreversibly to the tyrosine kinase domain of EGFR and other ERBB family members. Afatinib and dacomitinib are FDA-approved. In a phase I/randomized phase II study, afatinib showed good safety but limited single-reagent activity in GBM patients.¹¹⁵ In preclinical studies, dacomitinib showed efficacy in GBM.¹¹⁶ Whether these inhibitors can achieve therapeutic dose in the tumors of GBM patients awaits more studies.

Third generation EGFR inhibitors such as rociletinib and AZD9291 are currently being tested pre-clinically in GBM. These new drugs efficiently overcome resistance caused by the EGFR T790M gatekeeper mutation in non-small cell lung cancer, improving survival.^{117, 118} In preclinical models, AZD9291 demonstrated lower potency in inhibiting EGFRvIII compared to afatinib.¹¹⁷ Although the T790M mutation is infrequent in GBM patients, AZD9291 can efficiently cross the blood-brain barrier, making it an attractive candidate for inhibiting EGFR in GBM. AZD9291 is now in a phase I/II clinical trial in GBM (NCATS 1-UH2-TR001370-01)

EGFR/EGFRvIII Antibodies—FDA-approved anti-EGFR antibodies include cetuximab (C225; Erbitux), panitumumab (ABX-EGF; Vectibix), and nimotuzumab. All of these antibodies bind to the L2 domain, preventing ligand binding and/or dimerization of EGFR.¹¹⁹ Cetuximab binds to EGFRvIII and induces its internalization and mitochondrial localization. However, It fails to inhibit the activity of EGFRvIII.¹²⁰ Nimotuzumab inhibits growth of U87MG.EGFRvIII cells in vitro.¹²¹ So far, these antibodies have not shown promise in treating GBM patients.¹²²

Compared to EGFR^{WT}, EGFRvIII is not responsive to antibodies targeting the L2 domain because of the deletion mutation in the ligand-binding domain. mAb806 (ABT-806), a monoclonal antibody which only targets overexpressed-EGFR or co-expression of EGFR and EGFRvIII due to its binding affinity properties, was safe in GBM patients in a phase I

clinical trial.¹²³ This antibody did not show significant binding to wild-type EGFR in normal human tissues.¹²³

Antibodies can be conjugated to toxins or radioactive isotopes to enhance tumor killing. 125I mAb 425, either singly used or in combination with TMZ, was well-tolerated in patients and prolonged survival in a phase II clinical trial.¹²⁴ Bispecific antibodies (bisAbs) contain two different binding specificities fused into one molecule.¹²⁵ They can be engineered to bispecific T-cell engagers (BiTEs), which bind to the CD3 T-cell coreceptor to recruit cytotoxic T cells. A BiTE named bscEGFRvIIIxCD3, designed to redirect T-cells to tumors expressing EGFRvIII, showed potent killing of EGFRvIII-expressing GBM in vitro and in mice.¹²⁶ Injection of bscEGFRvIIIxCD3 intravenously achieved complete cure in up to 75% in NSG mice with U87.EGFRvIII intracranial xenografts.¹²⁶ Whether this BiTE can be used in patients awaits further study.

An important question for using antibodies to treat GBM is whether they can be effectively delivered to the tumors. Antibodies typically neither cross the blood-brain barrier effectively nor achieve adequate penetration depth inside the tumor,¹²⁷ although invasive surgery can disrupt the blood-brain barrier temporarily. Recently, studies show that the blood-brain barrier can be opened using non-invasive methods.¹²⁸ Advancement in modulating the permeability of the blood-brain barrier and new drug delivery technology will be helpful to solve those problems.

Anti- EGFRvIII vaccines—Vaccines can activate the host immune system, provide durable responses and therefore hold promise for therapy. Anti-GBM vaccines, such as GlioVac, a mixture of inactivated tumor cells, are under clinical evaluation.¹²⁹ As EGFRvIII is tumor-specific, a targeted vaccine of EGFRvIII is appealing. Rindopepimut (CDX-110) consists of a 14-mer peptide that spans the mutation site of EGFRvIII conjugated to the immune adjuvant keyhole limpet hemocyanin (KLH). CDX-110 specifically eliminates tumor cells that express EGFRvIII.¹³⁰ This vaccine was proven to be safe, immunogenic, and tumor-specific in a phase I clinical trial.¹³¹ In a multicenter phase II trial, together with TMZ, CDX-110 treatment of a relatively healthy group of GBM patients significantly improved overall survival of EGFRvIII+ GBM compared to less healthy historical controls.¹³² However, CDX-110 failed in a double-blind randomized phase III trial (“ACT IV”) that had an active control arm.^{27, 132, 133}

