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Epidermal growth factor receptor targeting and challenges in glioblastoma

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With the evolution of technology, there is now a deeper understanding of glioblastoma as an inter- and intraheterogeneous disease comprising a multitude of genetically and epigenetically different cancer cells. Greater characterization of glioblastoma at the molecular level has improved its initial pathophysiological staging and classification. With this knowledge comes the hope that more efficacious therapies to combat this highly lethal disease are on the horizon. One possibility for intervention is represented by the targeting of epidermal growth factor receptor (EGFR), which is amplified and mutated in a large subset of patients. In this review, we provide a brief overview of EGFR and its mutated form, EGFR variant III, describing the downstream cellular pathways activated by each receptor, available animal models, therapeutic strategies to inhibit the receptor, and possible intervention routes to efficiently target this receptor and prevent the emergence of resistant mechanisms which to date have hampered a successful therapeutic outcome.

Keywords: EGFR, EGFRvIII, glioblastoma, glioma, therapy.

Classification of Glioblastoma

Glioblastoma (GBM) is the most prevalent and aggressive malignant glioma, leaving patients with a median survival of 15 months following a rigorous course of radiation and concomitant temozolomide.[1] In the past, glioma grade was determined histopathologically based on the presence or absence of nuclear atypia, mitotic activity, and necrosis or microvascular proliferation. Recently, 4 separate studies have developed new classification schemes which integrate key genetic alterations with histopathological data and clinical outcome.[2] Taken together, gliomas may now be categorized into different molecular subgroups based on the tumor mutation status of isocitrate dehydrogenase (IDH) and the codeletion or loss of heterozygosity at chromosome 1p/19q (corresponding to mutations in *FUBP1* and *CIC*) combined with a mutation in *TERT* or *ATRX*, conferring telomere maintenance by telomerase or the alternative lengthening of telomeres, respectively. In this sense, GBM is molecularly categorized as tumors which have wild-type IDH expression (although secondary GBM is typically mutated in IDH), lack 1p/19q deletion, and have either an *ATRX* or a *TERT* mutation. Prior to these studies, GBM was initially classified into one of 4 major subgroups based on transcriptional data from The Cancer Genome Atlas: proneural, classical, mesenchymal, or neural. The proneural subgroup is associated with amplification or mutation of platelet-derived growth factor receptor, mutations in IDH 1 and IDH 2, and/or a disruption of the tumor suppressor p53. The classical subgroup is associated with amplification of chromosome 7 and deletion of chromosome 10, corresponding to epidermal growth factor receptor (EGFR) mutation/amplification and loss of the *Ink4a/ARF* locus, respectively. The mesenchymal subgroup shows high expression of the mesenchymal markers CHI3L1 (chitinase 3-like protein 1; also known as YKL-40) and MET, often accompanied by an inactivating mutation or deletion of the tumor suppressor neurofibromatosis type 1. Finally, tumors placed in the neural subgroup have a differentiated phenotype with neural, astrocytic, or oligodendrocytic gene expression patterns.[3] While this classification system may have been useful for deciding the treatment of GBM patients, given the highly heterogeneous nature of GBM, it was not uncommon for a single tumor to show a gene expression pattern which aligns with more than one of these subgroups.

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Epidermal Growth Factor Receptor and EGFR Variant III Signaling

Although the classical subgroup has been reported to account for ~25%–30% of GBM, recent large-scale sequencing efforts revealed that 57% of GBM patient samples contain various EGFR mutations often co-occurring with EGFR rearrangement and/or focal amplification.[4] EGFR is a transmembrane glycoprotein and the first of 4 ErbB receptor tyrosine kinases described, together with ErbB2 (Her2/Neu), ErbB3, and ErbB4. Wild-type EGFR is classically activated through ligand binding by factors such as epidermal growth factor (EGF), transforming growth factor alpha, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, and epigen.[5] In GBM, however, activation of EGFR may occur independently of ligand through cross-talk between receptor tyrosine kinases at the cell surface or between EGFR mutants and the wild-type receptor.[6–8]

