

Molecular alterations of *KIT* oncogene in gliomas

Ana L. Gomes^a, Jorge S. Reis-Filho^b, José M. Lopes^c, Olga Martinho^a, Maryou B.K. Lambros^b, Albino Martins^a, Fernando Schmitt^{a,c}, Fernando Pardal^d and Rui M. Reis^{a,*}

^a *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal*

^b *The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, SW3 6JB, UK*

^c *Medical Faculty of Porto University and IPATIMUP, Porto, Portugal*

^d *Department of Pathology, S. Marcos Hospital, Braga, Portugal*

Abstract. Gliomas are the most common and devastating primary brain tumours. Despite therapeutic advances, the majority of gliomas do not respond either to chemo or radiotherapy. KIT, a class III receptor tyrosine kinase (RTK), is frequently involved in tumorigenic processes. Currently, KIT constitutes an attractive therapeutic target. In the present study we assessed the frequency of KIT overexpression in gliomas and investigated the genetic mechanisms underlying KIT overexpression. KIT (CD117) immunohistochemistry was performed in a series of 179 gliomas of various grades. *KIT* activating gene mutations (exons 9, 11, 13 and 17) and gene amplification analysis, as defined by chromogenic *in situ* hybridization (CISH) and quantitative real-time PCR (qRT-PCR) were performed in CD117 positive cases. Tumour cell immunopositivity was detected in 15.6% (28/179) of cases, namely in 25% (1/4) of pilocytic astrocytomas, 25% (5/20) of diffuse astrocytomas, 20% (1/5) of anaplastic astrocytomas, 19.5% (15/77) of glioblastomas and one third (3/9) of anaplastic oligoastrocytomas. Only 5.7% (2/35) of anaplastic oligodendrogliomas showed CD117 immunoreactivity. No association was found between tumour CD117 overexpression and patient survival. In addition, we also observed CD117 overexpression in endothelial cells, which varied from 0–22.2% of cases, being more frequent in high-grade lesions. No *KIT* activating mutations were identified. Interestingly, CISH and/or qRT-PCR analysis revealed the presence of *KIT* gene amplification in 6 glioblastomas and 2 anaplastic oligoastrocytomas, corresponding to 33% (8/24) of CD117 positive cases. In conclusion, our results demonstrate that *KIT* gene amplification rather than gene mutation is a common genetic mechanism underlying KIT expression in subset of malignant gliomas. Further studies are warranted to determine whether glioma patients exhibiting KIT overexpression and *KIT* gene amplification may benefit from therapy with anti-KIT RTK inhibitors.

Keywords: Gliomas, glioblastomas, anaplastic oligoastrocytomas, KIT, mutations, amplification, CISH, quantitative real-time PCR

1. Introduction

Gliomas are the most frequent primary brain tumours accounting for approximately 70% of all neoplasms [22]. Histologically, gliomas are divided in several entities, with astrocytic tumours being the most prevalent type, followed by oligodendroglial and mixed oligoastrocytic tumours. According to the World Health Organization (WHO), tumours are classified into four grades of malignancy, grade I pi-

locytic astrocytomas generally behave in a less aggressive fashion, whereas grade II–IV are biologically malignant diffusely infiltrating the adjacent brain parenchyma and progressing ultimately to glioblastoma (WHO grade IV) [22]. Glioblastomas are not only the most aggressive, but also the most prevalent type of glioma, and despite all advances in neurosurgery, radio- and chemotherapy, its prognosis is still dismal. Currently, the median survival time of glioblastomas patients is approximately one year, and has changed little in the last three decades [25]. Therefore, novel, targeted therapies hold key for significant improvements in the survival of these patients.

The *KIT* gene maps to 4q12 and is a member of the platelet derived growth factor receptor (PDGFR)

*Corresponding author: Rui M. Reis, Escola de Ciências da Saúde, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Tel.: +351 235 604825; Fax: +351 253 604820; E-mail: rreis@eecsau.de.uminho.pt

family of tyrosine kinases [41]. In recent years it has become an important molecular target in cancer therapy [12]. *KIT* receptor regulates several crucial carcinogenic mechanism including cell proliferation, differentiation, migration and apoptosis [17]. Constitutive activation of *KIT* has been involved in several types of human cancer, including gastrointestinal stromal tumours (GISTs), where mutations in *hotspots* exons 9, 11, 13 or 17 are the causal mechanisms for such activation [17,21]. The clinical importance of *KIT* is linked with the development of specific receptor tyrosine kinase (RTK) inhibitors such as Imatinib and more recently Sunitinib, proven to be effective in the treatment of advanced patients with GISTs harbouring *KIT* gene mutations [6,23].

