EGFR as a Target for Glioblastoma Treatment: An Unfulfilled Promise

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Abstract The receptor for epidermal growth factor (EGFR) is a prime target for cancer therapy across a broad variety of tumor types. As it is a tyrosine kinase, small molecule tyrosine kinase inhibitors (TKIs) targeting signal transduction, as well as monoclonal antibodies against the EGFR, have been investigated as anti-tumor agents. However, despite the long-known enigmatic EGFR gene amplification and protein overexpression in glioblastoma, the most aggressive intrinsic human brain tumor, the potential of EGFR as a target for this tumor type has been unfulfilled. This review analyses the attempts to use TKIs and monoclonal antibodies against glioblastoma, with special consideration given to immunological approaches, the use of EGFR as a docking molecule for conjugates with toxins, T-cells, oncolytic viruses, exosomes and nanoparticles. Drug delivery issues associated with therapies for intracerebral diseases, with specific emphasis on convection enhanced delivery, are also discussed.

Key Points

- Targeting the EGFR signal transduction pathway faces the issue of redundant alternative signaling pathway activation and rapid adaptation.
- EGFR expression is highly variable within a glioblastoma.
- Intracompartmental cell surface targeting with large effector molecules or viral agents holds most promise to overcome the therapeutic deadlock.

1 Introduction

Targeting the receptor for epidermal growth factor (EGFR) has been rewarding in cancer and many pharmaceuticals are approved alone or in combination with chemotherapy for colorectal cancer, non-small-cell lung cancer, and pancreatic cancer, among others, but not for gliomas [1]. The approved agents are mostly tyrosine kinase inhibitors (TKIs) interfering with the receptor signaling, or monoclonal antibodies targeting the receptor at the cell surface to interfere with ligand binding (Fig. 1). It remains unresolved why EGFR targeting has not been successful for glioma as it should be ideally suitable in the context of this disease. EGFR was the first molecule to be linked to oncogenesis in glioblastoma [2]. During the time of the first oncogene descriptions, its gene became linked to a viral oncogene—v-erb B. Massive amplification of that gene was found in glioblastoma [3] and somatic copy number alterations are present in 43% of patients [4]. In addition, subsequently numerous mutations including constitutively active truncations and an in-frame deletion leading to constitutive activation of the intracellular tyrosine kinase were described as well as the numerous diverse intracellular signaling consequences [5]. Overall, about 60% of glioblastoma patients have some kind of genomic alteration affecting this pathway [4]. Of particular interest became the vIII mutation, which results in a molecule with an altered amino-acid sequence, giving rise to a unique site of antigenicity [6]. In many correlative analyses of EGFR status in clinical trials for glioblastoma, it was reported to be prognostically relevant [7], although a larger meta-analysis failed to confirm that overall [8]. In all large genome-wide cancer studies it turned out to be a key molecule for glioma [9] as well as for other tumor entities.
Therefore, because of being a ‘signature molecule’ for glioblastoma, EGFR was thought to be an ideal target for therapy [10, 11].

There are many possible explanations besides drug delivery issues for the still disappointing exploration of EGFR as a target for brain tumors, including a multitude of adaptive mechanisms [12], alternate pathways adaptation, and loss of relevance in later disease stages. These come to bear mostly with agents interfering with receptor signaling in the attempt to interrupt the activation of proliferative or migratory programs. These are mostly small molecules, so-called TKIs or monoclonal antibodies.

Alternatively, the EGFR has also been exploited as a target to deliver therapeutics to the tumor which are intrinsically toxic and thus independent from the activated signaling pathway, or trigger other processes like immune activation. Such constructs can be targeted toxins with an EGFR-binding ligand linked to a toxic molecule, which then relies on receptor internalization for specific delivery, or chimeric antigen receptor T cells which also recognize the EGFR as a docking molecule. The paradigmatic approaches are briefly summarized in Table 1.

Whatever the nature of the EGFR targeting agent, all face the problem of delivery, so aside from the adaptive mechanisms mentioned, delivery problems across the blood–brain barrier is another often-cited explanation for failure of EGFR targeting for glioblastoma.