The most common EGFR mutant, EGFR variant (v)III, has an extracellular domain truncation from exons 2 to 7 and is constitutively active in GBM.[9] While activation of wild-type EGFR results in signaling primarily through the pathways of signal transducer and activator of transcription 3 and mitogen-activated protein kinase, the mutant EGFRvIII receptor preferentially signals through the pathway of phosphatidylinositol-3 kinase (PI3K)/Akt.[10] Signal propagation from these receptors differs as well. For example, although wild-type EGFR has no influence on microRNA (miR)-9 expression, EGFRvIII has been shown to exert its tumorigenic influence through the specific inhibition of miR-9 leading to disinhibition of the miR-9 target, transcription factor Forkhead box protein 1. Also, unlike wild-type EGFR, cells expressing EGFRvIII are altered in their receptor trafficking. Classically, activation of wild-type EGFR results in receptor internalization and subsequent signal termination; however, EGFRvIII fails to internalize and therefore has increased stability in the plasma membrane, contributing to its sustained tumorigenic signaling.[10,11] The receptor response to ligand differs as well. As described above, wild-type EGFR is primarily activated by ligand such as EGF. Although EGFRvIII does not bind ligand, it has been shown that addition of EGF to cell culture media disrupts the physical interaction between EGFRvIII and MET, resulting in an inhibition of MET phosphorylation, and a reduction in its oncogenic signaling.[12]

Animal Models

For decades, signaling pathways have been dissected using established cell lines; however, it has become apparent that these models do not accurately resemble the tumor biology in vivo. For example, in vitro there is no growth differential between cells expressing wild-type EGFR compared with EGFRvIII; however, when these cells are orthotopically implanted into mice, cells expressing the mutant EGFRvIII form tumors at a significantly greater rate compared with tumors expressing wild-type EGFR.[13,14] Indeed, the molecular and pathophysiological heterogeneity of GBM is better addressed by patient-derived xenografts grown as neurospheres and expanded in vivo in recipient animals. These cell models more accurately represent the human GBM, due to their molecular and histopathological similarity to the disease, such as increased vascularity, invasion, and necrosis, and therefore represent a valid tool for effective drug design and testing.[13] Although orthotopic implantation of human tumors in mice can be a powerful tool to investigate GBM biology, genetically engineered mouse models resulting in spontaneous tumor growth have also been used. Several studies have described the role of EGFR in the induction of glioma-like tumors. Interestingly, while expression of wild-type or mutant EGFR alone is not sufficient to induce gliomagenesis in mice, combining EGFRvIII with *Ink4A/Arf* ablation or wild-type EGFR with *Ink4A/Arf* ablation plus phosphatase and tensin homolog or p53 loss results in a high penetrance of malignant glioma.[14,15] As animal models of GBM become more available, it is important to remember that the significant heterogeneity of this disease is not completely recapitulated in the mouse. Based on the intratumoral heterogeneity of EGFR mutations revealed through RNA sequencing,[4] it may be more timely and cost-effective to look to alternative models of GBM, such as those in the fly. As an example, aberrant expression of EGFRvIII in *Drosophila* has been used to investigate EGFR signaling pathways in GBM. In this study, the authors found that constitutive coactivation of the EGFR-Ras and PI3K pathways in the fly glia produced transplantable invasive glial cells which mimicked human glioma. This study revealed a role for novel kinases in glioma pathogenesis which may represent important therapeutic targets in human GBM.[16] Although several EGFR point mutations and other EGFR transcripts have been described (Fig. 1), there have been few studies which describe their oncogenic potential, and their effect on downstream signaling pathways and surrounding cancer cells remains to be elucidated.[6,17]

