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Targeting EGFR for Treatment of Glioblastoma: Molecular Basis to Overcome Resistance

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Abstract

Glioblastoma (glioblastoma multiforme; GBM; WHO Grade IV) accounts for the majority of primary malignant brain tumors in adults. Amplification and mutation of the epidermal growth factor receptor (EGFR) gene represent signature genetic abnormalities encountered in GBM. A range of potential therapies that target EGFR or its mutant constitutively active form, ΔEGFR, including tyrosine kinase inhibitors (TKIs), monoclonal antibodies, vaccines, and RNA-based agents, are currently in development or in clinical trials for the treatment of GBM. Data from experimental studies evaluating these therapies have been very promising; however, their efficacy in the clinic has so far been limited by both upfront and acquired drug resistance. This review discusses the current status of anti-EGFR agents and the recurrent problem of resistance to these agents that strongly indicates that a multiple target approach will provide a more favorable future for these types of targeted therapies in GBM.

Keywords

Epidermal growth factor receptor; EGFR-targeted therapy; Glioblastoma; therapeutic resistance

INTRODUCTION

Malignant gliomas, the most common primary intracranial brain tumors in adults, are among the deadliest of human cancers because they are highly invasive and neurologically destructive [1]. The median survival of patients with the most aggressive of these, WHO grade IV glioblastoma (GBM), is 12–15 months with a 5-year survival rate that remains at less than 5%, despite the use of intensive treatment modalities (i.e. surgical resection, radiotherapy, and chemotherapy) [1]. Thus, the development of novel efficacious therapies is greatly warranted to substantially improve the poor prognosis of patients afflicted with GBM.

One avenue towards achieving this has been a concerted effort at providing a global description of the genetic abnormalities that are present in GBM tumors [2, 3]. The most common genetic aberration associated with malignant glioma is amplification of the epidermal growth factor receptor (EGFR, also referred to as ERBB1 or HER1), with a frequency of about 50% [1]. EGFR is a member of the HER superfamily of receptor tyrosine kinases together with ERBB2, ERBB3, and ERBB4 [4]. The structure of each of the

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members comprises: a ligand-binding ectodomain with 2 cysteine-rich regions; a single transmembrane region; and, a cytoplasmic tyrosine kinase (TK) domain [5]. Binding of a cognate ligand to the ligand-binding site of HER receptors induces receptor homo- or heterodimerization, resulting in a conformational change that activates the intracellular TK domain. This results in autophosphorylation of the cytoplasmic tail of the receptor, induction of downstream signaling (through the phosphatidylinositol 3-kinase (PI3K)/Akt and the ras-raf-mitogen-activated protein kinase (MAPK) pathways, among others) [4], and transcription of genes controlling pleiotropic cellular responses [4]. The most common ligands for HER receptors are members of the EGF family of growth factors (i.e., heparin binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin) and transforming growth factor α [6]. Interestingly, there is no known ligand for ERBB2, which is believed to undergo ligand-independent activation [4]. HER receptors are localized at the surfaces of many types of epithelial, mesenchymal, and neuronal cells such that signal transduction from these receptors into the intracellular compartment actuates many cellular processes including cell differentiation, metabolism, proliferation, and survival [7].

EGFR was the first receptor discovered to possess tyrosine kinase activity and to be sequenced [7]. In 1984, it was revealed that the sequence of EGFR was closely related to that of a known oncoprotein, the *erbB* tyrosine kinase, previously discovered to be associated with the onset of erythroleukemia [8]. Since then, EGFR has been shown to be frequently overexpressed or hyperactivated in a number of epithelial tumors [5]. The downstream signaling effects of these aberrations lead to impaired apoptosis, and/or enhanced proliferation, angiogenesis, necrosis, and treatment refractoriness, suggesting a causative relationship between receptor dysregulation and the pathobiology of many cancers. Several possible mechanisms are attributed to receptor dysregulation. These include gene amplification and intrinsic alterations of the receptor structure as a result of mutation. Indeed, many EGFR mutants have been described, and the most common mutant form associated with GBM is ΔEGFR (also named EGFRvIII, or de2-7EGFR) [9]. Other mutant forms, such as EGFRvII and EGFRvV are also found in GBM, but are infrequent and their clinical relevance is undefined [10]. Mutant ΔEGFR, arises through an in-frame deletion of 801 bp from the extracellular domain of EGFR and possesses ligand-independent constitutive (but low) tyrosine kinase activity [11]. Additionally, low level autophosphorylation of ΔEGFR results in defective receptor internalization due to reduced interaction with Casitas B-lineage (Cbl) proteins, resulting in increased stability of the receptor at the cell surface and amplified mitogenic effects [12, 13].

EGFR gene amplification and mutations are also found in breast, lung, and prostate cancers [14]. In spite of this, therapies that have been effective for solid tumors originating from these tissues have shown limited efficacy against GBM. EGFR-specific inhibitors have been approved for use in patients with non-small cell lung carcinoma (NSCLC), and are currently in clinical trials for GBM [15, 16, 17]. However, the clinical experience has been that many GBM patients do not respond to these therapies and those that do eventually show progression [13]. Thus, while knowledge of the inherent genetic alterations is pertinent in determining rational therapeutic targets, the biology of glioma has so far rendered it inadequate for predicting a durable drug response in GBM patients. In this review, we will focus on the current status of EGFR-targeted therapies as potential treatments for glioblastoma. We will then discuss the adeptness of GBMs at escaping the need for receptor function when challenged with receptor-targeted therapeutics, and how this complexity should be considered when exploring strategies to overcome the problem of therapeutic resistance.

