Epidermal growth factor receptor variant III (EGFRvIII) is the most prevalent EGFR mutant found in glioblastoma (GBM). Due to a truncation of large portion of the extracellular region, EGFRvIII is unable to bind any ligand and constitutively signals to downstream effector molecules. It is tumor-specific, highly oncogenic and usually co-expressed with EGFR wild type (EGFRwt). EGFR tyrosine kinase inhibitors (TKIs) have proven ineffective in GBM and different mechanisms account for the occurrence of resistance to such inhibitors. Among these, EGFR TKIs induce a switch to platelet-derived growth factor receptor β (PDGFRβ) expression and signaling, thus rendering the tumors addicted to such receptor for continued growth and resistance to treatment. In our recent investigation, we showed the ability of a nuclease-resistant RNA aptamer, named CL4, to bind and inhibit EGFRvIII thus hampering proliferation, migration and invasion of EGFRvIII-positive GBM cells. Importantly, both CL4 and EGFR TKIs cooperate with a previously validated anti-PDGFRβ aptamer in inhibiting cell growth. Here, we highlight the potential of the EGFRvIII aptamer to hamper the EGFRvIII functional interplay with other receptor tyrosine kinases (RTKs) and cell surface proteins responsible for GBM development and progression. The utility of CL4 as targeting ligand for drug-delivery approaches is also discussed. Overall, aptamer-based molecules have significant implications for managing GBM in the near future.

Keywords: aptamer; glioblastoma; EGFRvIII; PDGFRβ; drug resistance; EGFR TKIs; combined treatment

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Gliomas are the most common primary central nervous system tumors that arise from neoplastic transformation of glial cells, non-neuronal cells that provide support and protection for neurons in the brain. Depending on the cellular origin, they are classified by the World Health Organization as astrocytoma, oligodendroglioma, mixed oligoastrocytoma, and ependymoma. Additional stratification of tumor grade, including low-grade (I or II) or high-grade (III or IV), is determined by their growth potential and aggressiveness reflecting their malignant behavior, response to treatment and survival \[1,2\]. Therefore, the most malignant and the most frequent subtype is the grade IV astrocytoma or glioblastoma multiforme (GBM), accounting for nearly 50% of all gliomas and approximately 20% of all brain tumors \[3\], with an incidence of 2-3 GBM cases per 100,000 people/year in Europe and North America \[4\]. The vast majority of GBMs appears de novo, without recognizable precursor lesions (primary GBMs), while a minority progresses from
pre-existing lower grade gliomas (secondary GBMs).

Despite available therapeutic strategies, consisting mainly of maximal surgical resection, radiotherapy plus concomitant and adjuvant temozolomide, patients with newly diagnosed GBM generally die in less than a year, with 5-year survival rate of less than 5% [5]. Thus, GBMs are almost always fatal and novel methods to more efficiently treat these tumors are required, taking into account their high heterogeneity and molecular complexity.

Over the last years, innovative therapies are being explored in order to target major signaling pathways that are altered in GBM cells. Among them, amplification of the epidermal growth factor receptor (EGFR) gene, which results in over-expression of the receptor, has been described in approximately 50% of all GBMs and represents one of the most frequent genetic alterations associated with these tumors. In addition, half of EGFR-amplified tumors expresses the oncogenic mutant EGFR variant III (EGFRvIII, EGFR type III, δe-2-7 or ΔEGFR), which contains an in-frame deletion of 801 bp of coding sequence from exons 2 to 7 of the wild type EGFR (EGFRwt) gene. This mutation results in a loss of 267 amino acids from the extracellular region of EGFR, which is involved in ligand binding. Thus, EGFRvIII is unable to bind any known EGFR ligands and constitutively signals to downstream effector molecules [6, 7].

The expression of EGFRvIII is heterogeneous in GBMs and is usually detected only in a minority of tumor cells within a tumor mass. It has been shown that the cells expressing EGFRvIII alone, in the absence of the wild type receptor, secrete elevated levels of IL-6 family cytokines which promote the proliferation of adjacent cells expressing high levels of EGFRwt, thus maintaining the tumor cell heterogeneity [8]. Nevertheless, EGFRvIII is rarely found in the absence of amplified EGFRwt and the co-expression of both receptors within a tumor increases tumorigenicity in vivo and confers a worse prognosis than EGFRwt expression alone [9-11].

