Efficacy of Osimertinib Plus Bevacizumab In Glioblastoma Patients With Simultaneous EGFR Amplification And EGFRvIII Mutation

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Abstract

Background: Amplification of *EGFR* and its active mutant *EGFRvIII* are common in glioblastoma (GB). While *EGFR* and *EGFRvIII* play critical roles in pathogenesis, targeted therapy with EGFR-tyrosine kinase inhibitors (TKIs) or antibodies has shown limited efficacy. To improve the likelihood of effectiveness, we targeted adult patients with recurrent GB enriched for simultaneous *EGFR* amplification and *EGFRvIII* mutation, with osimertinib/bevacizumab at doses described for non-small cell lung cancer (NSCLC).

Methods: We retrospectively explored whether previously described *EGFRvIII* mutation in association with *EGFR* gene amplification could predict response to osimertinib/bevacizumab combination in a subset of 15 patients treated at recurrence. The resistance pattern in a subgroup of subjects is described using a commercial NGS panel in liquid biopsy.

Results: There were ten males (66.7%), and the median patient's age was 56 years (range 38-70 years). After their initial diagnosis, 12 patients underwent partial (26.7%) or total resection (53.3%). Subsequently, all cases received IMRT and concurrent and adjuvant temozolomide (TMZ; the median number of cycles 9, range 6-12). The median follow-up after recurrence was 17.1 months (95% CI 12.3-22.6). All patients received osimertinib/bevacizumab as a second-line intervention with a median progression-free survival (PFS) of 5.1 months (95% CI 2.8-7.3) and overall survival (OS) of 9.0 months (95% CI 3.9-14.0). The PFS6 was 46.7%, and the overall response rate (ORR) was 13.3%. After exposure to the osimertinib/bevacizumab combination, the main secondary alterations were *MET* amplification, *STAT3*, *IGF1R*, *PTEN*, and *PDGFR*.

Conclusions: While the osimertinib/bevacizumab combination was marginally effective in most GB patients with simultaneous *EGFR* amplification plus *EGFRvIII* mutation, a subgroup experienced a long-lasting meaningful benefit. The findings of this brief cohort justify the continuation of the research in a clinical trial. The pattern of resistance after exposure to osimertinib/bevacizumab includes known mechanisms in the regulation of *EGFR*, findings that contribute to the understanding and targeting in a stepwise rational this pathway.

Introduction

Glioblastoma (GB) or grade IV glioma according to the 2016 WHO classification of brain malignancies, is the most common primary malignant tumor of the central nervous system (CNS), with an estimated incidence of 10/100.000 population, occurring frequently in people aged between 55–60 years (1, 2). The prognosis of GB remains poor despite first-line therapy, and the median overall survival (OS) is 12–15 months (2), while the 5-year survival does not exceed 10% of patients(3). After first line therapy, GB almost always recurs with poorer prognosis (median PFS of 1.5–6 months and median OS of 2–9 months) (4–6), especially due to intra-tumor heterogeneity, on cellular and molecular levels (7). Once GB progresses after first-line therapy, treatment options are limited, and recurrent GB (rGB) medical care remains a challenge. Loco-regional therapy (surgery and reirradiation) may be evaluated in selected cases while traditional systemic therapy has shown limited efficacy. In recent years, with greater knowledge of the underlying molecular features, a multitude of new drugs and combination regimens have been tested for efficacy in rGB patients (8–10).

Currently, astrocytic gliomas with a wild-type IDH and histone H3 status, necrosis and/or microvascular proliferation are classified as IDH-wild-type, WHO grade 4 glioblastomas (1, 11). In the absence of necrosis or

microvascular proliferation, such tumors should be evaluated for glioblastoma-associated EGFR gene amplification, TERT promoter mutations and/or the +7/-10 signature (11, 12). If one of these alterations is detected, they are classified as IDH-wild-type glioblastomas given their association with a poor prognosis, even in the absence of necrosis and microvascular proliferation (1, 13). MGMT promoter methylation has limited diagnostic value for rGB but can guide therapy, whether to choose chemotherapy with alkylating or other agents in IDH-wild-type gliomas (2). Next-generation sequencing (NGS) based gene panels could enable the assessment of all or most genetic and chromosomal aberrations relevant for diagnosis using a single assay (14, 15). Furthermore, array-based DNA methylation profiling and RNAseq has emerged as a powerful novel diagnostic method that is independent of histology and useful in the routine diagnostic and therapeutic work-up (16, 17).