GLIOBLASTOMA

Epidemiology

Each year approximately 6 to 12 out of 100,000 individuals are diagnosed with primary malignant brain tumors in the United States [18, 19]. These tumors represent ~ 2% of all cancers diagnosed each year and are the cause of 2% of all deaths from cancer in the U.S., with a death rate of ~13,000 Americans per year [18]. The World Health Organization (WHO) classification of Tumours of the Central Nervous System distinguishes gliomas based primarily on their histological appearances, where the grade indicates the level of malignancy [20, 21]. As already mentioned, the most malignant form is Grade IV glioblastoma, and is characterized by small areas of necrotic tissue surrounded by anaplastic cells [21, 22]. Tumors of this grade also display a number of hyperplastic blood vessels that facilitate rapid proliferation [21, 22]. Specifically, glioblastomas accounts for 60–70% of malignant gliomas diagnosed in American adults between the ages 46–74, and is more frequently diagnosed in men than in women [18]. Two major subclasses (primary and secondary) of GBM have been established based on the clinical properties and the chromosomal and genetic aberrations that are unique to each [1, 5]. Primary GBM appears to arise *de novo* from normal glial cells, or their precursors, and commonly occurs in patients above the age of 45 years [19]. In contrast, secondary GBM arises from the progressive transformation of lower grade tumors, are much less frequently encountered and are primarily seen in younger patients [1]. Given that the histopathology between primary and secondary GBM are identical at the grade IV stage, an exact estimation of frequency between the two is difficult to make. Nevertheless, primary GBM is believed to account for 95% of all GBMs, while only ~5% are believed to occur secondarily [23].

Genetic Alterations

The frequency of EGFR gene amplification and overexpression is more prevalent in the more common primary GBM compared to the secondary form [24]. In contrast, the frequency of inactivated p53 between the two mostly occurs in the less common secondary GBMs [24]. Thus, it appears that dysregulation of a variety of genetic pathways characterizes malignant gliomagenesis, with one subclass governed by overexpression or amplification of an oncogene and the other by inactivation of a tumor suppressor gene in the presence of a metabolic defect caused by mutations of the IDH1 and IDH2 genes [25]. Some of the other common genetic abnormalities encountered in GBM include deletion of $p16^{Ink4a}$, phosphatase and tensin homolog (PTEN) mutations, loss of heterozygosity (LOH) at 10q, and amplification of the platelet-derived growth factor (PDGF) and receptors (PDGFRα/β), further highlighting the complexities of this disease that make it a challenge to provide effective therapies [1, 22].

Dysregulation of EGFR has also been shown to enhance tumor growth, migration, angiogenesis, and metastatic spread [26]. Additionally, EGFR overexpression is a poor prognostic factor and correlates with decreased overall survival in GBM patients [27]. Most glial tumors that overexpress EGFR concomitantly overexpress the constitutively active mutant variant, ΔEGFR, with a frequency of ~40–50% [11]. Interestingly, ΔEGFR is tumor-specific, as it has not been found in normal tissues [14]. ΔEGFR overexpression correlates with induced tumor formation, increased proliferation, and inhibition of apoptosis [11, 28]. Similar to the wild-type (wt) receptor, the presence of ΔEGFR confers a less favorable prognosis for GBM patients [22]. Data from *in vitro* studies suggest that the cellular responses manifested due to the increased presence of wtEGFR and ΔEGFR result primarily from downstream activation of the MAPK/ERK and PI3K-Akt pathways, and in some cases, the co-expression of matrix metalloproteinases (MMPs) [29, 30]. Furthermore, it has been demonstrated *in vivo* that treatment with the ΔEGFR-specific monoclonal

antibody (mAb 806) significantly decreases tumor growth and increases apoptosis [31]. This antibody functions relatively specifically to ablate ΔEGFR-mediated signaling, therefore, it seems that some glioma cells may require the intracellular signaling imparted by ΔEGFR for survival and should be susceptible to inhibitors of this process. Both wtEGFR and ΔEGFR are bonafide oncogenes that are prevalent in GBM and this nominates them as attractive targets for therapeutic strategies [9, 11]. Indeed, several EGFR-targeted therapies have been developed and are currently in various stages of clinical trials for the treatment of malignant glioma [9, 17].

CURRENT TREATMENT OPTIONS FOR GLIOBLASTOMA

Standard of Care

Standard treatment for almost all GBM cases begins with surgical resection with the goal of gross total tumor removal to alleviate GBM symptoms and to facilitate treatment of any residual tumor [32]. Despite recent technological developments in surgical techniques, the vast majority of patients are not cured by surgical resection. Tumors often infiltrate the normal brain parenchyma, and this invasive nature necessitates the use of adjuvant radiotherapy that is either combined with or is subsequently followed by chemotherapy [1]. Radiotherapy involves the administration of usually 50 to 60 Gy of irradiation to the whole brain following surgery [33]. For several decades, nitrosoureas, particularly carmustine (BCNU) and lomustine (CCNU), were the most common chemotherapeutics used alone or in combination with radiotherapy [32, 34]. Pivotal clinical trials conducted in the 1970s and 1980s evaluating the benefits of adjuvant therapy demonstrated that patients with malignant glioma treated after surgery with radiotherapy, or radiotherapy in combination with chemotherapy, displayed an increase in overall survival [35, 36]. Additionally, in 1999, an alkylating agent, temozolomide (TMZ), became commercially available as a treatment for glioblastoma, and has become a part of the standard treatment regime for most cases [37] largely because of a large randomized clinical trial, where it was shown that the median survival in newly diagnosed GBM patients treated with radiation plus TMZ was 14.6 months as opposed to 12.1 months for the radiation only group [38].

Despite these optimizations to the adjuvant therapy regimens, the overall survival rate for GBM patients has remained relatively the same as it was ten years ago. Tumors inevitably recur, and once they do, life expectancy drastically diminishes and only limited therapeutic options are available. Major advances in molecular and cell biology have afforded the development of novel second and third line therapies for cancer patients in the realm of targeted therapy. So far, targeted therapy has been used effectively against breast tumors driven by growth factor receptor dysregulation, and in the case of GBM, therapies directed against a number of growth factors and receptors (i.e. VEGF, EGFR, PDGFR) are in various stages of clinical development [39, 40, 41, 42, 43]. The most common and most clinically advanced of these therapies target EGFR and the ligand-independent mutant ΔEGFR. Table 1 lists the current EGFR/ΔEGFR targeted agents for the treatment of glioma, which include monoclonal antibodies, tumor-antigen specific vaccines, small molecule inhibitors, and RNA-based therapies [5, 9].

