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Analysis of EGFR Overexpression, *EGFR* Gene Amplification and the EGFRvIII Mutation in Portuguese High-grade Gliomas

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Abstract. *Background: Patients with malignant gliomas do not respond to any current therapy. Epidermal growth factor receptor (EGFR) controls several oncogenic processes, being frequently up-regulated in gliomas due to overexpression, gene amplification and gene mutation. EGFR inhibitors are being tried in gliomas, yet the molecular determinants of therapeutic response are unclear. Materials and Methods: EGFR overexpression, EGFRvIII mutation and EGFR amplification were determined by immunohistochemistry and chromogenic in situ hybridization (CISH) in 27 primary glioblastomas (GBM), 24 anaplastic oligodendrogliomas (AO) and four anaplastic oligoastrocytomas (AOA). Results: EGFR overexpression was associated with EGFR amplification, being found in 48% and 53% GBM, 33% and 40% AO and 75% and 67% AOA, respectively. EGFRvIII was found in 22% GBM, 8% AO and was absent in AOA. No association was observed between EGFR alterations and patient survival. Conclusion: We characterized, for the first time, EGFR molecular alterations in Portuguese patients with malignant glioma and identified a subpopulation of patients presenting putative biomarkers for EGFR-based therapies.*

Gliomas are the most frequent primary central nervous system (CNS) tumors (1). According to the World Health Organization (WHO), gliomas are histologically divided into astrocytic, oligodendroglial and mixed oligoastrocytic tumors, and are classified into four grades of malignancy (1, 2). Oligodendrogliomas and oligoastrocytomas are stratified into grade II and grade III (anaplastic) tumors; on the other hand astrocytomas can be subdivided into grade II, grade III and grade IV (2). The most malignant form (WHO grade IV), glioblastoma (GBM), is also the most frequent glioma subtype (1, 2). GBMs can be divided into primary glioblastomas, which arise *de novo* and are molecularly characterized by epidermal growth factor receptor (*EGFR*) overexpression/amplification, and secondary glioblastomas, which are derived from lower-grade astrocytomas and are characterized by *TP53* mutations (3). The prognosis of patients with GBM is very poor, with survival usually less than twelve months (1, 4). Recently, the introduction of temozolomide-based chemotherapy in concomitancy with radiotherapy, led to increased GBM patient survival (15 months) (4). However, these results are far from being satisfactory and there are still patients that do not respond favorably to any described therapy. Therefore, it is necessary to understand the molecular features of gliomas in order to identify novel and effective therapeutic targets.

Receptor protein tyrosine kinases (RTKs) are major regulators of cell growth signaling and their importance in tumorigenesis and progression has been extensively investigated (5). Epidermal growth factor receptor (EGFR)/HER1 is a member of the class I epidermal growth factor family, which also includes HER2, HER3 and HER4. EGFR is a transmembrane glycoprotein that is stimulated by growth factors, namely the transforming growth factor-α (TGF-α) and EGF ligands, which bind to the extracellular domain of the receptor (6). Ligand binding to EGFR activates the receptor through dimerization leading to signal transduction and activation of downstream intracellular pathways, mainly RAS/RAF/MAPK, PI3K/AKT and STATs, that regulate cellular proliferation, differentiation, migration and survival (5). Several mechanisms of aberrant EGFR activation have been reported in cancer cells, namely

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overexpression of the ligand EGF and of the receptor, *EGFR* gene amplification and activating mutations. Moreover, dysregulated EGFR activation is known to act oncogenically, stimulating the growth and spread of cancer cells (5). Therefore, due to the paramount role of EGFR in tumorigenesis, several therapeutic strategies are being developed to target and inhibit signalling *via* the receptor (7-9). Monoclonal antibodies (mAbs) bind to the extracellular domain of EGFR inhibiting this region and blocking ligand binding. Cetuximab (IMC-C225, Erbitux) and Panitumumab (ABX-EGF, Vectibix) are EGFR-binding mAbs currently approved for the treatment of colorectal cancer (10). Alternatively, EGFR tyrosine kinase inhibitors (TKIs) bind to the intracellular tyrosine kinase domain, blocking kinase activity as well as the downstream signaling. Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva), are EGFR TKIs already approved for non-small cell lung cancer (NSCLC) patients (11).