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase of the ERBB family. Upon binding to ligands which include epidermal growth factor (EGF), Transforming growth factor alpha (TGF- α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), epigen or epiregulin, EGFR forms homodimers or heterodimers with other ERBB family members (18). Dimerization of EGFR leads to transphosphorylation (autophosphorylation) of its C-terminal tail, which serves as the docking site for SRC homology 2 (SH2) domain-containing signaling proteins including growth factor receptor-bound protein 2 (GRB2), phosphoinositide 3-kinase (PI3K), SRC homology 2 domain-containing transforming protein 1 (SHC1) and signal transducer and activator of transcription (STAT) proteins (18–20). These signaling proteins regulate downstream physiological and pathological processes. The EGFR pathway represents a particularly attractive therapeutic target in GB because is dysregulated in most human malignant gliomas through overexpression, amplification, and activating mutations.

Amplification of the *EGFR* gene occurs in $\approx$ 55% of IDH-wild-type GB patients and is associated with high levels of EGFR protein (21, 22). In contrast, a separate group of *EGFR* deletions and point mutations is found frequently in GB. *EGFR* deletions in GB include *EGFRvI* (N-terminal deletion), *vII* (deletion of exons 14–15), *vIII* (deletion of exons 2–7), *vIV* (deletion of exons 25–27), *vV* (deletion of exons 25–28), among which *vII* and *vIII* are oncogenic (23). In addition, point mutations in the extracellular region of *EGFR* (R108K, A289V/D/T, G598D among others) keep the receptor in an active state and are identified in 24% of GB cases (22, 23). Among *EGFR* mutants found in GB, the most common is *EGFRvIII*, occurring after amplification of *EGFR* and is felt to represent a late event (24, 25).

Several classes of EGFR inhibitors have been developed, including small-molecule tyrosine kinase inhibitors (TKIs), antibodies, immunotoxin conjugates, and antisense oligonucleotides. Because intracranial distribution of many agents is limited, the use of small-molecule TKIs offers a theoretical advantage (26). However, several early trials in unselected patients with GB have reported limited efficacy with EGFR-selective TKIs. Reasons why this lack of efficacy include limited brain penetration of drugs (first-generation TKIs do not cross the BBB -Blood-Brain Barrier - effectively), complex tumor heterogeneity (there are at least four characterized major molecular forms of EGFR), redundant signaling pathways, and limited potency in first and second-generation drugs because they aim targets that proved to be different in GB than other type of EGFR-amplified tumors (27–29). Osimertinib, a third-generation EGFR-TKI that effectively penetrates the BBB, was recently found to be > 10 times more efficient than the first-generation EGFR inhibitors in inhibiting GB proliferation, inducing cell cycle arrest which significantly inhibited colony formation, migration, and invasion through downregulation of EGFR/ERK signaling (30). Furthermore, osimertinib inhibits the constitutive activity of *EGFRvIII* with high potency (< 100

nM) in a tyrosine kinase-dependent manner (31). In addition, it has been recently found that cells expressing mutant *EGFRvIII* are prone to starvation and hypoxia-induced cell death (32). This research supports the idea that using anti-VEGF agents that produce a low-oxygen environment could promote *EGFRvIII*-mutant hypoxia-induced cell death. Albeit some initial equivocal results, there is sufficient biological evidence to consider that patients with EGFR-amplified and *EGFRvIII*-mutant GB could benefit from combined therapy with osimertinib and bevacizumab.

Methods

We conducted a retrospective review of rGB patients with simultaneous *EGFR* amplification and *EGFRvIII* mutation, that were treated with a fixed dose of osimertinib (80 mg/d) and bevacizumab (15 mg/kg, every three weeks) from March 2016 through September 2020 at Clínica del Country (Bogotá, Colombia). The endpoints were progression-free survival (PFS) at six months (PFS6), overall response rate (ORR), overall survival (OS) and safety. Two pairs of locally certified neuroradiologists evaluated responses using RANO criteria (33). Concerns about the response to radiation therapy or changes caused by it were settled via consensus among the observers. During treatment, all patients were treated according to best international practices.

Baseline demographic and clinical data for each patient were collected by individual chart review from the institution's electronic medical record system. Charts were scrutinized for treatment dose adjustments, discontinuations, and toxicities as defined by the Common Terminology Criteria for Adverse Events (CTCAE v5) (https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcae_v5_quick_reference_5x7.pdf). In general, both drugs were administered until disease progression or intolerance occurred. When significant toxic effects were discovered, osimertinib treatment was halted and resumption was set at 40 mg per day. When there was significant toxicity, bevacizumab was discontinued but, whenever possible, resumed at the same dose. If patients experienced severe adverse effects (gastrointestinal perforation [any grade], thromboembolism, pulmonary hemorrhage [grade 2] or other hemorrhages, allergic reaction, or cardiac toxic effects [grade 3]), bevacizumab treatment was discontinued. Bevacizumab was also halted if patients did not recover from an adverse effect that required suspension within 42 days (i.e., serum creatinine > 1.5 mg/dL, proteinuria greater than 2+, or hypertension [grade 4]).