That leads to the issue of local intraparenchymal or, ideally, compartmental delivery.

2 Compartmental Selectivity of EGFR Targeting

Direct compartmental targeting of EGFR in the brain in the context of intrinsic brain tumors is attractive from a drug safety standpoint because of its high degree of selectivity for the disease in the brain. In normal brain cells, be they neurons, normal glial cells, or microglia, EGFR is of little
relevance. It was found on endothelial cells of the brain in elderly patients [13] and is thought to play a role earlier in development with a predominantly neural expression, mostly in the cerebellum. The molecule could also be detected in the adult hippocampus [14], so there may also be expression on neuro-glial stem cells. Most of the neurobiological context of the EGF/EGFR system has been worked out in rodents [15] and that should inform many of the reservations with strategies against EGFR in the brain in humans, which are almost exclusively pursued in the context of intrinsic brain tumors. In the rodent brain, for example, presence of a functioning EGFR is thought to be necessary for the interaction of neural stem cells with their environment to maintain their self-renewal capabilities [16]. There is little direct evidence in humans for this [17], but in a rare investigation of human tissues over a wide age range, it appeared as if EGF and EGFR are relevant for a microglial sustained persistence or proliferation of sub-ventricular zone neuroglial stem cells [18]. Circumstantial support may come from the limited experience using EGFR TKIs in addition to radiation for brain metastases, which resulted in increased survival but was associated with increased neurocognitive decline [19], possibly an effect on stem-cell-based regenerative capacities. In contrast, a more localized approach with EGFR inhibition using HER-2/EGFR TKIs concurrent to stereotactic radiosurgery for brain metastasis significantly decreased 12-month incidence of local failure [20] without such sequelae.

Apart from vascular endothelial cells and neuroglial stem cells, EGFR is also expressed on meningeal cells and consequently on meningiomas [21]. The expression of EGFR has nevertheless not been found to be related to the clinical course [22] and no therapy has emerged from that finding [23].

Taken together, the (EGF-TGFα/tumor growth factor-alpha)/EGFR system appears to have a rather limited role in the adult central nervous system (CNS) so that a high degree of selectivity of the EGFR expression to glial neoplasia within the brain exists. Therefore, compartmental drug delivery arises as a valid, major strategy to increase efficacy of EGFR-based therapies.

### 3 EGFR Targeting Agents

#### 3.1 Small Molecule Kinase Inhibitors

Among the many agents developed to target the EGFR, the so-called small molecule TKIs interfere with the signal transduction cascade of its tyrosine kinase activity [24]. Despite the availability of several compounds that are approved for a broad spectrum of diseases, none is approved for glioblastoma, which is a result of numerous negative clinical trials. For the leading representatives of this group, erlotinib, gefitinib, afatinib, and lapatinib, trials have not shown efficacy either alone or in combination. Erlotinib showed no efficacy and unacceptable side effects.

| Approach | Reagents | Paradigmatic clinical trial | References |
|----------|----------|----------------------------|------------|
| Targeting signal transduction | Tyrosine kinase inhibition | Gefitinib, erlotinib (EGFR TKI) | Phase II | [135] for review |
| | Specific | Lapatinib (EGFR and HER-2 TKI) | Preclinical | [136] |
| | Broad | Osimertinib (TKI directed against resistance-associated EGFR mutation T790M) | Preclinical | [136] |
| Receptor blockade | Monoclonal antibodies | Cetuximab | Phase II | [50] |
| | | Nimotuzumab | Phase III | [61] |
| | Receptor targeted toxin | TP-38 | Pre-Phase I, 20 patients | [41] |
| | EGFRvIII-specific antibodies | D2C7-(scdsFv)-PE38KDEL | Phase I/II | [76] |
| | Vaccination | MR1(Fv)-PE-38, single-chain antibody | Preclinical | [121] |
| | | PEPVIII-III-KLH (CDX-110) | Phase III | [107] |
| | Boron neutron capture | Boronated anti-EGFR monoclonals or boronated EGF | Preclinical | [137] |
| | anti-EGFRvIII CAR T cells | Humanized anti-EGFRvIII CAR T | Phase I | [88] |
| | Radio-immunotherapy | (125)I-mAb 425 | Phase II | [56] |