In high-grade gliomas, *EGFR* is the most frequently amplified oncogene, being present in 40% of primary GBM and poorly characterized in anaplastic astrocytomas and anaplastic oligodendrogliomas (AO) (12-14). Importantly, *EGFR* gene amplification is known to be associated with protein overexpression in gliomas (2). Additionally, in about half of GBM cases with *EGFR* amplification, the event is coupled with *EGFR* gene mutations, with EGFRvIII (also known as ΔEGFR and del2-7EGFR) the most common in GBM (2, 15, 16). This EGFR mutant oncoprotein lacks a portion of the extracellular ligand-binding domain as a result of a genomic deletion involving exons 2 to 7, resulting in a mutant protein unable to bind EGFR ligands, yet constitutively activated in a ligand-independent manner leading to overproliferation of cancer cells (6, 17). EGFR represents an attractive therapeutic target in malignant gliomas and even though there are no anti-EGFR agents approved for glioma treatment at present, there are several ongoing clinical trials evaluating the efficacy of mAbs and TKIs in gliomas (7, 18, 19).

In the present study, we aimed to characterize the most common mechanisms (*EGFR* overexpression, gene amplification and EGFRvIII mutation) involved in EGFR activation in a Portuguese cohort of high-grade (astrocytic, oligodendroglial and mixed) gliomas and to identify the subset of patients that would potentially benefit from EGFR-targeted therapies.

Materials and Methods

Patients and tumor samples. Sixty-two formalin-fixed, paraffin-embedded samples of sporadic gliomas from 55 patients were retrieved from the Department of Pathology of the S. João Hospital, Porto and S. Marcos Hospital, Braga, Portugal, as well as the available patients' clinical data, as described elsewhere (20). Tumor samples were classified according to the WHO classification of CNS tumors (2): as 31 primary GBM (WHO grade IV), 26 AO (WHO grade III), one oligodendroglioma (O) (WHO grade II) and four anaplastic oligoastrocytomas (AOA) (WHO grade III) (Tables I and II). Twenty-seven (49.1%) patients were male and 28 (50.9%) were female; the mean age was 56.1 years (range 27-79 years) (Tables I and II).

EGFR and EGFRvIII immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were used in the immunohistochemical analysis. Previously documented mouse monoclonal anti-EGFR (clone 31G7, 1:100; Zymed® Laboratories Inc., South San Francisco, CA, USA) and mouse monoclonal anti-EGFRvIII antibody (clone G100, 1:100; Zymed®Laboratories Inc.) were used as primary antibodies (18, 21). Anti-EGFRvIII antibody specifically recognizes EGFRvIII and does not cross-react with the wild-type form (18).

Immunohistochemistry for EGFR was performed as described elsewhere (22). Briefly, after deparaffinization, sections were rehydrated and washed. Antigen retrieval was achieved by 20 min incubation at 37ïC with a bacterial protease extracted from *Streptomyces griseus* (Sigma-Aldrich Co., St. Louis, MO, USA). Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 10 min and samples were then incubated with Ultra V block (Lab Vision Corporation, Fremont, CA, USA) for 10 min at room temperature (RT). After incubation with primary antibody, overnight at RT, the secondary biotinylated goat anti-polyvalent antibody (Lab Vision Corporation) was applied for 10 min followed by 10 min incubation with Streptavidine Peroxidase (Lab Vision Corporation) at RT.