Chimeric antigen receptors (CARs)—CARs are engineered receptors containing a single-chain variable fragment (scFv) derived from monoclonal antibodies coupled to the transmembrane and the intracellular activation domains of the surface receptors of immune cells, such as T cells and NK cells. Cells expressing these CARs recognize and kill target cells that express the antibody-targeted molecule. EGFRvIII-specific CAR-T cells, when administered systemically, suppressed tumor growth and enhanced survival of mice in preclinical GBM models.¹³⁴ Currently, CAR-T cells targeting EGFRvIII are under phase I study to determine safety and feasibility for EGFRvIII positive GBM patients (NCT02209376).¹³⁴ Recently, CAR-modified NK cells also showed potent killing of GBM cells.¹³⁵ CAR modified immune cells are able to cross the blood-brain barrier in patients and preclinical models, which shows promise in GBM therapy.¹³⁶

RNA-based therapies—Current RNA-based therapies targeting EGFR/EGFRvIII include antisense oligonucleotides, RNA interference (RNAi), ribozymes and adjuvant microRNA (miRNA) based therapies. The principle is to reduce mRNA levels of EGFR/EGFRvIII to inhibit tumor growth. Although robust decreases in EGFR/EGFRvIII expression and tumor proliferation were observed in several studies in vitro, in vivo efficacy of RNA-based therapies still needs to be improved.^{137, 138} The blood-brain-barrier, inefficient tumor-targeted delivery, and off-target effects represent obstacles that need to be overcome.

Factors contributing to resistance to EGFR/EGFRvIII inhibition in GBM

Many chemicals or antibodies targeting EGFR/EGFRvIII are not efficient at crossing the blood-brain barrier, which limits their efficacy. Additionally, EGFR/EGFRvIII are tyrosine kinases at the upstream end of signal transduction pathways (Figure 4). Mutations or deregulation of downstream molecules and upregulation of redundant receptor tyrosine kinases may bypass EGFR/EGFRvIII inhibition. PTEN negatively regulates PI3K signaling downstream of EGFR.^{139, 140} Patients with *EGFR* amplification and with an intact *PTEN* gene showed a radiographic response (but ultimately did not show improved survival) when treated with EGFR inhibitors. Similar patients whose tumors showed loss of *PTEN* did not respond radiographically to EGFR inhibitors.¹⁴⁰ SFK and FGFR-dependent phosphorylation of PTEN at Y240 also confers resistance.¹⁴¹ Moreover, EGFR inhibition by erlotinib increased nuclear expression of the promyelocytic leukemia (PML) tumor suppressor, prevented cell death and promoted tumor cell survival.¹⁴² As RTKs usually activate similar downstream pathways, in GBM, upregulation or activation of receptors such as the IGF-1 receptor (IGF-1R), MET, and PDGFR β is also a mechanism contributing to resistance to EGFR/EGFRvIII inhibition.^{106, 143–145}

GBMs are highly heterogeneous. They usually contain a mixture of cells with *EGFR*, *MET*, or *PDGFA* amplification.^{105, 146} Therefore, targeting a single RTK may not be sufficient to inhibit GBM.¹⁰⁴ In addition, RTK amplification is usually located at extrachromosomal double minute structures, which confers another layer of resistance by increasing tumor heterogeneity and drug resistance.^{28, 147} In response to EGFR TKI treatment, EGFRvIII from extrachromosomal DNA was eliminated. However, after drug withdrawal, extrachromosomal DNA with EGFRvIII reemerged.²⁸ A better understanding of the dynamics of EGFR/EGFRvIII containing extrachromosomal DNA promises to provide insights into EGFR/EGFRvIII-targeted therapies. The heterogeneity in GBM also extends to tumor-infiltrating cells, among which microglia/macrophages make up the largest population, contributing at least one third of the total tumor mass.¹⁴⁸ Despite the fact that almost all EGFRvIII+ GBM tumors co-amplify *EGFR*, and that co-expression of those two molecules dramatically promotes tumor growth, how EGFR and EGFRvIII cooperate to regulate infiltration of immune cells in the tumor microenvironment remains largely unknown. A recent study performed parallel RNAi screens in vitro and in vivo in GBM. Hits in-vitro identified genes regulating cell metabolism, whereas the in-vivo screen identified genes that promoted survival of GBM cells in the tumor microenvironment, reinforcing the importance of targeting tumor-stroma cross talk.¹⁴⁹