This study was conducted in compliance with the Declaration of Helsinki (34) and with the International Conference on Harmonization Good Clinical Practice Guidelines. The protocol was approved by an institutional review board (ONCOLGroup Platform - Registration No. 2018/21904, Cayre, Clínica del Country, Bogotá, Colombia). All included patients provided signed informed consent and accepted the tumor tissue analysis. An Institutional Review Board and Privacy Board waiver was obtained to facilitate retrospective collection of clinical, pathologic and molecular data.

Genomic profiling

In all cases, the genomic profiling was done using validated methods. Studies were conducted using an NGS-based assay (FoundationOne®) performed in a laboratory accredited by CLIA and The College of American Pathologists (Foundation Medicine, Cambridge, MA, <http://www.foundationmedicine.com>). An expert pathologist (PA) reviewed hematoxylin and eosin (H&E) stained slides to confirm the GB diagnosis and ensure adequate formalin-fixed, paraffin-embedded (FFPE) specimen quality. Samples were considered to have

acceptable quality if: sample volume > 1 mm³, nucleated cellularity > 80% or > 30,000 cells, and > 20% tumor nuclei. When required, microdissection was performed to enrich samples with < 20% tumor nuclei. In brief, 50 ng of DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks of tumor samples. Hybridization capture of 3,320 exons from up to 315 cancer-related genes and select introns of 28 genes commonly rearranged in cancer were applied to the extracted DNA. The samples were sequenced to high, uniform coverage, with a mean sequencing depth of 5643, as previously described (35). GAs, including base substitutions, small insertions or deletions (indels), rearrangements, and copy number alterations, were determined and reported for these samples. *MGMT* (O-6-Methylguanine-DNA Methyltransferase) promoter methylation status was obtained from diagnostic tumor specimens by methylation-specific PCR bisulfite-treated DNA as previously described (36).

The commutations found at diagnosis were recorded in all patients. In 8 cases, an F-One liquid test was performed at the time of progression to osimertinib/bevacizumab; 5 of them (62%) had positive results with novel alterations described in the results.

Statistical design and analysis

Patient characteristics were summarized using median, interquartile range, and minimum/maximum values for continuous variables and frequency (%) for categorical variables. PFS was defined as the time interval between the date of first osimertinib/bevacizumab dose and the date of disease progression or death from any cause, whichever occurred first. Patients who were alive without disease progression at the time of the last follow-up were censored. OS was defined as the time interval between the first Osimertinib plus bevacizumab dose and the date of death due to any cause. Patients that were alive were censored at the last follow-up. The PFS, PFS6, and OS with 95% CI were estimated using Kaplan-Meier curves. A Cox regression model was used to estimate the adjusted HR stratified by response to prior chemoradiotherapy. The difference in the ORR was assessed using the χ^2 test. Statistical analyses were conducted with SPSS 26.0 version (Statistical Package for the Social Sciences, IBM, Armonk, NY, US).

Results

From March 2016 through September 2020, 61 patients with GB who harbored EGFR gene amplification were diagnosed in two reference centers for the treatment of brain tumors in Bogotá, Colombia. Like previous reports (37), *EGFRvIII* was detected in 40% (N = 24) of this EGFR-amplified GB cohort. *EGFR* amplification and mutation status were determined from the initial diagnostic tumor specimen in 47 (77%) patients and from recurrent, immediate pretreatment tumor tissue in 14 (23%) cases (Fig. 1). All tumors were *IDH1/2* wild type, consistent with previously described *EGFR* amplification and *IDH* mutation inverse correlation (38, 39). Seven patients (46.7%) had *MGMT* promoter methylation and the mean number of commutations found in the tumor tissue at the time of diagnosis was 3 (SD +/- 1) (**Supplementary Table 1**). Table 1 includes the baseline patient demographics and disease characteristics.

Table 1. Baseline patient demographics and disease characteristics.