The role of *KIT* in the CNS is far from being well understood. During normal brain development, *KIT* is reported to be expressed in different regions of the CNS [15,20,28] and to influence oligodendrocytic differentiation [18]. In CNS neoplasms, some studies have showed expression of *KIT* in medulloblastoma [8], neuroblastoma [5] and glioma [7,14,19,32, 38], however, its oncogenic role in these tumours remains largely unknown. Nevertheless, encouraging results were obtained in mouse models of glioblastoma treated with Imatinib, where an increase in the sensitivity to ionizing radiation was observed and a concomitant increase in median survival was attained [13]. In addition, clinical trials are ongoing using Imatinib for the treatment of recurrent glioblastoma patients [30, 31].

The aims of this study were first to define the frequency of *KIT* overexpression in a large series of gliomas including astrocytic, oligodendroglial and oligoastrocytic tumours, and secondly to determine whether *KIT* activating gene mutations or gene amplification would be the underlying mechanism driving *KIT* overexpression. These results would help to clarify the role of *KIT* in gliomagenesis and possibly provide a molecular basis for target therapy using RTK inhibitors in these highly lethal neoplasms.

2. Material and methods

2.1. Material

One hundred and seventy-nine formalin-fixed, paraffin-embedded cases of gliomas were retrieved at the pathology archives of the Department of Pathology

of Hospital S. João, Porto and of Hospital S. Marcos, Braga, Portugal. Cases were reviewed by two pathologists (JML, FP) and classified according to the WHO criteria [22]. The cohort includes 105 astrocytic, 64 oligodendroglial and 10 oligoastrocytic tumours [22]. The mean age of patients was 50.0 ± 16.2 (range, 2–79), with a female/male ratio of 0.84.

2.2. Immunohistochemistry assay

Representative 3 μm -thick sections were subjected to immunohistochemical analysis. *KIT* (CD117) expression was assessed by immunohistochemistry according to the streptavidin-biotin peroxidase complex system, using a primary antibody raised against CD117 (dilution 1:50; clone A4502, DAKO Corporation, Carpinteria, CA, US), as previously described [32]. Briefly, deparaffinized and rehydrated slides were subjected to 10 minutes incubation in 3% hydrogen peroxide in methanol, in order to inhibit endogenous peroxidase. No antigen retrieval was used. After incubation with the primary antibody at room temperature for 2 hours, the secondary biotinylated goat anti-polyvalent antibody was applied for 10 minutes followed by incubation with the streptavidin-peroxidase complex. The immune reaction was visualized by 3,3'-Diaminobenzidine (DAB) as a chromogen (UltraVision Detection System Anti-polyvalent, HRP/DAB; Lab Vision, Fremont, CA, US). Appropriated positive and negative controls were included in each run. Positive controls included a gastrointestinal stromal tumour with previously characterised CD117 overexpression. Negative controls included omission of the primary antibody and negative DAKO control (N1699, DAKO Corp., Carpinteria, California, USA). All sections were counterstained with Gill-2 haematoxylin.

Stained tumour samples were analysed according to a previously described semi-quantitative method [32] and without knowledge of the clinical findings. Only CD117 membranous with or without cytoplasmic immunoreactivity in neoplastic cells was considered specific. Both the distribution and intensity immunoreactivity were semi-quantitatively scored as follows: (–) (negative), (+) ($\leq 5\%$), (++) (5–50%) and (+++) ($> 50\%$). Samples with scores (–) and (+) were considered negative, and those with scores (++) and (+++) were considered positive.

2.3. *KIT* gene mutation analysis

DNA isolation was obtained from 10 µm-thick unstained tissue sections as previously described [4]. To identify areas containing tumour tissue, a serial H&E stained section was used. Microdissected tumour tissue was carefully collected into a microfuge tube using a sterile needle. After microdissection, all samples contained at least 85% of neoplastic cells. The dissected tissue was deparaffinized by a serial extraction with xylene and ethanol (100%–70%–50%) and allowed to air-dry. DNA was extracted using Qiagen's QIAamp[®] DNA Micro Kit (Cat. No. 56304, Hilden, Germany), following manufacture instructions. DNA samples were stored at –20°C for further analysis. Exons 9, 11, 13 and 17 were screened for *KIT* activating gene mutations as previously described [32]. Briefly, PCR reaction was carried in a final volume of 25 µl, under the following conditions: 1 × Buffer (Bioron, Germany); 1.5 mM MgCl₂ (Bioron, Germany); 200 µM dNTPs (Fermentas, USA); 0.5 µM primers (DNA Technology, Denmark) and 1 unit of Super Hot Taq Polymerase (Bioron, Germany).