For the EGFRvIII reaction, sections were deparaffinized, incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and rehydrated. Antigen retrieval was achieved by microwaving sections in 10 mM citrate buffer (pH 6.0) three times for 5 min at 700 W. Sections were then incubated with Ultra V block (Lab Vision Corporation) for 20 min at RT. After incubation with primary antibody overnight at 4ïC, the biotinylated "universal" secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min followed by R.T.U. Vectastain®Elite ABC reagent (Vector Laboratories) incubation for 45 min at 37ïC. Both EGFR and EGFRvIII sections were incubated with the chromogen 3,3'-diaminobenzidine (DAB) (Ultravision Detection System Anti-polyvalent, HRP/DAB; DAKO Corporation, Carpentaria, CA, USA), for 10 min at RT. Haematoxylin counterstaining was performed in a Leica Auto Stainer XL (Meyer Instruments Inc., Houston, TX, USA).

A specimen of human skin and a human glioblastoma with documented expression of EGFRvIII were used as positive controls for EGFR and EGFRvIII, respectively (22,23). Neoplastic cells with membranous and/or cytoplasmic intense immunoreactivity were considered positively stained. Both the distribution and intensity of the immunoreactivity were semi-quantitatively scored as follows: – (0%), + (<10%), ++ (10-50%), and +++ (>50%). Samples with scores (–) and (+) were considered negative, and those with scores (++) and (+++) were considered positive as described elsewhere (22,23).

Chromogenic in situ hybridization (CISH). The presence of *EGFR* gene amplification was assessed with CISH using Spot-Light amplification probes for *EGFR* (Zymed®Laboratories Inc.). CISH was performed using Spot-Light CISH Polymer Detection Kit (Zymed®Laboratories Inc.) in accordance with the manufacturer's protocol, and as described elsewhere (22). Amplification was defined

Table I. *Results of EGFR analysis in glioblastomas.*

Case No.	Age (years)/ gender	IHC - EGFR	CISH - *EGFR* amplification	IHC - EGFRvIII
12	27/F	+	Not Ampl	–
13	54/F	+++	Ampl	–
14	41/M	–	Not Ampl	–
19	61/F	–	Not Ampl	–
20	77/M	+++	Ampl	–
21	67/F	–	Not Ampl	–
22	50/F	+	Ampl	++
23	73/M	+	Not Ampl	–
24	57/M	+++	Ampl	–
24†	57/M	+++	Ampl	++
26	28/M	+++	NC	–
29	51/F	+++	Ampl	–
30	69/M	–	NC	–
30†	69/M	–	ND	–
32	53/M	+++	NC	++
32†	53/M	++	ND	+
33	59/F	+++	Ampl	+
36	56/F	+++	Ampl	+++
37	66/M	–	NC	–
38	68/F	–	ND	–
40	60/F	–	NC	–
41	58/F	–	Not Ampl	–
42	60/M	+++	Ampl	–
43	73/M	+++	Ampl	–
45	66/F	++	Not Ampl	+++
48	54/M	+++	Not Ampl	–
48†	54/M	+	Not Ampl	–
94	66/F	+	NC	–
96	79/M	+	Ampl	–
97	72/M	–	Not Ampl	–
231	62/F	++	NC	+++

ND, not determined; NC, not conclusive; Ampl, amplified; Not Ampl, not amplified; †, recurrence.

Table II. *Results of EGFR analysis in anaplastic oligodendrogliomas and oligoastrocytomas.*

Case No.	Age (years)/ gender	IHC - EGFR	CISH - *EGFR* amplification	IHC - EGFRvIII
Anaplastic oligodendrogliomas				
60	65/M	++	Not Ampl	–
62	47/F	+	Not Ampl	–
64	65/F	+	NC	–
66	50/M	+	Not Ampl	–
68	36/F	–	Not Ampl	–
70	60/F	–	NC	–
71	53/M	++	Not Ampl	–
72	65/M	+++	Ampl	++
73	47/M	–	Not Ampl	–
76	70/F	–	Not Ampl	–
81	64/F	+	NC	–
83	54/M	+	Not Ampl	–
110	44/M	–	Not Ampl	–
110†	44/M	–	Not Ampl	–
112	42/F	+	Ampl	–
115	40/M	++	Not Ampl	–
116	51/M	–	Ampl	+++
118	64/F	++	Ampl	–
119	68/F	+	Ampl	–
163	51/M	+++	Ampl	–
168‡	36/M	+	ND	ND
168†	36/M	–	Not Ampl	+
170	73/M	–	Not Ampl	–
172	45/M	+++	ND	ND
172†	45/M	+++	Ampl	–
202	65/F	+++	Ampl	–
259	52/F	–	NC	–
Anaplastic oligoastrocytomas				
63	46/F	+	Not Ampl	–
203	36/F	++	NC	–
205	33/M	++	Ampl	–
206	55/F	+++	Ampl	+