EGFR-Targeted Therapy

Monoclonal Antibodies—Cancer immunotherapy seeks to manipulate a person's own immune system to recognize and specifically destroy tumor cells, using target-specific antigenic proteins and peptides [44]. Although, tumor immunotherapy has shown some success in the treatment of renal cell carcinoma, melanoma, and hematologic cancers, the application of this approach to glioma presents more of a challenge [44]. Limitations include possible hindrance in the drug's ability to cross the blood-brain barrier (BBB) and induction

of potential autoimmunity, which could lead to severe and undesired effects, such as central nervous system toxicity [44, 45].

In the early 1900s, Paul Erhlich was the first to propose the process of using monoclonal antibodies to target tumors, and later advances in antibody technology afforded the production of human monoclonal antibodies [45, 46]. One way to inhibit EGFR-mediated signaling is to disrupt receptor-activating ligand binding [45]. Blocking ligand binding to its cognate receptor could normalize growth rates, induce apoptosis, and increase tumor susceptibility to chemotherapeutic agents. Monoclonal antibodies, both unconjugated and conjugated, directed towards wtEGFR and ΔEGFR have been developed for therapeutic use in GBM. The most developed of the unconjugated antibodies is cetuximab (Erbitux®; Merck KGaA), which functions to prevent EGFR-mediated signal transduction by interfering with ligand binding and EGFR extracellular dimerization [45]. Additionally, cetuximab is also believed to trigger EGFR receptor internalization and destruction [47]. Pre-clinical data from *in vitro* studies suggest that treatment with cetuximab alone has a minimal impact on glioma tumorigenicity; however, it is synergistic with cytostatic drugs and radiotherapy [47, 48]. Mouse xenograft (intracranial and subcutaneous) studies have demonstrated that treatment with cetuximab decreases proliferation and increases cell death and overall survival [49]. Despite the fact that antibodies are large in molecular weight, these data suggest that cetuximab can traverse the blood-brain barrier. Currently, a phase I/II clinical trial is ongoing to study the efficacy of combining radiotherapy, TMZ and cetuximab, together known as GERT, to treat patients with primary GBM [50]. Other unconjugated monoclonal antibodies that are in initial stages of clinical development for GBM include panitumumab (Vectibix®; Amgen) and nimotuzumab (Theraloc®; YM BioSciences Inc.), which all function similarly to cetuximab [5, 45]. More recently, cetuximab has been shown to be effective against GBM tumors harboring a novel exon 27 deletion mutation in the carboxyl-terminus domain of EGFR, EGFR-CTD [51]. Particularly, cetuximab was effective in impairing tumorigenicity and prolonging the survival of xenograft mice with oncogenic EGFR-CTD deletion mutants [51]. Thus, while it appears that cetuximab alone has had limited clinical effectiveness among GBM patients, these data suggest that it may be a more promising therapeutic for patients harboring this specific EGFR mutation.

With respect to conjugated antibodies, the specific binding properties of antibodies are exploited to deliver the toxic effects of either conjugated toxins or radioisotopes. ^{125}I-MAb 425 is one of the most advanced of the radioisotope-conjugated monoclonal anti-EGFR antibodies. Various phase II clinical trials have reported that ^{125}I-MAb 425, either administered alone or concomitant with radiotherapy or temozolomide, significantly improves median survival in GBM patients [52, 53, 54]. The ligand-conjugate toxin composed of EGF and diphtheria toxin, DAB389EGF, represents another means to specifically transport toxins to EGFR, which involves the use of cognate ligands as vectors. Additionally, treatment with DAB389EGF was associated with significant tumor regression in subcutaneous glioblastoma xenografts [55]. Some monoclonal antibodies have been engineered to specifically target ΔEGFR. One such antibody is mAb806, which attenuates receptor autophosphorylation by binding to the short cysteine loop of the extracellular domain that is always exposed in ΔEGFR, and may also weakly target amplified wt EGFR, which transiently exposes this epitope during the switch from the inactive to the ligand-activated conformation [31, 56]. Pre-clinical data show that mAb806 strongly inhibits the growth of tumor xenografts that express ΔEGFR and more weakly those that express wt EGFR. In the clinic, it is well tolerated and displays excellent biodistribution and specificity for its target in GBM patients [57, 58]. These promising results have prompted a Phase I clinical trial with a humanized version of mAb806 (*ABT-806*; Abbott).

Vaccines—Antitumor vaccines have also been developed with the promise of precisely eradicating tumor cells while limiting toxicity. To date, vaccines in clinical development for the treatment of glioma consist of different combinations of dendritic cells (DCs), peptides, adjuvants, and even autologous tumors [44]. The most promising of these vaccines are dendritic cell and peptide-based; other combinations have resulted in either no significant improvement over standard therapy or the induction of several adverse events [44]. The ΔEGFR-specific vaccines are directed against a novel glycine epitope at the fusion junction that arises as a consequence of the in frame deletion of exons 2–7 from the extracellular domain of wtEGFR [59]. The proposed mechanism of action for achieving tumor regression is believed to initiate with capture of the peptide by the antigen-presenting complex (APC). The peptide is then relocated into the most proximal lymph nodes, where the antigenic peptide is presented to circulating cytotoxic T-lymphocytes (CTLs), which are then activated upon recognition, and finally infiltrate the tumor to eliminate the respective cancer cells [60, 61]. Tumorigenicity studies in rodents show that intracerebral treatment with a ΔEGFR synthetic peptide conjugated to keyhole limpet hemocyanin (PEP-3-KLH/ CDX-110®, Celldex Therapeutics) reduces tumor size and increases overall survival [62]. Although major clinical responses were rarely obtained from previous peptide-based vaccine studies, the PEP-3-KLH vaccine in combination with TMZ has been shown to improve both progression free survival (14.2 months vs 7.3 months) and median survival (26 months vs. 15. 2 months) in GBM patients [5, 60]. Additionally, a vaccine comprised of DCs pulsed with the PEP-3-KLH peptide has been tested in the clinic and was shown to increase overall median survival time (22.8 months vs. 15.2 months) without severe adverse events [63]. From these results, it has been proposed that vaccination with PEP-3-KLH is safe and capable of inducing ΔEGFR-specific immune responses in patients with newly diagnosed primary GBM.