Conclusions and future directions

EGFR and *EGFRvIII* amplification are frequently observed in GBMs, contributing to tumorigenesis and progression. However, therapies directed against *EGFR* and *EGFRvIII* have not yet shown clear benefit clinically. Inefficient blood brain barrier penetration, as well as intratumoral heterogeneity, compensatory signaling pathways and secondary mutations all contribute to resistance. Immunotherapy targeting *EGFRvIII* may effectively kill *EGFRvIII*-positive tumor cells, but leaves behind residual tumor cells that retain *EGFR*-dependence, independently of *EGFRvIII*. Combination therapies are needed to improve outcome. A better understanding of GBM heterogeneity, crosstalk among tumor cells and stromal cells, along with improved understanding of the *EGFR/EGFRvIII* signaling network, its interplay with other pathways, better drugs, improved brain penetration and clarifying mechanisms of resistance will improve our ability to target this pathway.

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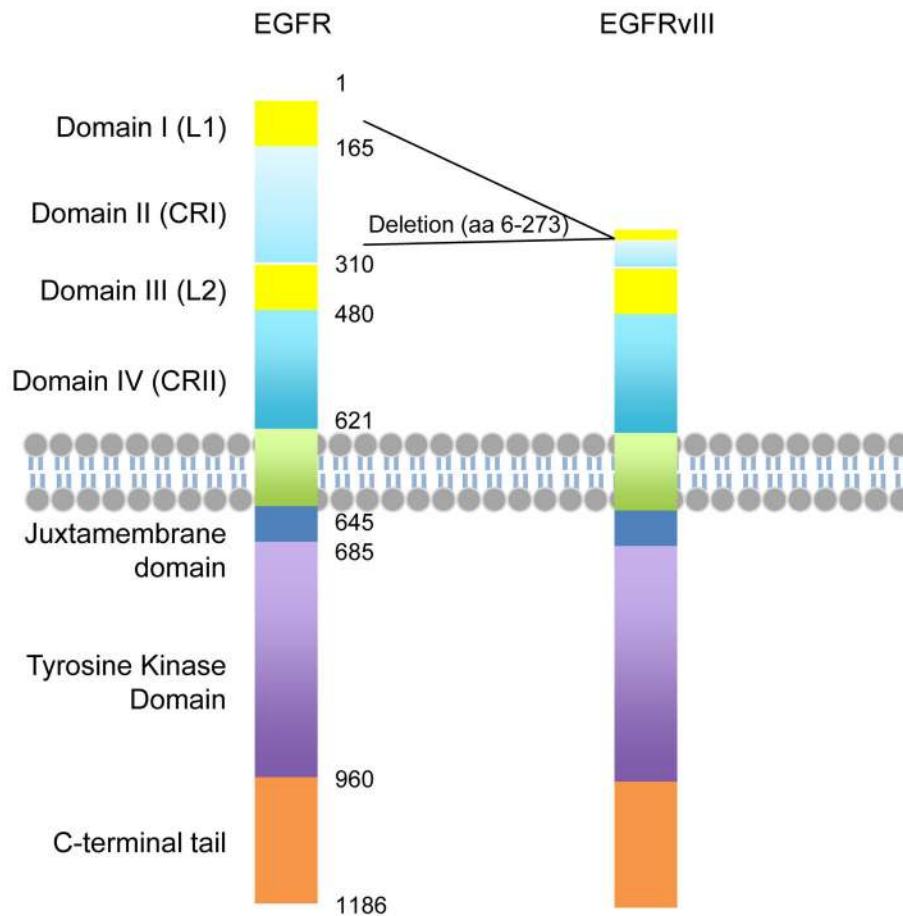


Figure 1. Functional domains of EGFR and EGFRvIII

EGFR is a transmembrane tyrosine kinase receptor. The extracellular region includes four domains, L1, CR1, L2 and CR2. L1 and L2 are Leucine-rich domains that directly bind ligands. EGFRvIII is with the deletion of almost the entire L1 and CR1 domains, resulting in deficiency in ligand binding. The transmembrane and intracellular regions of EGFR and EGFRvIII are identical.