CAR chimeric antigen receptor, EGFR epidermal growth factor receptor, TKI tyrosine kinase inhibitor, vIII variant III
as a single agent in newly diagnosed glioblastoma [25] and later studies combining it for recurrent glioblastoma with mechanistic target of rapamycin (mTOR) blockers or bevacizumab were unsuccessful [26–28]. Gefitinib did not result in improved overall survival in a phase II trial in recurrent glioblastoma [29] or in a phase I/II trial in combination with radiation in newly diagnosed glioblastoma [30]. Afatinib as a single agent has shown only limited efficacy in one trial in patients with recurrent glioblastoma [31]. Lapatinib has shown very limited efficacy as a single agent in an early clinical trial in combination with pazopanib, an oral anti-angiogenic TKI [32], which as a single agent also showed no efficacy in recurrent glioblastoma [33].

One of the issues is brain penetration, which has been a specific aim for the chemical design of new reagents for which there is only preclinical promise [34]. Brain penetration is difficult to measure reliably and only recently complex mass spectrometric methods have become available that, at least in animal models, promise to give precise estimates of substance distribution and metabolism. At least for erlotinib it could be shown that it distributes inside an intracranial U87 xenograft [35]. Earlier correlative pharmacokinetic studies of two clinical trials with erlotinib and gefitinib, from which tissue was available from recurrence/progression surgery, showed that erlotinib only accumulated 5–7% of the plasma concentration in the tumor. Gefitinib, in contrast, was sequestered in the tissue and reached 2- to 3-fold plasma concentrations so that, at least for gefitinib, lack of concentration could not have been the cause of insufficient efficacy [36].

Another issue is the lack of specificity, as the TKIs often act on several tyrosine kinases, albeit with differential efficacy. The adaptive capacity of glioblastoma cells, which activate a multiplicity of redundant pathways, is rapid and therefore easily overcomes inhibition of just one of them [37, 38]. This causes toxicity in the absence of efficacy as the normal cells lack that redundancy of pathway activation.

### 3.2 Monoclonal Antibodies

The drug development of monoclonal antibodies recognizing EGFR for the treatment of glioblastoma is more complex as they can serve not only as agents interfering with ligand binding, thus inactivating signaling, but can be used in the context of targeting drugs via conjugates and internalization or work by immunological mechanisms. Under normal circumstances, a cognate ligand binds to EGFR and that association leads to recruitment of that complex to clathrin-coated pits with subsequent internalization and downregulation of the receptor from the cell surface [39, 40]. In that context, antibodies to EGFR show a broad spectrum of effects. They can be either blocking antibodies which prevent ligand binding and can be receptor activating or non-activating, and induce internalization or not. Antibodies which are blocking and internalized are ideal for downregulating the receptor. They are also suitable to ferry conjugates like toxins into the cells. Cell surface binding antibodies which are not internalized will either disrupt ligand-mediated signaling or lead to activation of immunologic mechanisms like antibody-dependent cell-mediated cytotoxicity (ADCC).

Several antibodies developed against EGFR have been used against glioblastoma. Cetuximab was first approved for colorectal cancer and is an antibody-blocking ligand binding without receptor activation [41]. In a preclinical study with glioblastoma xenografts, the intraparenchymal delivery of cetuximab together with systemic application of an anti-angiogenic antibody against the receptor for vascular endothelial growth factor (VEGF) resulted in a marked reduction of tumor cell migration/invasion seen after VEGF inhibition [42]. The relevance of EGFR for glioma cell migration has long been known [43] and accordingly, the highest expression of EGFR was demonstrated on the infiltrative edge of tumors [44, 45]. Whether this indicates a relationship between the migratory program of neural stem cells remains to be proven [46]. The direct connection between EGFR and migration of glioma cells offers a target opportunity. It was seen that CSN6, part of a regulator of the ubiquitin-proteasome-dependent degradation of cancer-related proteins such as p53, c-myc, and c-Jun, when overexpressed reduces the degradation of EGFR and thus enhances the migratory and invasive properties of glioblastoma [47].