Variable	N = 15 (%)
Age	
≥65 years	11 (73.3)
≤64 years	4 (26.7)
Sex	
Male	10 (66.7)
Female	5 (33.3)
Karnofsky index	
<80%	1 (13.3)
>80%	13 (86.7)
RPA scale	
5	14 (93.3)
6	1 (6.7)
Compromise of eloquent areas	
Yes	5 (33.3)
No	11 (66.7)
Corpus callosum infiltration	
Yes	2 (13.3)
No	13 (86.7)
Tumor location	
Frontal lobe	4 (26.7)
Temporal lobe	8 (53.3)
Parietal lobe	2 (13.3)
>2 lobes	1 (6.7)
Baseline neurological status	
Altered	4 (26.7)
Normal	11 (73.3)
Tumor diameter	
<3 cm	6 (40.0)
>3 cm	8 (53.3)
ND	1 (6.7)
Type of surgery	

Biopsy/subtotal	7 (46.7)
Total	8 (53.3)
Pseudoprogression	
Yes	4 (26.7)
No	11 (73.3)

There were 10 males (66.7%), and the median patient's age was 56 years (range 38–70 years). Following their initial diagnosis, 4 patients underwent partial (N = 4/26.7%) and 8 total resections (N = 8/53.3%). In three cases (20%) it was only possible to perform a biopsy due to the involvement of some eloquent area. Subsequently, all cases received IMRT, with 14 (93%) completing 60 Gy and concurrent and adjuvant temozolomide (TMZ; median number of cycles 9, range 6–12). In addition, four patients had completed 12 cycles of TMZ. The overall response rate (ORR) to first-line therapy was 40% (N = 6), with 40% achieving stable disease (N = 6), and three patients progressing (20%). Four patients (26.7%) presented pseudoprogression; in two cases, it was symptomatic and required steroid support and subsequent tapering. This finding was only seen in the subset of patients who had a partial response. The PFS for the first-line treatment was 9.7 months (95% CI 6.5–12.8) (**Supplementary Fig. 1**), and the median OS from diagnosis was 17.0 months (95% CI 12.8–21.1) (**Supplementary Fig. 2**). Following progression, six patients underwent resection of recurrent tumor, with five achieving optimal surgery (83%). In addition, three of them were treated with re-radiation using fractionated stereotaxic radiosurgery. OS by *MGMT* favored the methylated population [*MGMT* methylated 26.9 months (95% CI 25.1–28.6) vs. Unmethylated *MGMT* 14.1 months (95% CI 12.1–16.0; P = 0.001)], finding that was reproduced for the PFS achieved with chemoradiation followed by temozolomide [*MGMT* methylated 15.1 months (95% CI 13.2–16.9) vs. Unmethylated *MGMT* 7.6 months (95% CI 6.9–8.3; P = 0.001)].

Outcomes for osimertinib/bevacizumab combination

Fifteen patients who had simultaneous EGFR amplification and *EGFRvIII* variant received osimertinib plus bevacizumab as a second-line intervention. At the time of the study report, 80% (N = 12) of the patients had died, and the rest were in follow-up receiving active treatment. The median follow-up was 17.1 months (95% CI 12.3–22.6) and all had a Karnofsky index greater than 70% at the time of starting treatment. Only four patients had a neurological deficit considered mild to moderate. Seven (46.7%) of 15 patients achieved PFS6, the median PFS was 5.1 months (95% CI 2.8–7.3) (Fig. 2A) and the median OS from osimertinib/bevacizumab was 9.0 months (95% CI 3.9–14.0) (Fig. 2B). The median number of bevacizumab cycles was 7.0 (range, 3 to 22), one patient (6.7%) achieved a complete response, another had a partial response (6.7%), and transient stable disease was observed in 10 cases. The ORR was 13.3% and the clinical benefit was 80%. Figure 3 shows a representative case that achieved a good response and PFS after osimertinib/bevacizumab. In a subset of eight patients, a secondary molecular study was performed by NGS in liquid biopsy; five had positive results with various alterations found after disease progression to the osimertinib/bevacizumab combination. Only two cases preserved the *EGFR* amplification and one the *EGFRvIII* alteration. *MET* amplification was documented in two cases, and in one that lost both baseline *EGFR* and CDKN2A/B alterations, a secondary *EGFR* A289V (Mutant Allele Frequency Percentage - MAF%; MAF 6.1%) point mutation plus an alteration in *STAT3* T433I (MAF 1.7%) were found. Other genes related to osimertinib/bevacizumab resistance were IGF1R (MAF 0.56%), PTEN, and

PDGFR (MAF 1.6%). Figure 4 illustrates genomic findings in five selected patients comparing initial tissue and blood analysis after progression. Two of the patients with higher PFS (15.5 and 9.1 months) after the start of osimertinib/bevacizumab developed MET amplification, opening the possibility of considering the use of specific MET inhibitors as rescue therapy. MGMT promoter methylation also positively affected PFS for recurrent disease treated with osimertinib/bevacizumab [MGMT methylated 8.3 months (95% CI 4.7–11.8) vs. Unmethylated MGMT 4.1 months (95% CI 1.6–6.5; $P = 0.02$)].