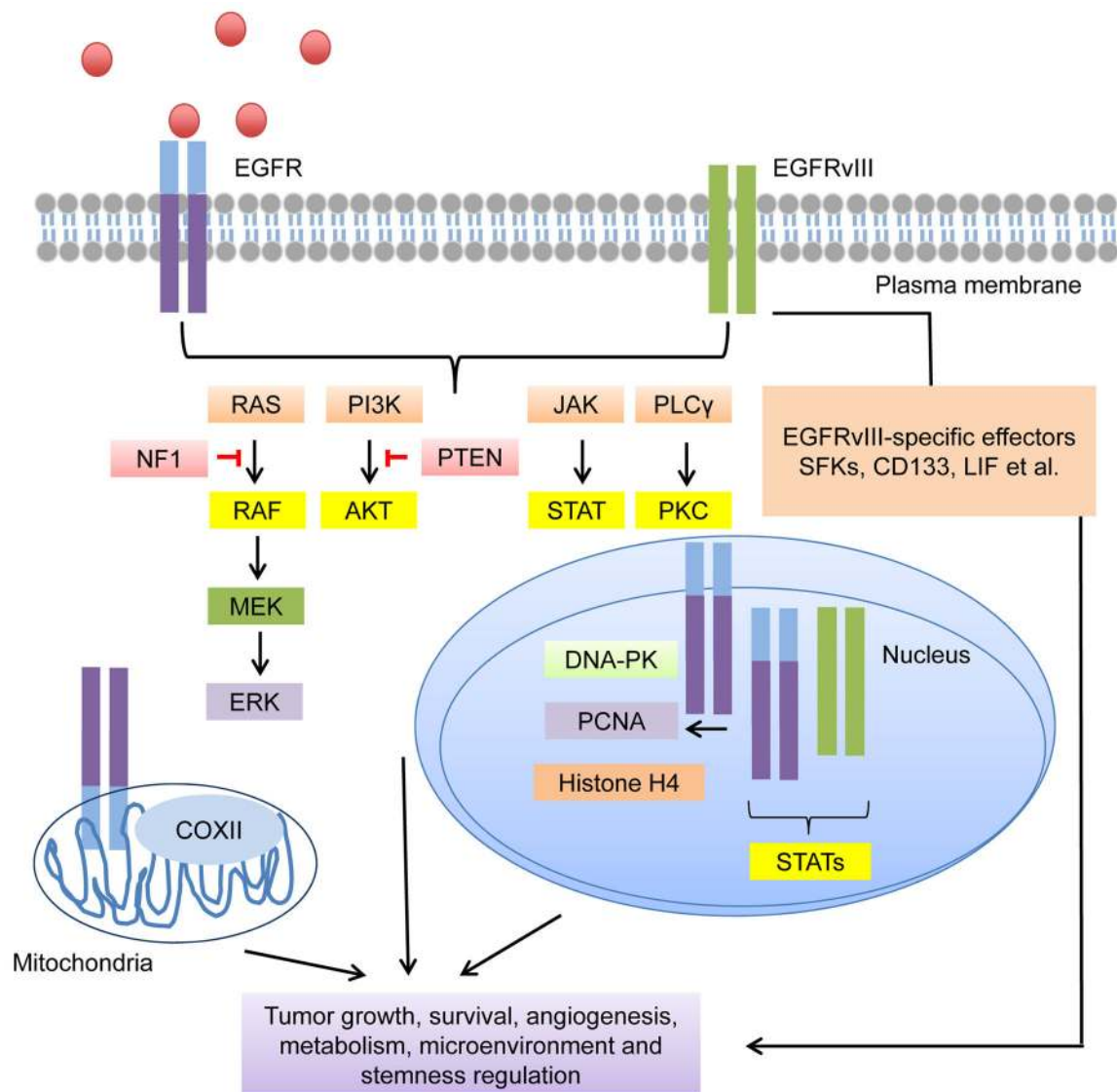


Figure 2. Signaling pathways mediated by EGFR/EGFRvIII

EGFR and EGFRvIII are able to transduce signals via classic RTK pathways including the RAS/RAF/MEK/ERK pathway, the PI3K/AKT pathway, the JAK/STAT pathway and the PKC pathway. The function of mitochondrial EGFR was also reported. Mitochondrial EGFR effector includes COXII. EGFR and EGFRvIII can also localize to the nucleus to activate a group of transcription factors and proteins involved in DNA damage responses, such as DNA-PK, PCNA, histone H4 and STATs. EGFRvIII has some unique signaling effectors. Activation of these signaling pathways and effector molecules together promote the fitness of the tumors.