Primers were previously described by Corless et al. [9]. Exon 11 PCR products were directly sequenced. Amplification of exons 9, 13 and 17 by PCR were followed by SSCP analysis in a 1 × MDE gel (Cambrex, USA). 8 µl of PCR product were incubated at 95°C for 10 minutes with an equal volume of formamide loading buffer (98% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue and xylene cyanol). SSCP gels were run at 20°C for exons 9, 13 and 17. Samples with an abnormal SSCP pattern were directly sequenced (Stabvida, Portugal). All cases were confirmed twice with a new and independent PCR amplification followed by direct sequencing.

2.4. Chromogenic *in situ* hybridization (CISH)

The presence of *KIT* gene amplifications was assessed by means of CISH with an in-house generated probes made up with three contiguous, FISH-mapped and end-sequence verified bacterial artificial chromosomes (BACs) (RP11-42B10, RP11-586A02 and RP11-273B19), which map to 4q12 (~ 55.0 Mb and ~ 55.5 Mb) according to Ensembl V39 – June 2006 build of the genome (http://www.ensembl.org/Homo_sapiens/index.html) [26]. The in-house probe was generated, biotin-labelled and used in hybridizations as previously described [26]. Heat pre-treatment of deparaffinized sections were incubated for 15 minutes

at 98°C in CISH pre-treatment buffer (SPOT-light tissue pre-treatment kit, Zymed) and digested with pepsin for 6 minutes at room temperature according to the manufacturer's instructions. CISH experiments were analysed by two of the authors (JSR-F & MBK) on a multi-headed microscope. Only unequivocal signals were counted. Signals were evaluated at 400× and 630× and 60 morphologically unequivocal neoplastic cells were counted for the presence of the gene probe signals. Amplification was defined as >5 signals per nucleus in more than 50% of cancer cells, or when large gene copy clusters were seen, in accord with previously described and validated cut-offs [26,33,34,39]. CISH hybridizations were evaluated with observers blinded to the immunohistochemistry results.

2.5. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed with LightCycler (Roche Molecular Biochemicals, Mannheim, Germany), using fluorescent hybridisation probes and fluorescence resonance energy transfer for the detection of PCR amplification product, following the manufacturer's instructions. Briefly, primers and probes were designed to amplify a 163 bp (exon 17 from *KIT* gene) and a 147 bp (*18S* gene) specific PCR product, where the *18S* was used as reference gene which was previously used in gliomas to study gene amplification by qReal-time [1–3]. PCR amplification was performed in a 10 µl reaction volume, under the following conditions: 1 × reaction master mix (Lightcycler FastStart DNA Master Hybridization Probes kit, Roche Molecular Biochemicals, Mannheim, Germany); 0,2 µM Probes (Roche Molecular Biochemicals, Mannheim, Germany); 0,5 µM primers; 4 mM MgCl₂ (Roche Molecular Biochemicals, Mannheim, Germany) and 1 µl (20 ng/µl) of DNA. The reaction was initiated by a desnaturation step for 10 min at 95°C, followed by 45 cycles with the following profile of amplification: incubation for 10 s at 94°C, specific annealing temperature (57°C for both genes) for 10 s and extension at 72°C for an amplicon dependent time (7 seconds for both genes), immediately followed by a cooling step for 2 min at 40°C. The following primers and probes were used. For *KIT* gene: 5'-TTTCTCCTCCAACCTAATAGTGT-3' (forward primer), 5'-GAATGGGTACTCACGTTTCC-3' (reverse primer), 5'-GTGATCCGACCATGAGTAAG GAGGATA-FL (donator probe), LC640-TTCTGGCT GCCAAGTCTCTGTG-3' (acceptor probe). For *18S* gene: 5'-GTAACCCGTTGAACCCATT-3' (forward

primer), 5'-CCATCCAATCGGTAGTAGCG-3' (reverse primer), 5'-CGGTCATAAGCTTGCCTTATTA-FL (donator probe), LC640-GTCCCTGCCCTTTGTACACACCG-3' (acceptor probe).