Apart from the paracrine interactions between cells expressing EGFRwt or EGFRvIII [8], different mechanisms of cooperation between the wild type and the mutant receptor have been reported that promote malignant progression. These include heterodimerization among the receptors associated with their phosphorylation [12, 13] and the phosphorylation of EGFRvIII by EGFRwt, in the absence of physical binding of the receptors, with the consequent nuclear translocation of EGFRvIII and signal transducer and activator of transcription 3 (STAT3) activation [14]. Further, a feed-forward loop has been demonstrated by which EGFRvIII is activated by co-expressed EGFRwt which is activated in turn by heparin-binding epidermal growth factor induced by EGFRvIII [13].

Despite the above findings suggest combinatorial targeting of both EGFR species, however, the results have so far been unsatisfactory in clinic given the high resistance of EGFRvIII to monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) [15, 16]. Indeed, the mAbs that are currently used to treat other tumors such as renal cell carcinoma, melanoma, and hematologic cancers [17, 18], resulted unsuccessful for GBM [19]. Further, while clinical results demonstrate the efficacy of EGFR TKIs in non-small cell lung carcinoma (NSCLC) patients [20], response rates in GBM patients are disappointing for such inhibitors [15]. It has been shown that the deletion in the extracellular region of EGFRvIII induces the receptor to adopt a conformation characterised by a lower kinase site occupancy compared to wild-type EGFR and the EGFR kinase mutants that are common in NSCLC. Thus, the first-generation EGFR TKIs, erlotinib and gefitinib, rapidly binds and releases the glioma associated mutant receptor thus limiting the inhibition of downstream signaling pathways [21, 22]. In contrast, the second-generation inhibitors, such as lapatinib, even if results more potent at binding and inhibiting EGFRvIII, has demonstrated intratumoral levels that result inadequate to induce inhibition of GBM tumor growth [22]. Apart from these pharmacological factors that dictate the low response of EGFRvIII to small molecule TKIs, multiple mechanisms of resistance to EGFR inhibition have been described and include: i) the loss of phosphatase and tensin homolog, which keeps the active signaling through the phosphatidylinositol 3 kinase pathway [23], ii) the co-activation of multiple receptor tyrosine kinases (RTKs) in the same tumor [24-26] and iii) the dependence on other non-amplified, non-mutated RTKs by a receptor "switching" mechanism [27]. The first clinical and biologic evidence for this last mechanism has been provided by Akhavan D et al. [27], that reported the existence of a transcriptional repressive mechanism by which EGFRvIII represses platelet-derived growth factor receptor β (PDGFRβ) expression. EGFR TKIs cause the de-repression of PDGFRβ allowing the tumors to become "addicted" to its expression and signaling for continued growth and resistance to targeted treatment.

All these studies provide plausible explanation of the failure of EGFR TKIs as monotherapy and suggest the need to develop more efficacious anti-EGFR/EGFRvIII therapies.

Highly selective compounds emerging for anti-cancer therapy are oligonucleotide aptamers used as ligands and inhibitors for disease-associated proteins. Similar to antibodies they interact at high affinity with their protein target, by recognizing a unique three-dimensional structure. Aptamers offer unique chemical and biological characteristics: small size, high stability, ready availability and lack of immunogenicity [28, 29].
By applying a SELEX approach on living cancer cells we have generated a 2′-fluoropyrimidine nuclease-resistant RNA-aptamer, which revealed a high specific ligand for the human EGFRwt. The aptamer, named CL4, binds to the domain IV of the extracellular region of the receptor thus interfering with ligand-dependent EGFR phosphorylation and activation of downstream signaling pathways in NSCLC and glioma [30, 31]. Further, by binding to EGFR, the aptamer prevents PDGF-BB-induced EGFR transactivation in cell lines and primary cell cultures of GBM [32]. We have recently shown that CL4 is able to bind to EGFRvIII mutant, in which the domain IV is still present despite the truncation, thus causing inhibition of receptor constitutive activity. Importantly, when applied to EGFRvIII-expressing GBM cell lines, highly resistant to EGFR TKIs, CL4 significantly inhibits cell viability and proliferation. We demonstrated that targeting EGFRvIII by CL4 causes upregulation of PDGFRβ (Figure 1), thus in agreement with the de-repression of PDGFRβ expression caused by EGFR TKIs [27]. Importantly, CL4 and gefitinib cooperate with a RNA aptamer, that we previously validated as ligand and inhibitor of human PDGFRβ [32], in inhibiting EGFRvIII-positive GBM cells growth.