In clinical trials, cetuximab failed to demonstrate efficacy either as a single agent for recurrent glioblastoma [48] or in combination with other reagents [49] or radiation [50] and therefore was clinically not developed beyond phase II. Nonetheless, the agent holds promise to be developed further and circumventing the delivery issue of large molecules by direct intracranial infusion, cetuximab effectively blocked glioblastoma cell invasion in an orthotopic xenograft model [42]. Another intracranial strategy being developed is also in preclinical stages where an adenoviral delivery of the corresponding antibody gene by direct intracerebral injection was used so that the antibody would be produced by the transduced cells to increase the local concentration of cetuximab [51]. A recent clinical trial established the safety of selective osmotic blood–brain barrier opening with intra-arterial mannitol infusion followed by intra-arterial infusion of cetuximab [52]. Other cetuximab-based developments include the boronation of the EGFR with antibodies [53] or later the construction of conjugates with cytotoxic platinum derivatives [54]. The boronated cetuximab compounds that were used in
Experimental boron neutron capture radiation therapy were found to be largely ineffective and the cetuximab-based bio-conjugates used with platinum compounds were also very limited in their efficacy. Instead, smaller peptide–platinum compounds were developed. An observation was made that anti-EGFR antibody treatment augments the effect of radiation [55], but most likely this has to be assumed to be an effect on the antibody-exposed vasculature and thus rather an indirect, tumor cell EGFR-independent effect. Direct use of radiolabeled monoclonal antibodies against EGFR have also been disappointing [56].

Another antibody with different properties, nimotuzumab, was developed into phase III. Nimotuzumab is also a ligand binding blocking antibody against EGFR without intrinsic stimulating activity [57]. It has lower affinity than cetuximab and thus binds more specifically to EGFR overexpressing cells. It has shown promising efficacy in multicenter studies [58] and phase II trials for high-grade glioma [59]. In a multicenter, prospective, open label, two-armed, randomized, phase III trial to test the efficacy of additional intravenous nimotuzumab to standard radiochemotherapy [60] (radiotherapy/temozolomide [RT/TMZ]) for newly diagnosed glioblastoma, it showed a signal of efficacy in patients with residual tumor which were O-6-methylguanine-DNA methyltransferase (MGMT) non-methylated. Unfortunately, the inclusion criteria were very broad and thus the study was not powered to prove such subgroup efficacy [61]. Nimotuzumab is currently used in clinical trials in pediatric brain-stem gliomas [62, 63]. In a clinical trial in newly diagnosed diffuse intrinsic pontine glioma (DIPG) from the University Milano, the combination of radiation plus nimotuzumab plus vinorelbine resulted in a median survival of 15 months [64].

In addition to the effects on receptor activity, purely immunological mechanisms are also conceivable when treating with antibodies by means of ADCC, being a well defined process [65]. The contribution of purely immunologic effector mechanisms to antibody-based treatment of glioblastoma is, however, unresolved and will remain so in the context of human clinical trials for some time as trials do not assess basic humoral or cellular immunologic parameters. The contribution to treatment of glioblastoma in most trial designs is most likely negligible. Even when discussing the mechanism of efficacy of other widely used monoclonal antibodies of extracranial cancer like herceptin, the role for ADCC is somewhat speculative [66] and strategies aiming at enhancing this effect are not clinically used yet [67]. Estimating immunological components like ADCC in gliomas would be pure guesswork in the presence of the well described immunosuppressive environment [68] and there is only one in vitro study report on ADCC in the context of cetuximab [69]. Even if on-treatment biopsies of patients were available to look at lymphocytic content and subtyping, they would probably be wide open to interpretation [70, 71].