The PCR was done in duplicate for each studied sample. A series of 10 normal DNA from healthy individuals was investigated to determine the confidence interval and the standard deviations of the calculated ratios for reference and target gene. Evaluation of data was performed using the $\Delta\Delta C_t$ method: $\Delta\Delta C_t = \Delta C_t$ Tumour DNA – ΔC_t Normal blood DNA. ΔC_t (threshold cycles) is the C_t of the reference gene minus the C_t of the target gene. Fold increase of the target gene *KIT* was calculated by $2^{(\Delta\Delta C_t)}$ and values >2 were defined as gene amplification.

2.6. Statistical analysis

The Statview software package (SAS Institute Inc., Cary, North Carolina, USA) was used for all calculations. Correlations between categorical variables were performed using the chi-square test and Fisher's exact test. Correlations between continuous and categorical variables were performed with analysis of variance (ANOVA). Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test. All tests were two-tailed, with a confidence interval of 95%.

3. Results

3.1. *KIT* (CD117) protein expression

CD117 overexpression was detected in 15.6% (28/179) of tumour cases (Table 1). Often, adjacent normal neuron depicted CD117 staining (Fig. 1A). Positive CD117 staining was found in almost all of the major types of gliomas analysed, specifically, 25.0% (1/4) of pilocytic astrocytomas, 26.3% (5/19) of diffuse astrocytomas, 20.0% (1/5) of anaplastic astrocytomas, 20.8% (16/77) of glioblastomas (Fig. 1B), 5.7% (2/35) of anaplastic oligodendrogliomas, and 33.3% (3/9) of anaplastic oligoastrocytomas (Fig. 1C). Four glioblastomas (case 9, 10, 11 and 20) recurred. Primary tumours of cases 9, 11 and 20 stained positively for CD117, and primary tumour of case 10 did not show CD117 overexpression. Recurrences of cases 9 and 11 were CD117 immunonegative, whereas recurrences of cases 9 and 19 stained positively.

Three anaplastic oligoastrocytomas exhibited CD117 staining in both astrocytic and oligodendroglial components. One anaplastic oligoastrocytoma presented weak CD117 immunostaining (+) solely in the astrocytic component. CD117 overexpression was also observed in endothelial cells of tumours, exhibiting variable immunoreactivity of neoplastic cells (Fig. 1D). CD117 positive endothelial cells were present in 5.3% (1/19) of diffuse astrocytomas, 16.9% (13/77) of glioblastomas, 7.0% (2/29) of oligodendrogliomas, 11.4% (4/35) of anaplastic oligodendrogliomas and 22.2% (2/9) anaplastic oligoastrocytomas. No CD117 positive endothelial cells were observed in pilocytic astrocytomas, anaplastic astrocytomas and oligoastrocytomas.

No statistically significant correlation was found between CD117 overexpression and age, histological type, WHO grade, or follow-up ($p > 0.05$). CD117 overexpression was, however, statistically correlated with male gender ($p = 0.0343$). When glial tumours were classified into the 3 major groups according to their histogenesis (astro, oligo, mixed), *KIT* expression was correlated with astrocytic lineage ($p = 0.0029$) (Table 2).

3.2. *KIT* gene status analysis

KIT mutations were evaluated in 28 CD117 overexpressing tumours and no alterations in exons 9, 11, 13 or 17 were identified (Table 1).

As gene mutation is only one of the possible mechanisms for CD117 overexpression, we also investigated whether *KIT* amplification could be the alternative underlying genetic mechanism for its overexpression. CISH rendered optimal results in 22 of the 28 CD117 positive gliomas, and revealed *KIT* amplification in 31.2% (7/22) of cases (Table 1). In all of them, either large gene clusters or an average of >10 signals/nucleus were observed. Of these 7 *KIT* amplified cases, 5 were glioblastomas (Fig. 2B), and 2 were anaplastic oligoastrocytomas (Fig. 2C) (Table 1). Three tumours (case 18, 20 and 25) showed the presence of *KIT* amplification in a non-modal clone, however these cases were not considered amplified by the criteria adopted. Interestingly, case 20 recurred as a GBM and the recurrence was solely composed of cells harbouring high level *KIT* gene amplification. Both primary and recurrence were positive (++) for CD117 staining.