Eleven patients (73.3%) received a third-line treatment, 9 were exposed to BCNU/ bevacizumab and 2 to nivolumab/bevacizumab. Seven patients achieved stable disease with a PFS of 4.1 months (95% CI 3.6–4.5). The best results with the third line were patients that achieved PFS6 with osimertinib/bevacizumab.

Safety and tolerability

Most treatment-related adverse events (AEs) were controllable with standard care, and the overall toxicity profile was consistent with previous reports in multiple solid tumors (Table 2). Common grade ≥ 2 AEs considered at least possibly related to osimertinib and bevacizumab combination included hypertension (40%), rash (33.3%), fatigue (33.3%), diarrhea (26.8%), paronychia (20.1%), and mild proteinuria (20.1%). Grade ≥ 3 related AEs included diarrhea (13.3%), fatigue (13.3%), hypertension (6.7%) and paronychia (6.7%). There was no severe bleeding or embolism, and one patient experienced transient interstitial grade 1 lung disease. Four patients (26.4%) experienced temporary dose interruption with osimertinib in one of the cases for 16 days due to grade 2–3 diarrhea. After correcting the event, he restarted with 40 mg with occasional stools. No patient required temporary or permanent suspension of bevacizumab.

Table 2
Toxicity profile of osimertinib/bevacizumab combination as a second line therapy in rGB.

Toxicity	N = 15 (%)		
Toxicity grade	All	Grade 1–2	Grade 3
Hypertension	6 (40)	5 (33.3)	1 (6.7)
Rash	5 (33.3)	5 (33.3)	-
Fatigue	5 (33.3)	3 (20.0)	2 (13.3)
Diarrhea	4 (26.8)	2 (13.3)	2 (13.3)
Proteinuria	3 (20.1)	3 (20.1)	-
Thrombocytopenia	1 (6.7)	1 (6.7)	-
Decreased albumin	2 (13.3)	2 (13.3)	-
Neutrophil decreased	1 (6.7)	1 (6.7)	-
Paronychia	3 (20.1)	2 (13.3)	1 (6.7)
Increased aspartate aminotransferase	2 (13.3)	2 (13.3)	-
WBC decreased	2 (13.3)	2 (13.3)	-
Creatinine increased	1 (6.7)	1 (6.7)	-
Increased alkaline phosphatase	1 (6.7)	1 (6.7)	-
Interstitial lung disease	1 (6.7)	1 (6.7)	-

Discussion

This study is a brief series of patients with EGFR-amplified and *EGFRvIII*-mutant GB that received combined therapy with osimertinib / bevacizumab for recurrent GB. This targeted intervention achieved a PFS6 of 46.7%, a higher outcome than lomustine or bevacizumab monotherapy, where the PFS6 ranged between 13% and 16% (40). Despite the sampling limitation, the PFS6 obtained with osimertinib/bevacizumab in this selected population was similar to that achieved with the lomustine/bevacizumab regimen in the BELOB trial (40). In addition, OS with osimertinib/bevacizumab was equal to that achieved in the control arm (9.0 months) of study EF-14, superior to regorafenib (7.4 months) (41), and slightly lower than that obtained with the tumor-treating fields (TTFields; 11.8 months) (42).

Many correlative analyses of EGFR status in clinical trials for glioblastoma found it to be prognostically significant, though a larger meta-analysis failed to confirm this. (43). In all large genome-wide cancer studies, it became a key molecule for glioma, which has favored the exploration of multiple molecules even without much success. Osimertinib is a 3rd generation irreversible EGFR tyrosine kinase inhibitor, targeting the ATP-binding pocket of the kinase domain by formation of a covalent bond with Cys 797. Additionally, osimertinib has been shown to have efficacy in brain metastases, conveying the ability to cross the blood brain barrier in clinically relevant concentrations superior to that of other inhibitors (in naïve patients with advanced EGFR-mutated Non-

small cell lung cancer and CNS metastases, the pooled ORR and DCR of osimertinib were 71% and 93%, respectively, and the combined median PFS was 12.21 months) (44). However, osimertinib has relatively low affinity for the kinase domain of both wild-type EGFR and *EGFRvIII*, as it was specifically designed to target the mutant T790M kinase domain (IC₅₀ 1 nM) and spare the wild-type variant (IC₅₀ 184 nM) (45). So far, no human trials with osimertinib in selected GB patients have been completed to date. There are some reports in the literature of efficacy, including one patient with EGFR A289V and C628F point mutations and EGFR copy number gain that received osimertinib at multifocal recurrence, with near-complete response in one lesion but discordant simultaneous progression at another site. The progressing lesion acquired an *EGFRvIII* mutation and continued to exhibit EGFR copy number gain (46).