### 3.2.1 Monoclonal Antibody Delivery Issues

As for the issue with antibody treatment of intracranial tumors, the blood–brain barrier as an obstacle to the passage of those large molecules into the brain/tumor parenchyma needs to be considered. However, there is mounting experience with monoclonal antibodies for intracerebral tumors [72, 73]. With bevacizumab, a humanized monoclonal antibody against VEGF to treat malignant glioma, dramatic imaging effects can be achieved [74]. These may be due to neutralizing the intravascular circulating VEGF and thus depriving the angiogenic tumor endothelium of it. The VEGF produced by tumor cells however, being in the interstitial compartment and acting on abluminal receptors, would still be active unless there was some penetration into the tumor, which therefore is assumed to take place. Bevacizumab has also been shown to be a very active agent against radiation necrosis in the brain [75]. The disappointing pivotal trials with bevacizumab are, however, reason to seek improved delivery across the blood–brain barrier by using focused ultrasound for transient barrier opening [76].

Monoclonal antibodies are also considered in the treatment of primary CNS lymphoma [77]. Rituximab, an antibody used for treatment of lymphoma, has been studied in this condition and it could be found in the cerebrospinal fluid after intravenous administration, although only at 0.1% of the systemic concentration, but that nevertheless proves some permeability [78].

### 3.3 Targeted Toxins

Toxins or conjugates based on antibody-based delivery are large molecules that will not sufficiently cross the blood–brain barrier and have too high a risk for unwanted side effects when administered systemically. Therefore, the need to devise new delivery methods has been recognized and direct intraparenchymal delivery methods are being developed [79] (for review). The paradigmatic method is direct slow catheter-mediated infusion with slow flow rates, which creates a positive pressure wave and can achieve high concentrations of large molecules on the other side of the blood–brain barrier. This so-called ‘convection-enhanced delivery’ (CED) [80] can potentially saturate large tissue volumes. Only one phase III trial using this method has been completed in glioblastoma. In this trial the IL-13 receptor was targeted with a compound in which IL-13 was fused to a truncated pseudomonas exotoxin.
(cintredikin besudotox) and given by convection over 5 days [81]. Unfortunately this treatment was not superior to its comparator, locally implanted carmustine wafers [82].

CED has also been used for a variety of toxin conjugates based on the EGFR. Targeting the EGFR with toxins is based on the concept of internalization after binding to the receptor (see above). TP-38 is a paradigmatic molecule of that class consisting of TGFrα conjugated with a truncated pseudomonas exotoxin similar to the cintredikin besudotox. This has been shown to be safe and effective in a phase I clinical trial where it was also intraparenchymally delivered by convection, but unfortunately it is currently not further developed [41]. More such molecules are, however, under development and await clinical testing [83–86].

3.4 EGFR as Effector Agent “Address”

3.4.1 CAR-T cells

Immunotherapy has become a major area of promise in the therapy of cancer. A relatively new technology is the engineering of T cells which recognize their target independently from MHC-mediated antigen presentation by expressing a chimeric antigen receptor, so-called Chimeric antigen receptor (CAR)-T cells [87]. This technology has been successfully used in many cancer paradigms, especially leukemia, but the lack of specificity of most antigens, which are also found on normal cells, leads to side effects. Glioblastomas express the already mentioned EGFRvIII, which has in itself a unique site of antigenicity and is a truly specific cancer antigen. It has thus been possible to engineer a CAR that recognizes the vIII receptor mutation via a humanized single-chain antibody variable fragment (scFv), which is fused with the key constituents of the intra-cytoplasmic signaling domains of the T-cell receptor [88]. When expressed in genetically engineered T cells, this leads to specific CAR-T cells against vIII-expressing glioblastoma cells. This is currently under investigation in early clinical trials [89]. Similar reagents have been under preclinical evaluation before [90, 91], and it is expected that there will be a multitude of reagents developed [92]. It is particularly attractive that there is apparent efficacy also against glioma-initiating or glioma stem cells [90].