A vaccine that recognizes wtEGFR has also been designed, in which an EGFR binding peptide is conjugated with a lytic-type peptide containing cationic-rich amino acids that kills the cancer cell by disintegration of the cell membrane [64]. The goal of this vaccine is to serve as an adjuvant for either treatment with monoclonal antibodies or tyrosine kinase inhibitors to eradicate the cancer cells that are intractable to signaling inhibition, which renders them resistant to these therapies. Recent *in vitro* studies show that the EGFR-lytic peptide destroys cancer cells as quickly as ten minutes after exposure and exerts strong cytotoxic activity on TKI-resistant glioma cells. Although, this therapy has not been tested specifically in a glioblastoma xenograft model, it was shown to be effective in suppressing the growth of both breast and pancreatic xenografts [64]. Futhermore, more recent evidence has demonstrated that a single replacement of histidine to arginine in the lytic peptide sequence enhances its anticancer activity [65]. Taken together, these data suggest a potential value for this therapy against GBMs that should be further explored.

In spite of these advancements, a number of obstacles involving the efficacy of epitope-specific vaccines remain, including the notion that targeting of a heterogeneously expressed tumor antigen may potentially select for the survival and proliferation of antigen-negative cells. In fact, in a more recent phase II clinical trial to assess the immunogenicity of the PEP-3-KLH peptide vaccine, tumor recurrence following a significant period of progression free survival was observed [66]. Indeed, 82% of the relapse tumors were completely ΔEGFR negative, demonstrating that this vaccine can effectively eliminate ΔEGFR-expressing cells; however, it currently lacks the efficacy that is needed to eradicate this disease [66].

Small Molecule Inhibitors—Small molecule tyrosine kinase inhibitors (TKIs) are the most clinically advanced of the EGFR-targeted therapies, and both reversible and irreversible inhibitors are in clinical trials. Some of the reversible inhibitors include erlotinib

(Tarceva®/OSI774; Genentech/Roche/OSI), gefitinib (Iressa®/ZD1839; AstraZeneca), lapatanib (Tykerb®; GlaxoSmithKline) and PKI166 (Novartis), and the irreversible inhibitors include canertinib (CI1033; Pfizer/Warner-Lambert) and pelitinib (EKB-569; Wyest-Ayerst) [42]. Mechanistically, these TKIs compete with ATP for binding to the tyrosine kinase domain of EGFR [42]. The irreversible and reversible nature of these inhibitors lead to the ablation of both phosphorylation of the receptor and downstream signaling [5]. Though a number of these inhibitors have been developed, gefitinib and erlotinib represent the best explored TKI inhibitors in the clinic for the treatment of GBM [5].

In pre-clinical *in vitro* studies, erlotinib was shown to inhibit anchorage-independent growth of glioblastoma cell lines [67]. More importantly, this inhibition was shown to correlate with suppressed induction of EGFR mRNA [67]. Additionally, long-term exposure to erlotinib was found to down-regulate the expression of both ΔEGFR and molecular effectors of tumor invasion in transformed glioblastoma cell lines [30]. The efficacy of erlotinib, however, is more characterized in other cancer cell types, where it has been shown to inhibit cell-cycle progression by inducing G_1/S phase arrest [68, 69]. Moreover, erlotinib is able to induce apoptosis in colon and pancreatic cancer cell lines by stimulating DNA fragmentation and decreasing the expression of the anti-apoptotic Bcl-2 family members, respectively [68, 69]. Data from *in vivo* studies demonstrate that erlotinib also displays anti-angiogenic activity by suppressing vessel formation in pancreatic tumor xenografts [69]. Erlotinib was first clinically tested for the treatment of advanced and metastatic NSCLC, and to date was shown to significantly improve median survival in these patients by 42.5% in a phase III randomized trial [5]. In contrast, in a recent phase II clinical trial for GBM therapy, erlotinib was well tolerated, but only demonstrated a modest effect over placebo [15]. These results underscore the notion that differences in tissue-specific biology and/or signaling networks coupled to EGFR greatly influence TKI efficacy.

Interestingly, the antitumor activity of gefitinib is independent of the expression level of EGFR, but is heavily determined by its ability to inhibit anti-apoptotic signals [70]. Similar to erlotinib, gefitinib was shown to be effective at inhibiting the *in vitro* growth of a variety of human cancer cell lines by similar mechanisms [71]. Treatment with gefitinib was shown to inhibit cell survival and proliferation and by inducing G_0/G_1 arrest in adenocarcinoma and pancreatic cancer cells, respectively [69]. Additionally, gefitinib is able to hinder the *in vivo* growth of human breast and ovarian tumor xenografts, but despite its success in other cancer cell types, gefitinib has been less effective against glioblastoma [71]. Gefitinib and erlotinib appear to work best against tumors expressing EGFR with mutations in exons 19 and 21 of the TK domain, but to date, such EGFR mutants have not been found in GBM [5]. Interestingly, in the same study testing the efficacy of cetuximab against GBM tumors harboring novel EGFR mutations, erlotinib was also shown to be very effective against these EGFR CTD mutant GBM tumors [51]. Unfortunately, the clinical efficacy of gefitinib reflects the pre-clinical studies, in which in one phase II clinical trial, gefitinib was well tolerated and displayed anti-tumor activity, but the median overall survival time in GBM patients was only 38.4 weeks from treatment initiation [16]. A more recent phase II trial revealed that gefitinib reaches high concentrations in tumor tissue and efficiently dephosphorylates its target; however, regulatory circuits that promote sustained downstream signal transduction independent of EGFR phosphorylation appear to be more dominant [72]. Thus, it appears that gefitinib alone may prove to have limited therapeutic value in treating GBM patients.