Previously, Lassman et al. evaluated EGFR expression/signaling on gefitinib and erlotinib using Western blot (anti-pEGFR Tyr 1068 antibodies) on GB datasets from NABTC 01–03 and 00–01 (47). Erlotinib and gefitinib treatment did not consistently affect EGFR activity pre-/post- erlotinib or gefitinib, whether by pEGFR, pERK, or pAKT. This suggested that erlotinib and gefitinib did not effectively inhibit EGFR phosphorylation or signaling in these tumors. Lack of effect on downstream signal transduction despite apparent appropriate inhibition appears to be mediated through alternative escape pathways, such as MET, IGF1R (insulin growth factor receptor 1), and PI3K (47).

In our study, the post-progression analysis of the liquid biopsy (performance of post-progression liquid biopsy 62%) found alterations in IGF1R, PTEN, AKT3, PDGFR, STAT3, and MET amplification, the latter associated with the best response after exposure to osimertinib/bevacizumab. In the same way, Mellinghoff *et al.* treated 49 recurrent glioblastoma patients with erlotinib (at doses from 150 to 500 mg) and gefitinib (from 150 to 1000 mg) and found that PTEN loss was associated with resistance to EGFR tyrosine kinase inhibitors (48). There is also substantial preclinical evidence that activation of the PI3K, MET, or the IGF1R pathways confers resistance in a similar manner to what occurs with first line osimertinib in patients with NSCLC and actionable mutations in EGFR (49–52).

There is increasing experimental and clinical evidence that activation of EGFR and downstream signaling pathways render specific subgroups of GBs vulnerable to hypoxia-inducing therapies. Hypoxia-induced cell death in GB models is preceded by ATP depletion, increase in glycolysis and higher levels of mitochondrial superoxides which have been associated with decreased metabolic flux and decreased NADPH/NADP⁺ ratio (53). The difficulty in effectively tackling tumor cells lies on the ample heterogeneity amongst various GB samples and models. There are different point mutations in the extracellular region of EGFR whose exact biological implications still elude us. A plethora of cell signaling pathways mediated by EGFR can be divided in those originating from plasma membrane: RAS/mitogen activated protein kinase/extracellular signal-regulated kinase (RAS/MAPK/ERK) pathway, PI3K/protein kinase B (PKB/AKT) pathway, Janus kinase/STAT (JAK/STAT) pathway and protein kinase C (PKC) pathway (4). The second group includes non-plasma membrane signaling pathways: nuclear (DNA-PK, PCNA, histone H4 and STAT proteins) and mitochondrial (COXII) (4).

Interestingly, interaction of JAK2 with EGFR within the JAK/STAT pathway has shown to confer resistance to EGFR inhibitors and when *EGFRvIII* is expressed, STAT3 forms a complex with *EGFRvIII* that drives malignant transformation and initiates mesenchymal transformation in high-grade gliomas (54–56). Active EGFR recruits and activates phospholipase C (PLC) which in turn activates PKC. PKCs downstream effectors include p53 and p21, cell growth regulators (RAS-RAF1 and others that may be tumor suppressants or oncogenes depending on

context). In GB, PKC isoenzymes show pro-tumorigenic functions. High levels of isoform PLC-gamma are correlated with survival and inhibition of PKC proved decreased viability of GB cells; hence PKC is a critical signaling target (57–59). EGFR can phosphorylate *EGFRvIII* to activate STAT3/5 in the nucleus and facilitate tumour progression. This could be one of many biological correlates that explains why patients with *EGFRvIII* have shortened survival, strictly dependent on its kinase activity. *EGFRvIII* phosphorylates SFKs which promotes mitochondrial localization of *EGFRvIII* and increases cell survival under low glucose conditions (60). *EGFRvIII* also activates c-SRC that promotes angiogenesis via VEGF secretion and growth of cells expressing wild-type EGFR via paracrine interleukin 6 (IL-6). These preclinical findings favor the use of osimertinib / bevacizumab, and in our case the presence of acquired mutations in STAT3 and AKT confirm the escape through these pathways (60). Figure 5 illustrates the *EGFRvIII* related pathways and angiogenesis in GB cells (see **Supplementary data to Fig. 5** for a detailed explanation).