ND, not determined; NC, not conclusive; Ampl, amplified; Not Ampl, not amplified; †, recurrence; ‡, oligodendroglioma grade II.

as more than 5 signals per nucleus in more than 50% of cancer cells, or when large gene copy clusters were seen. Signals were evaluated at x400 and x600 and at least 60 morphologically unequivocal neoplastic cells were counted for the presence of the gene probe signals. CISH hybridizations were evaluated in a blinded manner, by two independent observers, on a multi-headed microscope.

Statistical analysis. Statistical analysis was performed using SPSS for Windows (version 14.0; SPSS Inc., Chicago, IL, USA). The correlation between categorical variables was calculated for statistical significance using Pearson's chi-square test and the threshold for significance was $p \leq 0.05$.

Follow-up information was available for 40 out of 55 patients, with follow-up periods ranging from 1 to 93 months (median 11 months, mean 16 months). Survival duration was defined as the time between diagnosis and death. Associations among EGFR expression, amplification, EGFRvIII expression and patients' age with patients' survival were assessed using Cox proportional hazards regression analyses.

Results

EGFR protein overexpression. EGFR immunohistochemical analysis was performed on 62 glioma samples from 55 patients. Results are summarized on Table I and Table II. The EGFR neoplastic staining was membranous and/or cytoplasmic, without immunoreactivity of endothelial tumor cells (Figure 1 A and B). EGFR overexpression (2+/3+) was detected in 24 of 55 tumors (44%). A high percentage of positive cases was found in all histological types of gliomas analyzed, namely, 48% (13/27) of GBM (Table I, Figure 1A), 33% (8/24) of AO and 75% (3/4) of AOA (Table II, Figure 1B).