The reality is that these EGFR-specific tyrosine kinase inhibitors have been relatively ineffective against gliomas, with response rates only reaching as high as 25% in the case of erlotinib [9]. Though both TKIs are well tolerated and display some antitumor activity in

GBM patients, the recurrent problem of resistance to receptor inhibition has limited their efficacy [73, 74]. The presence of ΔEGFR with intact PTEN has been suggested as a molecular determinant of glioma sensitivity to TKIs [70], but many tumors that harbor this signature show only a modest response to TKIs or are resistant [70]. Furthermore, this signature is not predictive of TKI sensitivity in some serially passaged GBM xenografts [75]. These findings suggest that additional molecular determinants are relevant, and discovering them will be critically important to the rational design of more effective GBM therapies. Additionally, though the molecular weights of small molecule inhibitors are within the size limit for molecules that are allowed to cross the BBB, recent studies have shown that plasma concentrations of gefitinib and erlotinib following therapy were only 6–11% of the starting dose [5]. Thus, insufficient delivery to the target may be another cause of the disappointing clinical responses to these TKIs.

EGFR RNA-based Therapies—Interference with genetic transcription or translation is another mechanism through which receptor inhibition may be achieved, and some methods that have been developed over the last decade include antisense RNA, RNA interference (RNAi), and ribozymes [76]. Antisense oligonucleotides, such as OGX-011, are already in advanced clinical development for the treatment of NSCLC and prostate cancer, and thus far, the clinical responses are very promising [77]. Antisense RNAs hybridize to the sense mRNA of the target, resulting in inhibition of translation and protein synthesis. In an orthotopic xenograft model of human glioblastoma, intratumoral injection of a plasmid or viral vector expressing ΔEGFR-targeted antisense RNA was shown to cause a significant decrease in tumor growth [78]. In RNA interference methods, the suppression of homologous genes by small interfering RNAs (siRNAs) leads to sequence-specific target mRNA degradation. EGFR-specific siRNAs are directed against the TK domain, and were shown to cause 90% knockdown of EGFR mRNA in U251 glioma cells [79]. Furthermore, siRNA mediated-knockdown of EGFR resulted in G2/M arrest and reduced proliferation [79]. These findings were confirmed *in vivo* in an intracranial xenograft model, where treatment with EGFR-specific siRNAs increased overall survival by almost 90% [79]. However, the safety of siRNAs as therapeutics has been the biggest concern. In other *in vivo* studies, a vast number of mice fatalities were observed due to oversaturation of RNAi pathways [76]. One strategy in place to overcome this obstacle is to use the lowest possible concentration of siRNAs that provides therapeutic efficacy by designing exogenous siRNAs with increasing length [76]. This would introduce them into the RNAi pathway upstream of RISC directly at the step of Dicer cleavage, resulting in enhanced RNAi activity at lower concentrations [76]. Another strategy in place is the use of cyclodextrin-modified dendritic polyamine complexes (DexAMs) as a vehicle for translocating siRNAs [80]. Recently, DexAMs were shown to deliver EGFRvIII siRNAs efficiently and selectively to glioblastoma cells with minimal toxicity [80]. Futhermore, codelivery of EGFRvIII-siRNA and erlotinib in GBM was found to significantly inhibit cell proliferation and induce apoptosis in glioblastoma cells [80]. Finally, a third method of interference that has been explored involves the use of anti-ΔEGFR hairpin ribozymes. Ribozymes catalytically cleave certain RNA substrates in a sequence-specific manner, in which cleavage is mediated by a catalytic core [5]. In pre-clinical *in vitro* studies, treatment with anti-ΔEGFR hairpin ribozymes was shown to reduce ΔEGFR mRNA by 90% and inhibit anchorage-independent growth of U87MGΔEGFR glioma cells [5]. These encouraging pre-clinical outcomes along with the success of this approach in other cancers suggest that RNA-based therapies should be further explored in clinical trials for the treatment of GBM.

MALIGNANT GLIOMA AND THERAPEUTIC RESISTANCE

In spite of the advances in our current knowledge of glioma biology and genetics, this disease remains largely incurable. For the reasons discussed above, some EGFR-targeted

agents hold great potential, however, successful treatment of malignant gliomas continues to be a major therapeutic challenge due to both inherent and acquired resistance [81, 82]. Mechanisms causing resistance to EGFR TKIs have been studied extensively in a number of solid tumors. Some of the documented mechanisms include the acquisition of secondary EGFR point mutations, co-activation and/or amplification of other RTKs, and up-regulation of drug efflux pumps [82]. A few of these mechanisms have been established for NSCLC and breast cancer; however, mechanisms of resistance that are unique to glioma are not clearly defined [82, 83]. Malignant gliomas are recalcitrant by nature and are able to escape the need for receptor function by activating alternative compensatory pathways when challenged with receptor-targeted therapeutics. Though, the specific compensatory mechanisms that are essential to this escaper phenotype have not been delineated, a few generalizations (see Figure 1.) can be drawn from established knowledge in the field of glioma biology and therapeutic resistance [70].