Therapeutic Targeting: Tyrosine Kinase Inhibitors

The prevalence of wild-type and mutated EGFR in GBM makes EGFR an excellent target. Among the strategies of EGFR inhibitors currently available, tyrosine kinase inhibitors (TKIs) have shown promising results in non-small cell lung carcinoma (NSCLC) patients. TKIs are small-molecule ATP competitors which inhibit signaling by EGFR and its ErbB family members. Two classes of TKIs have been evaluated in clinical trials for GBM patients, but all have shown little success. These are the first-generation reversible inhibitors (erlotinib, gefitinib, and lapatinib) and the second-generation irreversible inhibitors (afatinib, dacomitinib, and neratinib). Considering the prevalence of EGFR amplification and mutation in GBM, their failure to achieve any therapeutic benefit was unexpected. However, many factors may have contributed to this failure, such as insufficient target inhibition, activation of resistance mechanisms, and tumor heterogeneity.[18] For example, it has recently been shown that dynamic regulation of EGFR expression by small circular extrachromosomal DNA elements, called double-minute chromosomes, facilitates an escape route for EGFR inhibition. These DNA elements can be downregulated by EGFR-targeted therapy in vitro and in vivo but can reemerge upon drug withdrawal, thus reestablishing EGFR expression.[19] Additionally, EGFR alterations are often accompanied by alterations in other receptor tyrosine kinases, and this redundancy has also been shown to provide an escape route from anti-EGFR therapeutic targeting.[20–22] Similarly, additional intracellular pathways are often mutated in GBM patients and, because the components of the p53, retinoblastoma, and PI3K pathways tend to be mutually exclusive, these pathways may offer different resistance routes to treatment (Fig. 2).