3.4.2 Nanoparticles

Nanoparticles are vesicular carriers that enable drug release at defined targets while protecting their content from degradation or elimination during transport, and have a long history of pharmacological development also in the context of glioblastoma [93]. Nanocarriers such as liposomes, polymeric nanoparticles, or lipid nanocapsules take advantage of the increasing size of fenestrations in the capillary endothelium of malignant gliomas leading to a partial loss of barrier integrity of the blood–brain tumor barrier [94, 95]. The resulting enhanced permeability and retention (EPR) effect [96, 97] enables large molecules and nanoparticles to become trapped in the interstitium of tumors when barriers become leaky. In addition to this non-specific effect, construction of nanocarriers including targeting antibodies or specific receptor ligands increases the likelihood of selective accumulation in tumors, albeit only in the coherent tumor and not in the area of infiltration. Consequently, the EGFR is a molecule ideal for such tumor targeting in the context of glioblastoma and numerous agents have been constructed.

Extensive preclinical studies have shown promising results, like the use of cetuximab conjugated liposomes, so-called immunoliposomes [98], but there is only limited experience with early clinical trials. Using minicells (400-nm nanoparticles derived from Salmonella typhimurium) that were loaded with doxorubicin and conjugated with panitumumab, EGFR-targeted delivery was evaluated in 14 patients with recurrent glioblastoma [99].

3.4.3 Oncolytic Viruses

Oncolytic viruses have great potential in cancer therapy as they can be designed for many specific characteristics of the respective tumor entities. There are, however, major safety concerns so between design and clinical evaluation, lengthy preclinical studies are mandated. As a result, decades may pass between the description of a promising viral agent and initial clinical trials [100]. Herpes simplex virus (HSV-1)-based constructs are easily manipulated and have already been shown to be safe in phase III clinical trials in melanoma (G207), and received regulatory approval. G47, a third-generation oncolytic HSV-1, is in phase II for glioblastoma [101]. To increase efficacy and safety of oncolytic HSV-1, an EGFR-targeted oncolytic HSV has been progressively developed, by engineering a fully replication-competent virus to specifically infect cells expressing the receptor, which led to highly efficient eradication in a preclinical orthotopic glioma model [102]. However, with persisting safety concerns, this was followed by further virus modification so that by introducing an miR-124 response element into a crucial viral replication gene, the virus is now capable of only reproducing in glioblastoma cells where miR-124 is absent in contrast to the high miR-124 expression in normal glial and neuronal cells of the brain [103]. This agent has promise for clinical trials which are anticipated in the near future.
4 EGFR as an Immunologic Target: Vaccination

EGFRvIII results from a partial gene deletion and this is seen within tumors in a high proportion of patients, albeit usually not in all cells of a tumor [104]. This highlights that intratumoral heterogeneity is a key obstacle to many EGFR-directed therapies. Specific antibodies to the intramolecular unique site of antigenicity have been developed in various immunotherapeutic paradigms [105] as already mentioned. A purely immunological approach to target the EGFRvIII has been attempted by vaccination approaches using the unique antigenic epitope arising within the mutant protein sequence [106]. A synthetic peptide containing that unique amino-acid sequence of the EGFRvIII (rindopenvimut) was simply coupled to keyhole limpet hemocyanine and injected intradermally, resulting in an immune response like in a typical vaccination [107]. This therapy has seen very promising phase I and II clinical trials [108], and a specific humoral and cellular immune response was measured in patients. Upon recurrence, 60–80% of the reoperated patients for recurrent tumors showed complete eradication of vIII-positive cells [109]. Unfortunately, however, the pivotal phase III trial for newly diagnosed glioblastoma failed to show overall efficacy [110], with the results still being evaluated for subgroup efficacy.