Cross-Resistance

Malignant brain tumors are typified by resistance to apoptosis and diffuse invasion into the normal brain parenchyma [84]. During invasion, brain tumors cells often arrest in mitosis, rendering them refractory to radiotherapy and chemotherapy, whose antitumor activity triggers DNA damage-induced apoptosis [85]. Additionally, once tumor cells disseminate into the normal brain regions, they are protected by an intact BBB, which can also significantly limit the efficacy of therapeutic agents that cannot traverse this barrier [85]. Furthermore, despite being robustly angiogenic, the tortuous vasculature in malignant brain tumors critically limits drug penetration [85]. Hence, overcoming the obstacles that lie within the inherent biology of gliomagenesis will be necessary if these tumors are to become completely eradicated. A primary mechanism by which some EGFR TKIs (i.e., gefitinib) exert their effects is also through induction of apoptosis [70]. In essence, brain tumor cells that have developed a way to tolerate chemotherapy and radiotherapy may also be intractable to these TKIs or vice versa, as a result of cross-resistance [86]. In fact, oncogenic ΔEGFR has been demonstrated to confer resistance to chemotherapy by inducing the expression of the anti-apoptotic protein Bcl-xL [86]. Surprisingly, despite sustained repression of ΔEGFR expression, some GBM tumors eventually recur [87]. These ΔEGFR-independent tumors were significantly less apoptotic and had restored Bcl-xL protein expression [87]. This result and other findings that demonstrate that some tumors are also maintained in the absence of EGFR kinase activity suggest that treatment with EGFR-targeted therapies alone may not reach maximum therapeutic efficacy [87, 88]. The most clinically advanced approach to inhibit Bcl-xL anti-apoptotic activity involves the use of a bi-specific antisense Bcl-2/Bcl-xL oligonucleotide [89]. In pre-clinical studies, this therapy has already been shown to be effective in inducing apoptosis in glioblastoma cell lines that overexpress these two proteins [90]. Thus, concomitant treatment with Bcl-xL-specific antisense oligonucleotides may enhance the efficacy of both chemotherapeutic agents and EGFR-directed TKIs in the treatment of GBM [91].

Tumor Heterogeneity

GBM tumors are considerably heterogeneous, and this intratumoral diversity represents a second probable cause of anti-EGFR therapeutic resistance. Within these tumors lie mixed cytological subtypes, regional differences in gene expression, and variable representation of key genetic mutations and chromosomal alterations [84]. The maintenance of individual cell growth within GBM tumors could result from (1) mutation and amplification of multiple RTKs, or, (2) deletions or decreased function in tumor suppressor genes. In essence, some tumors may be inherently resistant to EGFR-targeted therapy simply because they are not dependent on its receptor function. In fact, in other solid tumors such as colorectal cancer, activation of ERBB2 signaling was shown to be a mechanism of resistance to cetuximab

[92]. Indeed, other RTKs and GPI-linked receptors, such as IGFR1, MET, PDGFRα/β and uPAR, have been reported to be altered in GBM, and their ability to compensate for EGFR has been implicated in the persistent activation of downstream survival signaling, even in the presence of EGFR inhibitors [93, 94, 95, 96]. For example, treatment with either erlotinib or the MET inhibitor SU11274 alone had no discernible effect on inhibiting the activation of the PI3K/AKT pathway in U87MGΔEGFR cells. However, when used in combination, downstream signaling as measured by activated Akt and S6 ribosomal protein was significantly inhibited and anchorage-independent growth of these cells was also reduced [93, 97] Similarly, activation of c-Met expression in response to EGFR inhibition led to the survival of GBM tumor cells in a mouse model of glioblastoma and inhibiting MET reversed this phenotype [98]. These results indicate that co-activation of RTKs within the same tumor may confer resistance to EGFR TKIs. Persistent activation of the PI3K/Akt pathway may also be due to mutations in the tumor suppressor gene PTEN, which is a negative regulator of this pathway. The frequency of PTEN deletion or mutation in GBM is around 40–50%, indicating that there is a high probability that the antitumor activity of many EGFR inhibitors will ultimately be negated due to defects in components of the targeted pathway [93]. Co-expression of ΔEGFR and wild type PTEN has been shown to correlate with tumor sensitivity to EGFR TKIs; however, as mentioned before, there is considerable variability in patient response despite the presence of this genetic signature [70]. This suggests that multiple inputs to PI3K signaling may confer insensitivity to these therapies. More recently, PTEN-deficient glioblastoma cell lines were shown to employ the autophagic process as a survival pathway of escape [99, 100, 101]. Specifically, overexpression and accumulation of αB-crystallin, a heat shock protein that can impair caspase activation, following erlotinib treatment was observed in DBTRG-05 and U87 glioma cells, respectively [99]. Likewise, inhibition of autophagy or autophagosome maturation was shown to increase the death-inducing activity of both erlotinib and an mTOR inhibitor, PI-103, respectively [99, 100]. Thus, cooperation between TKIs and inhibitors of predictive compensatory mechanisms may be required to achieve a more robust anti-tumor effect against GBM.

Cooperative tumor cell-tumor cell interaction has also been linked to the maintenance of intratumoral heterogeneity in GBM [84]. Inda *et al.*, demonstrated that ΔEGFR-expressing cells release interluekin-6 (IL-6) in order to activate wtEGFR expressing cells through a paracrine cytokine signaling circuit [84]. The secretion of IL-6 was also correlated with enhanced tumorigenic growth of U87 glioma cells [84]. Prior to these studies, IL-6 had been implicated in drug resistance and survival signaling through both the BclxL and STAT3 pathways in prostate cancer [102]. Interestingly, these effects were significantly attenuated by transfection with anti-sense Bcl-xL oligonucleotides, further supporting the notion that RNA-based therapies should be more closely considered as a therapeutic option in GBM therapy [102]. More recently, IL-6, as part of the EGFR-ID3-IL-6 signaling axis, was shown to also promote tumor cell heterogeneity in GSC populations [103]. Taken together, it is clear that GBM tumors possess a number of intrinsic variables that would render single-based therapies inept, and understanding this variability will be important to the optimization of these agents for clinical use.