Recently, Struve *et al.* reported that *EGFRvIII* expression is associated with prolonged survival, but only in patients with MGMT promoter methylation (61). In addition, using isogenic cell lines with high *EGFRvIII* expression they demonstrate that *EGFRvIII* increases TMZ sensitivity through DNA double-strand breaks and a pronounced S/G2-phase arrest. As well, they demonstrated a greater expression of DNA mismatch repair (MMR) proteins in *EGFRvIII* positive cells and patient tumor samples, which was most pronounced for MSH2 and MSH6. *EGFRvIII*-specific knockdown reduced MMR protein expression, thereby increasing TMZ resistance (61). In opposition, Chi *et al.* found that the MGMT promoter methylation was not associated with clinical response to dacomitinib in a cohort of GB patients with EGFR amplification and the *EGFRvIII* mutation (28). In our study, MGMT promoter methylation significantly impacted OS ($P = 0.001$) (**Supplementary Fig. 3**) as well as PFS with initial treatment (chemoradiation therapy followed by temozolomide; $P = 0.001$) (**Supplementary Fig. 4**), and PFS with the osimertinib / bevacizumab combination ($P = 0.02$) (**Supplementary Fig. 5**), favoring the hypothesis that relates the overexpression of the *EGFRvIII* mutation with the response to the alkylating agent and the direct inhibition of EGFR.

Eskilsson *et al.* favored our hypothesis by finding a slight correlation between EGFR and VEGFA expression when considering only GB samples with EGFR amplification ($P = 0.028$), but not in those without EGFR amplification. Interestingly, strong correlation between EGFR and VEGFA expression in GB samples with EGFR amplification was limited to those that additionally harbored *EGFRvIII* ($P = 0.031$) and/or EGFR missense mutations ($P = 0.037$) (62). In addition, Chagoya *et al.* tested the efficacy of osimertinib in vivo and in vitro finding that *EGFRvIII* GBs are heterogeneous in terms of expression of *EGFRvIII*, by the extent of *EGFRvIII*s tyrosine kinase activity, and in the expression of several other key genes (31). To our knowledge, previous clinical trials did not consider the heterogeneity of EGFR positive GBs. For example, the failed *EGFRvIII*-vaccine trial that included all *EGFRvIII* positive GB patients (63) or the recent fall of depatuxizumab in the INTELANCE-1 study (64). Part of the answer may lie in the recent findings of the REGOMA study, where a molecular signature associated with prolonged survival in GB patients treated with regorafenib was found (65). Santangelo *et al.* reported elevated expression levels of HIF1A mRNA and CDKN1A mRNA and reduced expression levels of miR-93-5p, miR-3607-3p, and miR-301a-3p in tumor tissue at first surgery, can identify a subgroup of patients treated with regorafenib with favorable benefit. The selection of patients with EGFR amplification who have the *EGFRvIII* mutation and high levels of VEGF or HIF1 may be the best candidates to consider the use of the osimertinib/bevacizumab combination.

In conclusion, our findings are intriguing and support the idea of better understanding all signaling pathways implicated in *EGFR*-positive GB patients. To the best of our knowledge, this is the first report of a group of patients with *EGFR* amplification and *EGFRvIII* mutations who were treated with osimertinib/bevacizumab. Some had a positive impact on PFS and PFS6, which validates the transition to a confirmatory clinical trial. Even so, more preclinical studies are needed to determine the molecular signatures of *EGFR* GBs that may make these tumors sensitive to the combination.

Declarations

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Ethical approval

This study was reviewed and approved by ONCOLGroup Platform - Registration No. 2018/21904, Cayre, Clínica del Country, Bogotá, Colombia. All included patients provided signed informed consent and accepted the tumor tissue analysis. An Institutional Review Board and Privacy Board waiver was obtained to facilitate retrospective collection of clinical, pathologic and molecular data.

Data availability statement

The datasets presented in this article are not readily available because of the Colombian organic law of data protection that limits access to genetic information in an open format. Requests to access the datasets should be directed to the corresponding author, who will release it upon formal request to the Ministry of Health of Colombia following the requirements of Law 1581 of 2012, paragraph 201811601170851 of 2018.