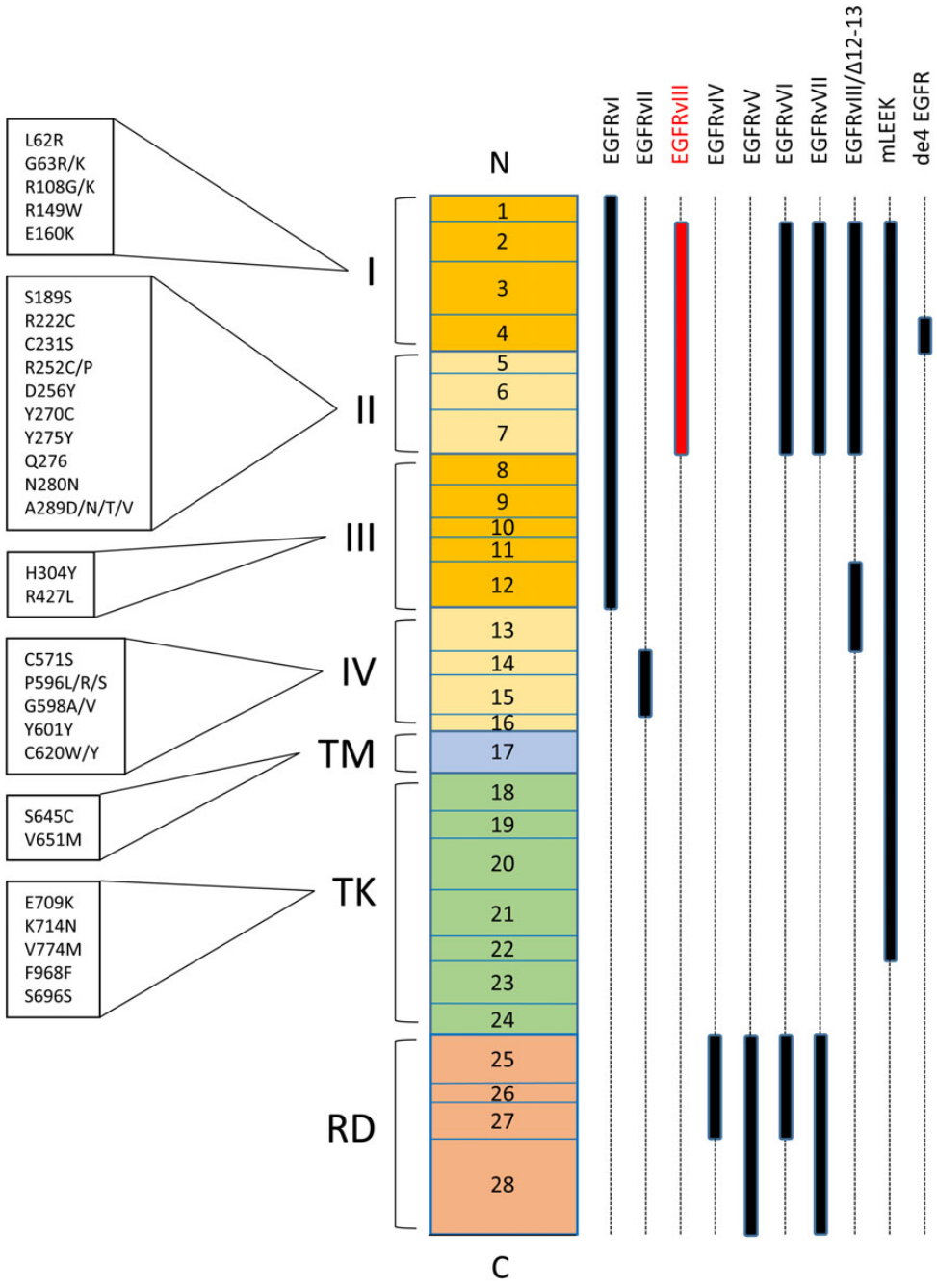

Fig. 1. EGFR domain organization and mutations found in GBM. Location of mutants within the EGFR. Exons 1–28 labeled as such. Boxes list commonly occurring point mutations by domain. The extracellular domain comprises I, II, III, and IV subdomains. TM, transmembrane domain. The intracellular domain comprises the TK domain and the regulatory/phosphorylation (RD) domain. Vertical black bars depict location of deletion mutants. EGFRvI : N-542 NH_2-terminal truncation; EGFRvII: exon 14–15 deletion; EGFRvIII (expressed in 30% of GBM patients): exon 2–7 deletion; EGFRvIV: exon 25–27 deletion; EGFRvV: C-958 COOH-terminal truncation; EGFRvVI: EGFRvIII + EGFRvIV composite; EGFRvVII: EGFRvIII + EGFRvV composite; EGFRvIII/Δ12–13: EGFRvIII + exon 12–13 deletion; mLEEK: exon 2–22 deletion; de4 EGFR: exon 4 deletion.

Therapeutic Targeting: Immune-mediated Therapy

Despite the failure of anti-EGFR targeting by TKI therapy, there have been several other targeting strategies which have performed well preclinically and are currently being tested in clinical trials. Although the brain has long been considered an "immune-privileged" organ, one which lacks true peripheral immune cell infiltration, recent evidence garnered through neurological autoimmune diseases, such as multiple sclerosis, has stimulated interest in the development of immune-mediated therapies for GBM.[23,24] GBM-specific amplification of EGFR and expression of EGFRvIII enable the exploitation of these receptors for immune-mediated therapies. In the general sense,

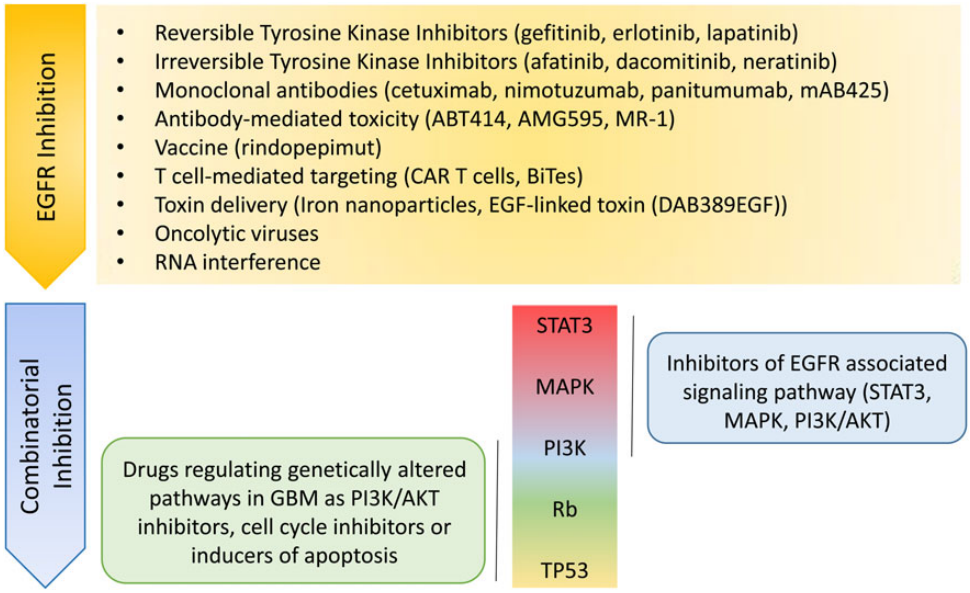

Fig. 2. Improving EGFR targeting with combinatorial therapy. EGFR inhibition has proved ineffective in initial clinical trials. Possible avenues for improving efficacy include better selection of the patient population, more potent inhibition of EGFR (by improving the drug delivery and pharmacokinetics properties), and a rational choice of drugs to combine with EGFR inhibitors, based on the understanding of signaling pathways and genetic alterations promoting tumor growth in each patient. STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase; Rb, retinoblastoma; TP53, tumor protein 53.