It is noteworthy that EGFRvIII is tumorigenic, in part, through its transactivation of other RTKs, including AXL, PDGFR, vascular endothelial growth factor receptor 3 and fibroblast growth factor receptor [24-26]. Further, many new studies are focusing on elucidating the cross-talk among EGFRvIII and the hepatocyte growth factor (HGF) receptor (MET), aimed to provide innovative strategies for treating EGFRvIII-expressing gliomas [25, 26, 33, 34]. Indeed MET is known to be active in glioma and to be activated by EGFRvIII by the formation of an EGFRvIII-MET heterodimer at the cell surface [25, 34] or independently from the physical association of the receptors. In this last mechanism of activation, the EGFRvIII-dependent c-jun-N-terminal kinase isoform 2 activation induces the secretion of HGF ligand leading to activation of MET, both in a paracrine and autocrine manner [35]. Preventing EGFRvIII-dependent transactivation could be a viable strategy for glioma therapy. Our findings showing the ability of CL4 aptamer to inhibit EGFRvIII and the value of aptamer-based inhibition of EGFR and PDGFRβ, strongly encourage us to further test whether the aptamer is effective in inhibiting the EGFRvIII-dependent activation of MET or eventually other RTKs with a crucial role in GBMs.

It has been recently demonstrated a functional interplay among EGFRvIII mutant and GBM microenvironment that promote tumor cell invasion. Indeed, it is now clear that tumor cells do not act alone but in close interaction with the extracellular matrix and with stromal cells in the tumor microenvironment (TME). The communication between tumor cells and TME regulates invasion and angiogenesis of different tumors including glioma. Great effort is indeed devoted to develop therapeutic agents interfering either with the recruitment of stromal cells into the TME, with tumor
cell-stromal interaction, or with specific pathways activated by the TME. It has been demonstrated that in the environment of hypoxia plus extracellular matrix vitronectin, integrin β3 interacts with EGFRvIII and activates a SRC/FAK/EGFRvIII signaling axis to promote GBM cell invasion [56]. In this scenario, the CL4 aptamer, by directly binding to the EGFRvIII receptor, could hamper EGFRvIII/integrin β3 complexes thus increasing the effect of integrin αvβ3 antagonist to inhibit GBM progression and metastasis.

Apart from the utility of aptamers as direct inhibitors of their target, aptamers have been also developed as carriers for cell-targeted delivery of therapeutic reagents or imaging agents in novel therapeutic/diagnostic applications [37]. Importantly, given the excellent targeting properties of the anti-EGFR CL4 aptamer, it has been recently used to specifically deliver nanoparticles (NPs) containing therapeutic anti-miRNA to tumors from human EGFR-positive breast cancer cells orthotopically implanted in nude mice [38]. Aptamer-based drug delivery is emerging as a promising approach to efficiently deliver chemotherapeutics through the blood brain barrier (BBB), which represents one main obstacle to the success of antineoplastic drugs [39, 40]. The high affinity and specificity of the anti-EGFR/EGFRvIII and anti-PDGFRβ aptamers prompt us to explore their ability to function as targeting agents for drug-loaded NPs across the BBB. Preliminary studies suggest that the anti-PDGFRβ aptamer can be bound to polymeric nanoparticles to act as targeting ligand for chemotherapeutics specifically to GBM cell lines (our unpublished observations) and we are currently investigating the aptamer-conjugated nanovectors in vivo, by using an orthotopic implanted glioma model.

Our findings suggest that the aptamer-based strategy to target EGFR/EGFRvIII and PDGFRβ might represent a novel therapeutic approach against GBM and suggest further preclinical settings by using the aptamers alone or in combination with conventional chemotherapeutics. Further, the results inspire attempts to explore the potential of the CL4 aptamer to hamper the interplay among EGFRvIII and other cell surface proteins responsible for GBM development and progression.

Conflicting interests
The authors have declared that no conflict of interests exist.

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Abbreviations

BBB: blood brain barrier; EGFRvIII: epidermal growth factor receptor variant III; EGFRwt: epidermal growth factor receptor wild type; GBM: glioblastoma multiforme; HGF: hepatocyte growth factor; MET, hepatocyte growth factor receptor; mAbs: monoclonal antibodies; NPs: nanoparticles; NSCLC: non-small cell lung carcinoma; PDGFRβ: platelet-derived growth factor receptor β; RTKs: receptor tyrosine kinases; STAT3: signal transducer and activator of transcription 3; TKIs: tyrosine kinase inhibitors; TME: tumor microenvironment.

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