5 Regulation of EGFR Gene Expression as a Target

In glioblastoma, the complex and interwoven receptor tyrosine kinase (RTK) signaling pathways can be regulated by microRNAs, members of the small non-coding RNA family, by controlling post-transcriptional gene expression by accelerating or blocking the mRNA decay. For example, decreased levels of miR-218 induce the expression of downstream RTK effectors such as phospholipase C-γ1 (PLCγ1) and S6 kinase (S6K1) that counteract the negative feedback loop and therefore increase the activation of both EGFR and platelet-derived growth factor receptor alpha (PDGFRα) [111]. The recent discovery that microRNAs can be found encapsulated in extracellular vesicles (EVs) and subsequently taken up by neighboring cells led to new ways to investigate how cells communicate and modulate gene expression through epigenetic rearrangements [112, 113]. Recent work demonstrated that EVs can carry and transfer EGFR [114] and that cell communication via EVs promotes intratumoral heterogeneity in glioblastoma [115]. In a positive way, miR-1 can interact with Annexin A2 (ANAXA2), one of the major EV proteins, to reduce glioblastoma tumorigenicity [116].

In cancer, with activation of the EGFR pathway like in non-small-cell lung cancer (NSCLC), most of the mutation are concentrated on the kinase domain, while in glioblastoma, mutations are mostly found in the extracellular (EC) domain leading to a poor response to EGFR inhibitors such as erlotinib [117]. In addition, recent work showed that methylation of the EC domain by protein arginine methyltransferase 1 (PRMT1) enhanced EGF binding and therefore dimerization. This led to increased activation of the receptor, counteracting the effect of cetuximab in a mouse model of colon cancer [118]. The expression of the mutant EGFRvIII is also under tight control by epigenetic mechanisms as the inhibition of histone deacetylation resulted in reduction of EGFRvIII expression, which is thought to explain the relatively low and sparse EGFRvIII expression in tumor regions with high EGFR amplification and rearrangement [119, 120]. Epigenetic mechanisms that control EGFR expression were found to be similar in the germinal matrix and gliomas, and it is probable that dysregulation of a locus-specific role for chromatin remodeling in EGFR expression in normal human neural development is involved in gliomagenesis. Strategies targeting these mechanisms have not entered any preclinical development yet [121].

6 General Aspects of EGFR Targeting and Intratumoral Heterogeneity

Many different kinds of EGFR alterations exist simultaneously to a various extent in any glioblastoma (Fig. 1). The complexity of genomic alteration in EGFR and therefore its targeting was recently highlighted by The Cancer Genome Atlas (TCGA). Based on the RNA sequencing analysis of 164 glioblastoma, Brennan and colleagues confirmed that somatic alterations in EGFR regroup into more than EGFR locus amplification associated with exon 2–7 deletion (EGFRvIII), but also exon 12–13 and 14–15 deletion in the extracellular domain as well as C-terminal deletion in exon 25–27 [122]. Additionally, point mutation and protein fusion can also be detected [9] [123]. Most of the minor EGFR variants can be found only in a restricted subpopulation (allelic fraction <10%) and a new single-cell analysis approach based on DNA [124] and RNA [125] sequencing demonstrated that when five alterations in EGFR are found in one tumor resection, up to 32 different subpopulation can be identified, creating a wide range of different targets and potential resistance (Fig. 1) [125]. Moreover, targeting RTK in glioblastoma has been proven difficult due to the co-amplification of multiple RTK (mostly EGFR, PDGFRα, and MET), resulting in intratumoral heterogeneity with many subpopulations with different RTK expression profiles.
This is the most likely cause for the disappointment with individual RTK targeting in clinical trials for glioblastoma. As specific subpopulations or clones can be responsible for tumor growth and propagation such as EGFRvIII by enhancing neighboring cell proliferation through IL6 and LIF secretion [128] and activation of the STAT3 pathway [129, 130], treatment with combinatorial RTK inhibitors and STAT signaling pathway might more efficiently target such glioblastoma-driving subpopulations. One aspect to consider in glioblastoma is that compared with other cancer types harboring EGFR amplification, no clonal resistance was observed after EGFR inhibitor treatment. In NSCLC, for example, patients acquire T790M point mutation in exon 20 after erlotinib or gefitinib treatment and therefore develop resistance. No such second-site mutations were discovered in glioblastoma, despite patients developing resistance to EGFR inhibitors. One hypothesis is that the presence of the EGFRvIII mutant increases activation of the PI3K-AKT-mTOR pathway, and when associated with alteration in the tumor suppressor PTEN leads to an increased inhibition of EGFR to achieve the same level of inhibition and overcome resistance [131]. More recently, single-cell analysis of the dynamic evolution of EGFR amplification showed that resistance to EGFR inhibitors in glioblastoma was mostly due to the elimination of the extrachromosomal DNA containing the EGFR amplification (double minutes). Through a highly adaptive process, glioblastoma tumor cells become resistant to lapatinib by losing their EGFR amplified double minutes, which could be shown to reappear after end of treatment [132].