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Author Contributions

AFC, OA, ARP, EJ, FH, DG, JFR, HC and JAM planned and coordinated the study. DJV, CP, FS, CO, AM, SB, NU, DP, LR, ZLZ, EJ and CS reviewed patient records and composed the database. LR reviewed all histopathology studies. JR, JA, JGR, NS and ARP performed DNA extraction and library preparation and MGMT methylation analysis. AFC, ARP, OA, CR, and RR performed all statistical analysis and data interpretation. AFC, ARP, OA and DJV wrote the initial draft of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

Andrés F. Cardona discloses financial research support from Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Foundation Medicine, Roche Diagnostics, Termo Fisher, Broad Institute, Amgen, Flatiron Health, Teva Pharma, Rochem Biocare, Bayer, INQBox and The Foundation for Clinical and Applied Cancer Research – FICMAC. Additionally, he was linked and received honoraria as an advisor, participate in speakers' bureau. He gave expert testimony to EISAI, Merck Serono, Janssen Pharmaceutical, Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Pfizer, Novartis, Celldex Therapeutics, Foundation Medicine, Eli Lilly, Guardant Health, Illumina, and Foundation for Clinical and Applied Cancer Research – FICMAC.

Oscar Arrieta reports personal fees from Pfizer, grants and individual fees from Astra Zeneca, grants and individual fees from Boehringer-Ingelheim, Lilly, Merck, Bristol Myers Squibb, Roche, outside the submitted work.

Christian Rolfo reports relation with Mylan, Archer Biosciences, Oncopass, Inivata, Merck Serono Novartis, MSD, Boehringer Ingelheim, Guardant Health, etc AstraZeneca as part of Speakers' Bureau. Also, he received research funding from Pfizer and had uncompensated Relationships with OncoDNA, Biomark, and Guardant Health.

Leonardo Rojas received honoraria as an advisor, participate in speakers' bureau from Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Astra Zeneca and Eli Lilly. Additionally, he was linked and received honoraria as researcher.

The other authors have no conflicts to declare.

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Supplemental Data

Supplemental figures & tables are not available with this version.

Figures

Figure 1

Flow of samples and patients who were tested for the presence of EGFR amplification and EGFRvIII mutation.

Figure 2

A. PFS to osimertinib/bevacizumab combination. B. OS to osimertinib/bevacizumab combination

Figure 3

Magnetic resonance imaging (MRI) scans of a representative case (patient #3) who achieved a good response and long PFS after starting treatment with osimertinib/ bevacizumab.

Figure 4

Genomic findings in five selected patients comparing initial tissue and blood analysis after progression to osimertinib/bevacizumab.

Figure 5

Visual representation of the signaling pathways activated by EGFR and EGFRvIII and their interaction with integrin, AKT/mTOR, and Wnt signaling. Activation upregulates different transcription factors involved in tumor cell proliferation, invasion, and angiogenesis, blocked by various EGFR- and EGFRvIII inhibitors. A. The PI3K/Akt pathway is known to control cell proliferation, growth, and apoptosis, but changes in this pathway can also increase cell invasion by inhibiting ECM detachment-induced cell death and stimulating MMP secretion. Expression of EGFRvIII increases the activation of Akt through downregulation of the cell cycle inhibitor p27 and enhances cell proliferation. In vivo, EGFRvIII has a strong association with the phosphorylation of mTOR, and it has been demonstrated that the mutant receptor might be an activator of PI3K in the absence of PTEN loss. B. The Stat family of transcription factors acts as important signaling components and transfers the signal transduction of various cytokines and growth factors like EGF from the extracellular environment to the nucleus. Stat3 is upregulated in many cancer types and promotes immune responses and differentiation and cellular transformation, proliferation, and invasion. Upon activation of cell surface receptors through binding of its ligands, the receptor-associated Janus kinase (JAK) and the Src kinase act as connecting links between the receptors and Stat3. Stat3 is phosphorylated and translocated into the nucleus, where it induces the transcription of multiple genes involved in the regulation of cellular and tissue processes, including those that function in cell motility. The binding of EGF to EGFR leads to an activation of Stat3 and subsequently a Stat3-

induced increased TWIST expression. Even though EGFR levels show a strong correlation with p-Stat3, the correlation of EGFRvIII with activated Stat3 seems to be even stronger. C. EGFRvIII in Integrin-FAK-ERK Signaling. It has been demonstrated that EGFRvIII constitutively activates the MAPK pathway in human glioma cells. Substrates of phosphatase and tensin homolog PTEN, a tumor suppressor in many cancer types, includes FAK and inhibits integrin- and growth factor-mediated MAPK signaling. PTEN phosphatase activity suppresses the invasion of EGFRvIII-expressing glioma cells. EGFRvIII could enhance the phosphorylation levels of FAK in glioma cells while forming a complex, which correlates with the increased catalytic activity of FAK comparable to stimulation by growth factors or integrins.