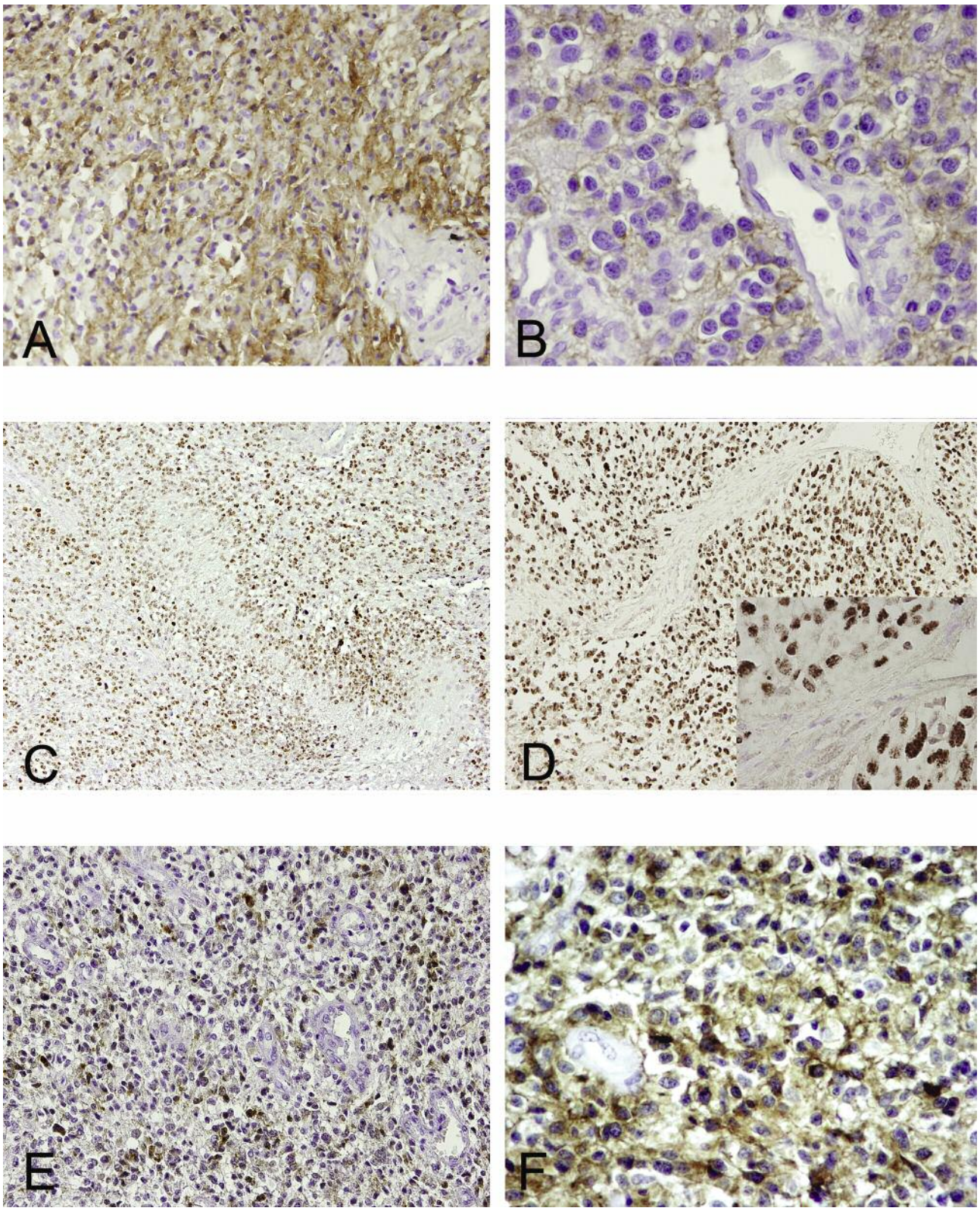

Figure 1. *EGFR cytoplasmic/membranous immunohistochemistry expression in GBM (A, x200) and AO (B, x400). EGFR amplification revealed by high nuclear signaling in GBM (C, x100) and AO (D, x100; inset, x600). EGFRvIII cytoplasmic/membranous immunohistochemistry expression in GBM (E, x200) and AO (F, x400).*

EGFR amplification. CISH analysis was possible in 54 tumors and was conclusive in 42 (78%) of the cases (Tables I and II). The pattern of EGFR signals observed using CISH was compatible with double minute amplification (Figure 1C and D). Overall, *EGFR* gene amplification was detected in 20 (48%) high-grade gliomas. Specifically, *EGFR* gene amplification was observed in 53% of GBM (10/19) (Table I, Figure 1C), 40% of AO (8/20) (Table II, Figure 1D) and 67% of AOA (2/3) (Table II). Of the 20 gliomas with *EGFR* amplification, 15 (75%) exhibited EGFR overexpression ($p=0.001$). All recurrent cases with available CISH analysis for both primary and recurrent samples, namely two GBM (cases 24 and 48) and one AO (case 110), showed the same *EGFR* amplification status.

EGFRvIII protein overexpression. EGFRvIII expression analysis was performed in 60 samples of 55 gliomas and results are summarized in Table I and Table II. EGFRvIII staining was predominantly cytoplasmic and was observed only in neoplastic cells, not in endothelial tumor cells (Figure 1E and F).