Despite all these issues, which over the years have shown how difficult EGFR is as a potential target for treating glioblastoma, many reagents are in development after taking these experiences into consideration. Mostly, EGFR is used as an address rather than a single molecule to be knocked out of the signaling pathways (Table 2). Understanding the consequences of EGFR targeting in glioblastoma and the adaptive mechanisms, combinatorial approaches are mandatory [37] and many will include an immunotherapeutic strategy like immune checkpoint inhibition or metabolic approaches [133, 134].

7 Conclusion

Although overexpression of EGFR and mutations of EGFR are one of the most characteristic features of glioblastoma, the cell-biological complexity of this target has outgrown therapeutic developments for decades. While the signaling properties of EGFR and the signaling cascades lose attraction for glioblastoma therapy, EGFR becomes

| Table 2 Ongoing trials targeting the EGFR in glioblastoma |
|----------------------------------------------------------|
| Drugs | Glioblastoma | Phase | Characteristics |
|-------|--------------|-------|-----------------|
| D2C7-IT Recurrent | Phase I | Single-chain fragment variable monoclonal antibody fragment immunotoxin with high binding affinity for both EGFRwt- and EGFRvIII-expressing glioblastoma cells |
| EGFR(V)-EDV-Dox Recurrent | Phase I | Nanotechnology delivery system plus panitumumab (monoclonal antibody against EGFR) |
| ABT-414 Newly diagnosed with EGFR amp | Phase II | Monoclonal antibody-drug conjugate (ADC) against EGFR |
| ABT-414 + TMZ Recurrent pediatric | Phase II | Monoclonal antibody-drug conjugate (ADC) against EGFR |
| anti-EGFRvIII CAR T cells Recurrent | Phase I | Autologous anti-EGFRvIII CAR-T cells with cyclophosphamide and fludarabine as lymphodepleting chemotherapy |
| Cetuximab + mannitol + SOC Newly diagnosed | Phase I/II | Monoclonal antibody against EGFR + brain–blood barrier disruption |
| ABBV-221 Glioblastoma | Phase I | Antibody-drug conjugate (ADC) targeting EGFR |
| Tesevatinib Recurrent | Phase II | Small molecule, ErbB2 receptor antagonist |
| Rindopepimut Recurrent | Expended access | Peptide vaccine that targets EGFRvIII |
| Lapatinib + SOC Recurrent | Phase II | Small molecule, EGFR and ErbB2 inhibitor |
| Sym004 Recurrent | Phase II | Mixture of two synergistic full-length anti-EGFR antibodies, which bind to two separate non-overlapping epitopes on EGFR |
| Abemaciclib, CC-115 or neratinib post-SOC Newly diagnosed | Phase II | CDK4 and 6 inhibitor; DNA-PK/TOR inhibitor; EGFR inhibitor |

CAR chimeric antigen receptor, EGFR epidermal growth factor receptor, EGFRwt epidermal growth factor receptor wild-type, TMZ temozolomide, vIII variant III, SOC standard of care

[126, 127].
attractive as a docking molecule, which by internalization after ligand binding to the extracellular domain will carry effector agents like monoclonal antibody conjugated toxins or oncolytic viruses into the cells. As EGFR has almost no role in the adult brain, the drug delivery issues which have hampered effective treatment by lack of blood–brain barrier permeability for large effector agents will be addressed by the development and refinement of intraparenchymal delivery methods. Solving the delivery issues of currently developed highly effective and compartmentally non-toxic molecules will eventually close the gap between effective extracranial EGFR targeting and the so-far disappointing attempts at targeting EGFR in glioblastoma.

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