Cancer Stem Cells

Although the genetic pathways that are involved in the development of malignant gliomas are now reasonably well-characterized [2, 3], the cellular origins of these tumors are poorly understood [18]. There is growing evidence that glioma stem cells are major contributors to resistance to standard treatments, and several studies have demonstrated that malignant brain tumors that were enriched with cancer stem cells (CSCs) were more resistant to radiotherapy and chemotherapy than other tumors due to their ability to alter both checkpoint and DNA

repair pathways [85, 104]. Many conventional therapies specifically target rapidly proliferating cells, while sparing the quiescent, tumor cell compartment. This arises from the notion that proliferation is the main problem for cancer treatment [105]. Indeed, rapidly proliferating cells make up the bulk of the tumor; however, if the CSCs that are left behind repopulate the tumor, then the question that inevitably arises is: are current anti-cancer therapies targeting the right population of cells? CSCs are thought to be the source of tumor relapse due to increased genetic stability, decreased oxidative stress, or the presence of multiple drug resistant transporters [106]. In the latter case, activation of these transporters could lead to increased efflux of anti-EGFR agents from the tumor, resulting in decreased intracellular concentrations of drug. Additionally, the genetic stability of this population would render them unperturbed by the pro-apoptotic signals generated by EGFR TKIs. The challenge would therefore remain in designing novel therapies that specifically target the CSC population or CSC-specific multidrug resistance transporters to eradicate these sources of tumor maintenance. More importantly, as a role for neural stem/progenitor cells (NSCs) in learning and memory is becoming accepted, understanding the biological differences between normal and cancer stem cells is required to develop such selective therapies in order to spare normal brain cells. In fact, a recent study testing the efficacy of bortezomib, a protease inhibitor, on treating multiple low- and high-grade glioma stem-like cell (GSC) cultures revealed that bortezomib reduces GSC populations by 80% with minimal effects on NSC populations [107]. Additionally, erlotinib was shown to produce similar results as bortezomib, while TMZ and cisplatin were shown to be more toxic to NSCs and less effective against GSCs [107]. Thus, it appears that combining newer, promising agents with TKIs, rather than with older chemotherapeutic agents, could result in more durable responses in GBM patients. The nonreceptor tyrosine kinase BMX has also been implicated in maintaining self-renewal and tumorigenic potential of GSC populations by activating STAT3 [108]. Furthermore, BMX knockdown potently inhibited STAT3 activation and growth of GSC-derived intracranial tumors, suggesting that BMX represents a GSC therapeutic target [108]. Thus, identifying more GSC-specific targets and testing the efficacy of additional selective drugs are clearly warranted in an effort to overcome the contribution of cancer stem cells to therapy resistance.

Tumor Microenvironment

Malignant glioma patients are known to be profoundly immunosuppressed, and even if systemic immune responses are generated by EGFR-specific monoclonal antibodies or peptide vaccines, they could be negated in the tumor microenvironment by a variety of immunosuppressive growth factors and cytokines [109]. In contrast to EGFR TKIs, the mechanisms of resistance to these therapies in any cancer are poorly defined [91]. Growth factors, such as TGFβ, have been shown to promote tumor escape from immunosurveillance, and high plasma levels of TGFβ correlate with a negative prognosis in a variety of cancers [45]. Additionally, the production of TGFβ can lead to the accumulation of $CD3^+$, $CD4^+$, $CD25^+$, $FOXP3^+$ T_{Reg} cells. Regulatory T cells (T_{Regs}) are a specialized subpopulation of T cells that function to actively suppress the immune system in order to maintain system homeostasis and prevent pathological self-reactivity. In essence, immunosuppressive cell populations, such as T_{Regs}, could be attributed to the attenuated efficacy of EGFR-specific immunotherapy agents by way of inhibiting antitumor immunity. In GBM, increased numbers of T_{Reg} cells have been found in the peripheral blood and also in the tumor microenvironment, and though a prognostic role of T_{Regs} present in glioma has not been thoroughly evaluated, T_{Regs} have been shown to be associated with poor clinical outcome in other systemic tumors [109]. In a syngeneic murine model of glioma, depletion of T_{Regs} resulted in complete tumor rejection and enhanced survival [110]. Given that STAT3 is required for TGFβ production, inhibitors of the JAK/STAT pathway have been explored as a potential strategy to overcome T_{Reg}-mediated immunosuppression [109].

Additionally, STAT3 is overexpressed in glioma and the STAT3 inhibitor WP1066, was shown to be effective in inducing apoptosis and inhibiting the *in vivo* growth of U87 glioma cells [111]. Currently, GC1008, a TGFβ-specific antibody, is in the initial stages of clinical development for metastatic kidney cancer and malignant melanoma, and given the role of TGFβ in immunosuppression, GC1008 or WP1066 in combination with EGFR-specific immunotherapy agents could potentially be more efficacious in the treatment of malignant glioma.

Finally, aside from a possible role for IL-6 in intrinsic resistance, other studies have shown that secretion of IL-6 by stromal cells into the tumor microenvironment may also promote tumor survival and block apoptosis, thereby resulting in chemotherapeutic resistance [112]. These effects were also shown to be via the JAK/STAT and Bcl-xL pathways [112]. More recently, TGFβ-dependent IL-6 secretion was shown to be responsible for the acquired resistance of lung tumor cells to the EGFR TKI erlotinib [113]. Thus, as an alternative approach, blocking IL-6-mediated signaling may enhance the efficacy of EGFR-targeted therapies.

PERSPECTIVES

The successful application of EGFR-targeted therapy for the treatment of glioblastoma has been proven to be very challenging. A deeper understanding of the intricate inter-relationships that underlie the pathobiology of this disease will be required to achieve stable therapeutic responses to targeted agents. Malignant brain tumors require a very complex signaling network that is not only driven by EGFR, and this complexity dictates tumor sensitivity to EGFR-targeted therapies. Therefore, blocking EGFR alone may not sufficiently translate into a clinical benefit for GBM patients. In essence, a departure from the status quo of single-based EGFR targeted therapy should be considered, which holds promise that substantial increases in long-term disease control can be achieved not only for glioma patients, but also for individuals afflicted with other cancers that are driven by these genetic lesions. With the development of novel combinatorial therapies, improvement in patient quality of life may be achieved through tailored, personalized choices of appropriate therapeutic modalities.