EGFRvIII overexpression was observed in 22% of GBM (6/27) (Table I, Figure 1E), 8% of AO (2/24) (Table II, Figure 1F) and was absent in AOA (0/4) (Table II). In GBM, all but one sample with EGFRvIII overexpression also exhibited EGFR overexpression (Table I, $p=0.114$). Similarly, with the exception of one case, all informative samples with EGFRvIII overexpression also showed *EGFR* gene amplification (Table I, $p=0.596$). Both AO cases with EGFRvIII overexpression depicted *EGFR* gene amplification, (Table II, $p=0.068$) one of them also with EGFR overexpression (Table II, $p=0.602$).

Clinical significance of EGFR overexpression, EGFR amplification and EGFRvIII overexpression. Association between patient age and EGFR overexpression was assessed for glioma, GBM and AO patients (data not shown). EGFR overexpression in GBM patients tended ($p=0.082$) to occur in younger patients. Multivariate analysis (Cox proportional hazards) was used to determine the association between patient age, EGFR overexpression, *EGFR* amplification or EGFRvIII overexpression and overall survival for GBM and AO. The median age of patients was calculated for GBM (60 years) and AO (51 years) and these values were used to split patients into younger and older groups. Survival time was calculated after one year for GBM patients and after five years for AO patients. No correlation was found between EGFR overexpression, *EGFR* amplification, or EGFRvIII overexpression and overall survival of GBM and AO patients (Table III). EGFR overexpression showed a tendency ($p=0.054$, Table III) for being associated with shorter survival in AO patients. There was a significant correlation ($p=0.014$, Table III) between being older and better survival in AO patients.

Table III. *Multivariate analysis for the effect of EGFR overexpression, EGFR amplification, EGFRvIII overexpression and age on survival of GBM and AO patients.*

Variable	Hazard ratio (95% confidence interval)	*p*-value
GBM (1-year survival)		
EGFR overexpression	3.655 (0.731-18.277)	0.114
EGFR amplification	1.073 (0.167-6.885)	0.941
EGFRvIII overexpression	0.732 (0.131-4.095)	0.723
Median age (<60 or ≥60 years)	1.222 (0.204-7.333)	0.826
AO (5-year survival)		
EGFR overexpression	36.927 (0.942-1447.576)	0.054
EGFR amplification	0.112 (0.007-1.710)	0.116
EGFRvIII overexpression	NS	
Median age (<51 or ≥51 years)	0.065 (0.007-0.570)	0.014

NS: Insufficient data for statistical evaluation.

Discussion

In this study, we characterized, for the first time, the presence of EGFR alterations in a series of high grade gliomas from Portuguese patients. We studied a total of 31 GBM and observed that the frequency of GBM presenting EGFR overexpression (48%), *EGFR* amplification (53%) and EGFRvIII overexpression (22%) were in line with the published literature (24-26). Similarly to several studies a positive correlation ($p=0.012$) was found between EGFR overexpression and gene amplification in GBM (14, 18, 24, 27). Additionally, all but one GBM with EGFRvIII overexpression presented gene amplification, which is also in agreement with previous studies (24, 25). It was previously reported that older patients with GBM have higher rates of EGFR overexpression and amplification (24, 28). We found no statistical differences between patient age and EGFR overexpression, *EGFR* gene amplification, or EGFRvIII overexpression in the same cases.

EGFR activation status in anaplastic oligodendrogliomas has been less frequently reported (13, 27, 29, 30). We analyzed 24 cases and observed EGFR overexpression in 33%, *EGFR* amplification in 40% and EGFRvIII mutation in 8% of anaplastic oligodendrogliomas. EGFR overexpression tended to correlate with gene amplification ($p=0.094$). Other authors described *EGFR* amplification in anaplastic oligodendrogliomas and frequencies varied from 0-42.5% (13, 27, 31). A possible reason for this discrepancy is the distinct methodologies used. Regarding EGFRvIII expression, results are few and contradictory. Wikstrand and colleagues analyzed 5 anaplastic oligodendrogliomas and described a frequency of 20% of EGFRvIII expression (30); however, in another study from the same group EGFRvIII was not found to be expressed in 25 anaplastic oligodendrogliomas (29).