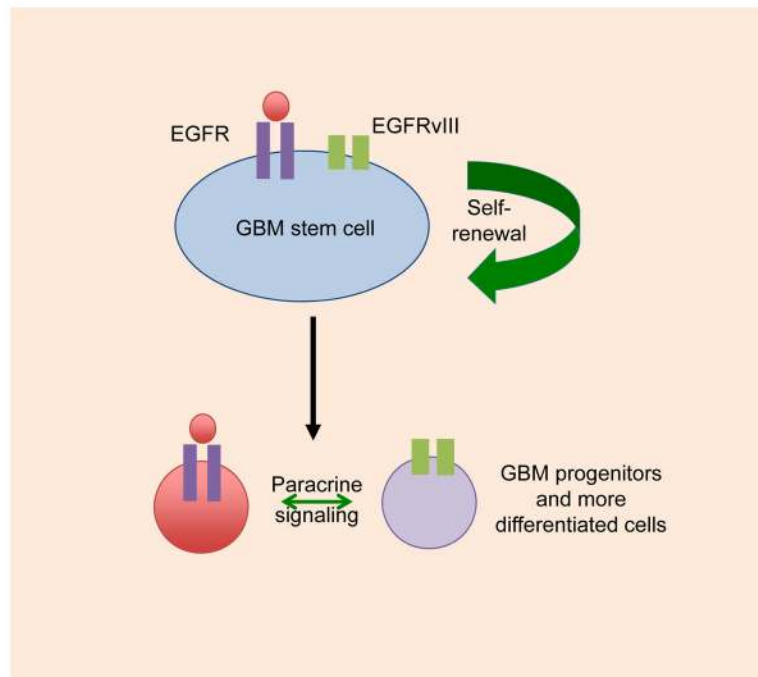


Figure 3. Hierarchical model for EGFR and EGFRvIII signaling

Rare cells in human GBM tumors that co-amplify *EGFR* and *EGFRvIII* show co-expression, with co-expression of EGFR and EGFRvIII driving malignancy more robustly, as compared to cells expressing EGFR or EGFRvIII alone. Most cells in human GBM tumors that co-amplify *EGFR* and *EGFRvIII* express predominantly EGFR or EGFRvIII, and cells expressing EGFRvIII can signal in a paracrine manner to cells expressing EGFR. A possible model incorporating these features is shown. See text for details.