monoclonal antibodies directed against EGFR inhibit its signaling by binding and locking the receptor in an inactive conformation.[25] Unarmed monoclonal antibodies that have been evaluated in clinical trials include cetuximab, nimotuzumab, pantimumab, and mAb425. Aside from directly inhibiting EGFR signaling, however, antibodies such as ABT414, AMG595, and MR1-1 specifically recognize the mutant EGFRvIII and are armed with cytotoxins enabling tumor cell destruction.[18,26]

The in-frame deletion of EGFRvIII generates a novel glycine residue at the junction of exons 1 and 8 creating an immunogenic epitope, and this can be exploited by other immune-mediated therapies as well. For example, the novel peptide-based vaccine CDX-110 (rindopepimut), which is in late-stage clinical development, is an EGFRvIII-specific peptide sequence conjugated to the highly immunogenic carrier protein keyhole limpet hemocyanin. Production of an immunological response against the peptide has been shown to specifically eliminate cells expressing the EGFRvIII receptor.[27] Another approach to treating GBM patients with an immune-mediated therapy is the use of chimeric antigen receptor (CAR) T cells, which are genetically modified T cells engineered for enhanced reactivity against tumor antigens. CAR T cells that have been engineered to target EGFRvIII have shown promise, and their use is currently being tested in clinical trials.[28] Additionally, the use of EGFRvIII-targeted bispecific T-cell engagers (BiTEs) which redirect T cells against EGFRvIII-expressing GBM are being investigated.[27] Other mechanisms targeting EGFR for the treatment of GBM include EGFR gene silencing by RNA interference, ribozyme-mediated cleavage of EGFR mRNA, and novel drug conjugates such as DAB389EGF, which links EGF to the diphtheria toxin.[27] Oncolytic viruses containing a single-chain variable fragment directed against EGFR and iron oxide nanoparticles conjugated to an EGFRvIII-specific antibody have also been used to increase GBM target specificity and enhance tumor visualization.[29]

Conclusions and Future Direction

In conclusion, a lesson may be learned from anti-EGFR targeting in NSCLC. Although TKI therapy for NSCLC has been effective, EGFR point mutations have been shown to be drivers of therapeutic resistance. Such mutations can be detected in relapse tumor tissue and more recently in circulating tumor DNA found in blood.[30] Although the presence of new EGFR point mutations following TKI therapy has not been demonstrated yet for GBM, similar to the T790M mutation in NSCLC, TK domain mutations do occur (Fig. 1). Therefore it can be hypothesized that a selective pressure from anti-EGFR drugs may lead to the expansion of resistance-conferring mutants. However, more posttherapy patient tumor sampling is required to adequately address this hypothesis. Perhaps the paradigm that large EGFR deletions are the main mutations in GBM needs to be updated to incorporate the most common EGFR extracellular point mutations. These point mutations are often heterogeneously expressed with EGFR deletions, which raises the issue of their role in potentiating GBM heterogeneity.[4,6] Indeed, the reported cross-talk between wild-type EGFR and EGFRvIII which leads to enhanced tumorigenicity is further complicated by multiple EGFR genetic aberrations (mutations, activating deletions, and amplification) coexisting within the same tumor.[31] As it has been reported for the mutant EGFRvIII, other mutant EGFRs may differ in their intracellular signaling pathways or affinity for EGFR-specific therapies. It is likely, therefore, that targeting EGFR therapeutically is negatively impacted by the heterogeneity of EGFR expression necessitating the appropriate

choice of drug or drug combination. Similar to what has recently been described in NSCLC,[32] with the increasing feasibility of large-scale GBM sequencing, greater insight into the presence of various EGFR mutants, and a more thorough understanding of additional pathway circuitry within each tumor, it is likely that more effective treatment regimens for GBM patients will be designed which incorporate EGFR TKIs with additional therapeutics targeting associated signaling molecules (Fig. 2).

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