The influence of EGFR overexpression, *EGFR* gene amplification and EGFRvIII overexpression in patient prognosis has been highly controversial for gliomas (24, 25, 28, 32-37). To clarify this issue, we performed a multivariate analysis and found no association between these EGFR alterations and patients' overall survival in our cohort.

EGFR is becoming an important therapeutic target, with some anti-EGFR drugs already being used in clinical practice and several novel EGFR inhibitors under development (38). Among the two major approaches, using EGFR-TKIs and mAbs, the former seems to be more suitable for gliomas due to their low molecular weight potentially being better at overcoming the blood–brain barrier (39). In NSCLC, the presence of activating mutations in the EGFR kinase domain was associated with selective EGFR-TKI sensitivity, allowing the selection of patients with a higher probability of clinical response to gefitinib and erlotinib (11). However, these mutations have never been found in glioma cell lines (11), or in glioma patients (11, 40, 41). Recently, Lee and colleagues reported EGFR activation in GBM due to missense mutations in the EGFR extracellular domain (42). They reported that transformed cells with the EGFR ectodomain mutations had increased sensitivity to erlotinib; however studying DNA samples from a previous clinical trial, these authors were unable to associate EGFR ectodomain mutations with clinical responses to EGFR TKI inhibitors (42).

The few clinical trials with gefitinib and erlotinib in gliomas included a small number of patients and variable molecular markers, insufficient for conclusive results regarding patients' response and *EGFR* molecular status. Rich and colleagues, in a phase II trial, described a monotherapy study with gefitinib in GBM patients and found neither objective tumor response nor association between EGFR expression, *EGFR* amplification, or EGFRvIII expression and gefitinib response (43). Franceschi and colleagues' recent phase II trial investigated the role of gefitinib in patients with high-grade gliomas and reported an 18% stable disease rate without any correlation of EGFR expression or gene status and tumor response (44). Vogelbaum *et al.* described a monotherapy with erlotinib in patients with recurrent GBM and found 25% with stable disease and 25% with partial response rates; although *EGFR* amplification was observed in about 50% of tumors, it was not associated with erlotinib response (45). In a phase II study with erlotinib in recurrent GBM, Cloughesy *et al.* described a 33% stable disease rate and EGFR expression associated with a slight tendency for better patient outcome (46). Haas-Kogan *et al.* reported that the presence of EGFR overexpression and gene amplification, associated with low levels of activated Akt, were associated with response to erlotinib, suggesting that these molecular alterations could be predictive markers for EGFR-TKI sensitivity in gliomas (18). Mellinghoff *et al.* described that coexpression of EGFRvIII and phosphatase and tensin homolog (PTEN) (RTK downstream negative regulator) was associated with a better response to gefitinib and erlotinib in recurrent GBM (19). In general, results with erlotinib seem to be more promising than with gefitinib, possibly due to its targeting of both EGFRvIII mutant and wild-type EGFR (39).

There have also been clinical trials involving EGFR-targeted mAbs in gliomas (9, 47). A phase I/II clinical trial using the EGFR mAb h-R3 in malignant glioma enrolled 29 patients with an objective response in 37.9% and stable disease in 41.4% of the cases (47). Another phase I/II trial using cetuximab in GBM is ongoing (9). There are several *in vitro* and *in vivo* studies reporting EGFR-targeted mAbs effects in glioma. Recently, Johns *et al.* studied the efficacy of two EGFR-specific mAbs (mAbs 806 and 528) against glioma cell line-derived xenografts expressing EGFR and EGFRvIII and reported that their efficacy was dependent on EGFR overexpression and receptor activation status (48). Currently, considerable efforts are being made to design anti-EGFRvIII strategies, such as mAbs and vaccines, since this mutated form constitutes a tumor-specific target that is not present in normal cells.

In conclusion, in the present study, we found that a high percentage of GBM and AO exhibited EGFR overexpression and amplification, as did a significant proportion of GBM and a small proportion of AO expressing EGFRvIII. Our results represent the first step for the identification of Portuguese glioma patients who could respond to specific therapies targeting EGFR.

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