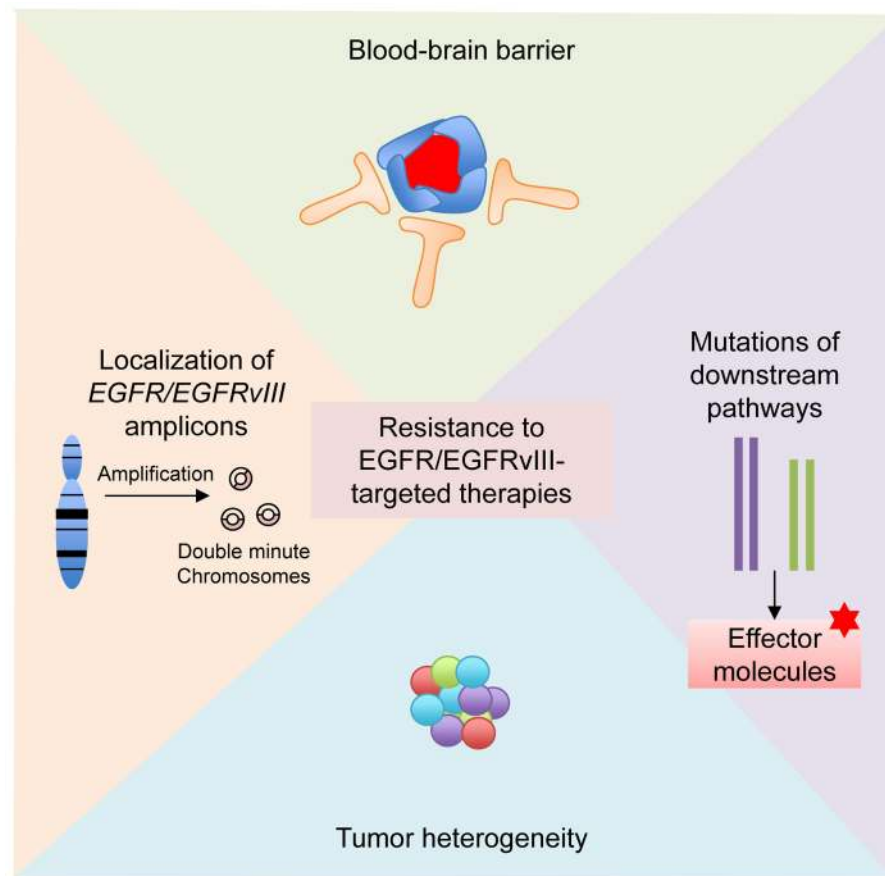


Figure 4. Factors contributing to resistance to EGFR/EGFRvIII-targeted therapies

Factors contributing to resistance to EGFR/EGFRvIII inhibition include blood-brain barrier penetrance (i.e. many antibodies and chemicals cannot across the blood-brain barrier), mutations of signaling molecules downstream of EGFR/EGFRvIII (i.e. *PTEN* mutation and *NF1* mutation, which maintain activation of downstream pathways despite upstream inhibition), tumor heterogeneity (distinct tumor cells can harbor different mutations or receptor kinase amplification, interaction between tumor cells and stromal cells in the tumor microenvironment) and extrachromosomal localization of *EGFR* and *EGFRvIII* amplicons (facilitating the cells' ability to evade EGFR inhibitors).

Table 1Mutations of *EGFR* and related pathways in GBM

“Percentage” indicates frequencies of mutations in GBM patients.

GENE Symbol	Chromosomal location	Alteration	Percentage
<i>EGFR</i>	Ch 7p	Amplification/mutation	57.4
			11.8 with EGFRvIII
<i>p110a</i>	Ch 3q	Mutation	18.3
<i>p85a</i>	Ch 5q	Mutation	6.8
<i>PTEN</i>	Ch 10q	Mutation/deletion	34.3
<i>NF1</i>	Ch 17q	Mutation/deletion	10

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Table 2

EGFR/EGFRvIII-targeted therapies

EGFR/EGFRvIII targeted therapy		Effects in GBM	References
Inhibitors	1st generation	Not very effective in GBM clinical trials.	108–109
2nd generation	Gefitinib	Good safety but limited single-reagent activity on GBM patients. Promising in combination with TMZ in a case report.	115
	Erlotinib		
	Lapatinib		
3rd generation	Afatinib	Promising in pre-clinical models.	116
	Dacomitinib		
	Rociletinib		
Antibodies	AZD9291	Effects on GBM to be evaluated.	
	Cetuximab	In phase I/II clinical trial	
	Panitumumab	Not very effective in GBM clinical trials.	119–122
	Nimotuzumab		
	mAB806		
	Targeting the EGFRvIII-specific sequence	Good safety. Anti-GBM effects to be evaluated.	123
Toxin or radioactive isotope conjugated	When used singly or in combination with TMZ, significantly prolonged patient survival.	124	
Bis.Abs	125I mAB425		
Vaccines	Rindopepimut (CDX110)	Promising in pre-clinical models.	126
	CAR-T cells targeting EGFRvIII	Significantly prolonged patient survival when co-administrated with TMZ. Failed phase III trial.	130–133
RNA-based therapies	Antisense oligonucleotides, RNAi, ribozymes and adjuvant miRNA based therapy	Promising in pre-clinical models. Currently under phase I clinical trial.	134–136
		Feasibility to be further evaluated in pre-clinical models.	137–138