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List of Abbreviations

GBM	Glioblastoma multiforme
WHO	World Health Organization
EGFR/ERRB1/HER1	Epidermal growth factor receptor
ERBB2	human epidermal growth factor receptor 2
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
TKI	tyrosine kinase inhibitor
RTKs	receptor tyrosine kinases
PI3K	phosphatidylinositol 3-kinase

Akt	protein kinase B
MAPK	mitogen-activated protein kinase
Cbl	Casitas B-lineage
NSCLC	non small cell lung carcinoma
IDH1	isocitrate dehydrogenase 1
IDH2	isocitrate dehydrogenase 2
PTEN	phosphatase and tensin homolog
LOH	loss of heterozygosity
PDGF	platelet-derived growth factor
PDGFR-α/β	platelet-derived growth factor receptor alpha/beta
ERK	extracellular-signal-regulated kinases
MMPs	matrix metalloproteinases
mAb	monoclonal antibody
BCNU	carmustine
CCNU	lomustine
TMZ	temozolomide
VEGF	vascular endothelial growth factor
RNA	ribonucleic acid
BBB	blood-brain barrier
GERT	cetuximab, radiotherapy and temozolomide
CTD	carboxyl-terminus domain
DCs	dendritic cells
mRNA	messenger ribonucleic acid
APC	antigen-presenting complex
CTLs	cytotoxic T-lymphocytes
ATP	adenosine triphosphate
RNAi	ribonucleic acid interference
siRNAs	small interfering ribonucleic acids
DexAMs	cyclodextrin-modified dendritic polyamine complexes
DNA	deoxyribonucleic acid
Bcl-xL	B-cell lymphoma-extra large
IGFR	insulin growth factor receptor
MET	hepatocyte growth factor receptor
uPAR	urokinase plasminogen activator receptor
mTOR	mammalian target of rapamycin
IL-6	interleukin-6

STAT3	Signal transducer and activator of transcription 3
ID3	inhibitor of differentiation 3
CSCs	cancer stem cells
NSCs	neural stem/progenitor cells
GSCs	glioma stem-like cells
BMX	bone marrow X-linked nonreceptor tyrosine kinase
TGFβ	transforming growth factor beta
CD	cluster of differentiation
FOXP3	forkhead box P3
T_{Regs}	regulatory T cells
JAK	janus kinase

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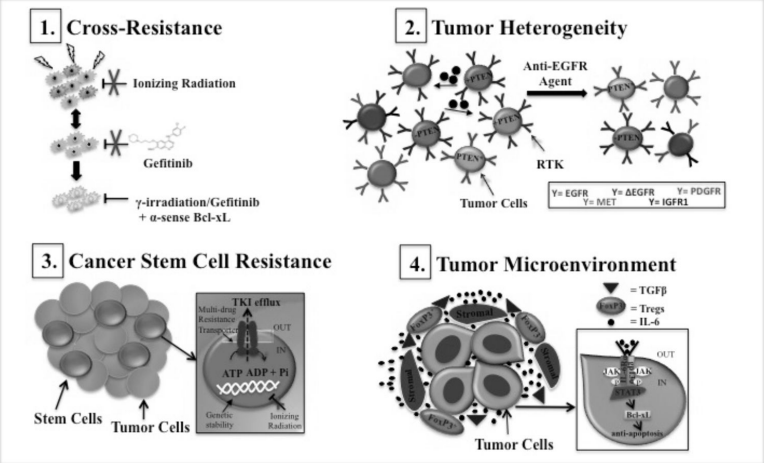

Figure 1.

Malignant glioma and therapeutic resistance. GBMs are adept at evading inhibition of EGFR receptor function through several possible mechanisms. (**1)** Brain tumor cells that are intractable to DNA damage-induced apoptosis may also tolerate apoptotic cues driven by TKI-mediated inhibition of EGFR. Combinatorial therapy using inhibitors of anti-apoptotic activity may overcome this cross-resistance. **(2)** Intratumoral diversity within GBM tumors may drive resistance to single based anti-EGFR agents due to: RTK co-activation, PTEN deletion/mutations, and tumor cell-tumor cell interactions via secreted molecules. PTEN* denotes mutation **(3)** Efflux of EGFR TKIs and increased genetic stability may lead to the maintenance of CSC populations and tumor relapse. **(4)** Enhanced immunosuppression mediated by circulating growth factors, cytokines and suppressor T cells can antagonize the systemic immune responses generated by anti-EGFR immunotherapies. Additionally, circulating IL-6 in the tumor microenvironment can facilitate resistance intracellularly via activation of the JAK/STAT3/Bcl-xL pathway.

Table 1

EGFR-Targeted Therapies in Clinical Trials for the Treatment of Malignant Glioma

Therapy	Substance	Brand Name	Target(s)	Company/Institution	Phase in Clinical Trials
Monoclonal Antibodies	Cetuximab	*Erbitux*	EGFR	Merck KGaA	Phase I/ II (as GERT)
	Panitumumab	*Vectibix*	EGFR	Amgen	Phase II (w/ Irinotecan)
	Nimotuzumab	*Theraloc*	EGFR	YM BioSciences Inc.	Phase III (w/ TMZ + radiotherapy)
	^{125}I-MAb 425		EGFR	Fox Chase Cancer Center	Phase II
	mAb 806	*ABT-806*	ΔEGFR (weakly EGFR)	Abbott	Phase I
Vaccines	PEP-3-KLH	*CDX-110*	ΔEGFR	Celldex Therapeutics	Phase II (w/ TMZ)
Tyrosine Kinase Inhibitors	Geftinib	*Iressa*	EGFR	AstraZeneca Pharmaceuticals	Phase II
	Erlotinib	*Tarceva*	EGFR/ AEGFR	Genentech Inc.	Phase II
	Lapatinib	*Tykerb*	EGFR/HER2	GlaxoSmithKline	Phase I/II

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