In addition, *KIT* gene amplification status was also evaluated by qRT-PCR (Table 1). The concordance be-

Table 1
KIT molecular analysis in CD117 expressing gliomas

Case no.	Diagnosis	CD117 score	<i>KIT</i> mutation				<i>KIT</i> amplification	
			Exon 9	Exon 11	Exon 13	Exon 17	CISH	Q real-time (Ratio)
1	PA (WHO I)	(++)	WT	WT	WT	WT	n.p.	0.6
2	DA (WHO II)	(++)	WT	WT	n.p.	WT	n.p.	n.d.
3	DA (WHO II)	(++)	WT	WT	WT	WT	Absent	0.3
4	DA (WHO II)	(++)	WT	WT	WT	WT	Absent	0.1
5	DA (WHO II)	(++)	WT	WT	WT	WT	n.p.	n.d.
6	DA (WHO II)	(++)	n.p.	n.p.	n.p.	n.p.	n.p.	n.d.
7	AA (WHO III)	(++)	WT	WT	WT	WT	Absent	0.2
8	GBM (WHO IV)	(++)	WT	WT	WT	WT	Present	42.3
9	GBM (WHO IV)	(++)	WT	WT	WT	WT	n.p.	0.8
	GBM (WHO IV)*	(-)	WT	WT	n.p.	n.p.	Absent	0.9
10	GBM (WHO IV)	(-)	n.d.	n.d.	n.d.	n.d.	n.d.	0.9
	GBM (WHO IV)*	(++)	WT	n.p.	n.p.	n.p.	Absent	n.p.
11	GBM (WHO IV)	(++)	WT	WT	n.p.	n.p.	Absent	n.d.
	GBM (WHO IV)*	(-)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	GBM (WHO IV)	(++)	WT	WT	WT	WT	Absent	n.d.
13	GBM (WHO IV)	(++)	WT	WT	n.p.	n.p.	Absent	n.d.
14	GBM (WHO IV)	(++)	n.p.	WT	WT	n.p.	n.p.	n.d.
15	GBM (WHO IV)	(++)	WT	n.p.	n.p.	n.p.	Present	49.4
16	GBM (WHO IV)	(++)	n.p.	n.p.	n.p.	n.p.	Absent	n.d.
17	GBM (WHO IV)	(++)	WT	n.p.	WT	WT	Absent	0.5
18	GBM (WHO IV)	(++)	n.p.	n.p.	n.p.	n.p.	Absent#	n.d.
19	GBM (WHO IV)	(+ + +)	WT	WT	WT	WT	Absent	1.1
20	GBM (WHO IV)	(++)	WT	WT	WT	WT	Absent#	5.3
	GBM (WHO IV)*	(++)	WT	WT	WT	n.p.	Present	3.8
21	GBM (WHO IV)	(++)	WT	WT	WT	WT	n.p.	2.4
22	GBM (WHO IV)	(++)	WT	WT	WT	n.p.	Present	33.2
23	GBM (WHO IV)	(++)	WT	n.p.	WT	WT	Present	n.p.
24	AO (WHO III)	(++)	WT	WT	WT	WT	Absent	n.d.
25	AO (WHO III)	(++)	WT	WT	WT	n.p.	Absent#	0.5
26	AOA (WHO III)	(++)	WT	WT	WT	WT	Absent	0.3
27	AOA (WHO III)	(++)	WT	WT	WT	WT	Present	‡astro: 3.4; oligo: 9.5
28	AOA (WHO III)	(++)	WT	WT	WT	WT	Present	72.2

*Recurrences; PA, pilocytic astrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; WT, wild type; n.d., not done; n.p., not possible; #, amplification not significant (see results); ‡, astro, astrocytic component, oligo, oligodendroglial component; Bold face values and words indicate *KIT* amplification.

tween both CISH and QReal-Time techniques was almost absolute. We confirmed the presence of *KIT* amplification in 6 out of 6 cases previously shown to harbour *KIT* amplification by CISH analysis. One additional case harbouring *KIT* gene amplification as defined by CISH could not be analysed by qRT-PCR (DNA of suboptimal quality). In 8 cases identified as negative for amplification by CISH were also negative for *KIT* amplification by qRT-PCR. The only discordant result was the detection of *KIT* amplification by qRT-PCR in primary tumour of case 20, which was

considered non-amplified by CISH according to the criteria used for gene amplification (see Section 1), but harboured a non-modal population (10% of tumour cells) with *KIT* amplification (Table 1). Furthermore, using qRT-PCR, we were able to assess *KIT* amplification status in four additional tumours, revealing another tumour with *KIT* amplification (case 21). Using this technique we could also analyse independently both astrocytic and oligodendroglial components of an anaplastic oligoastrocytoma positive for *KIT* gene amplification by CISH (case 27). In agreement with

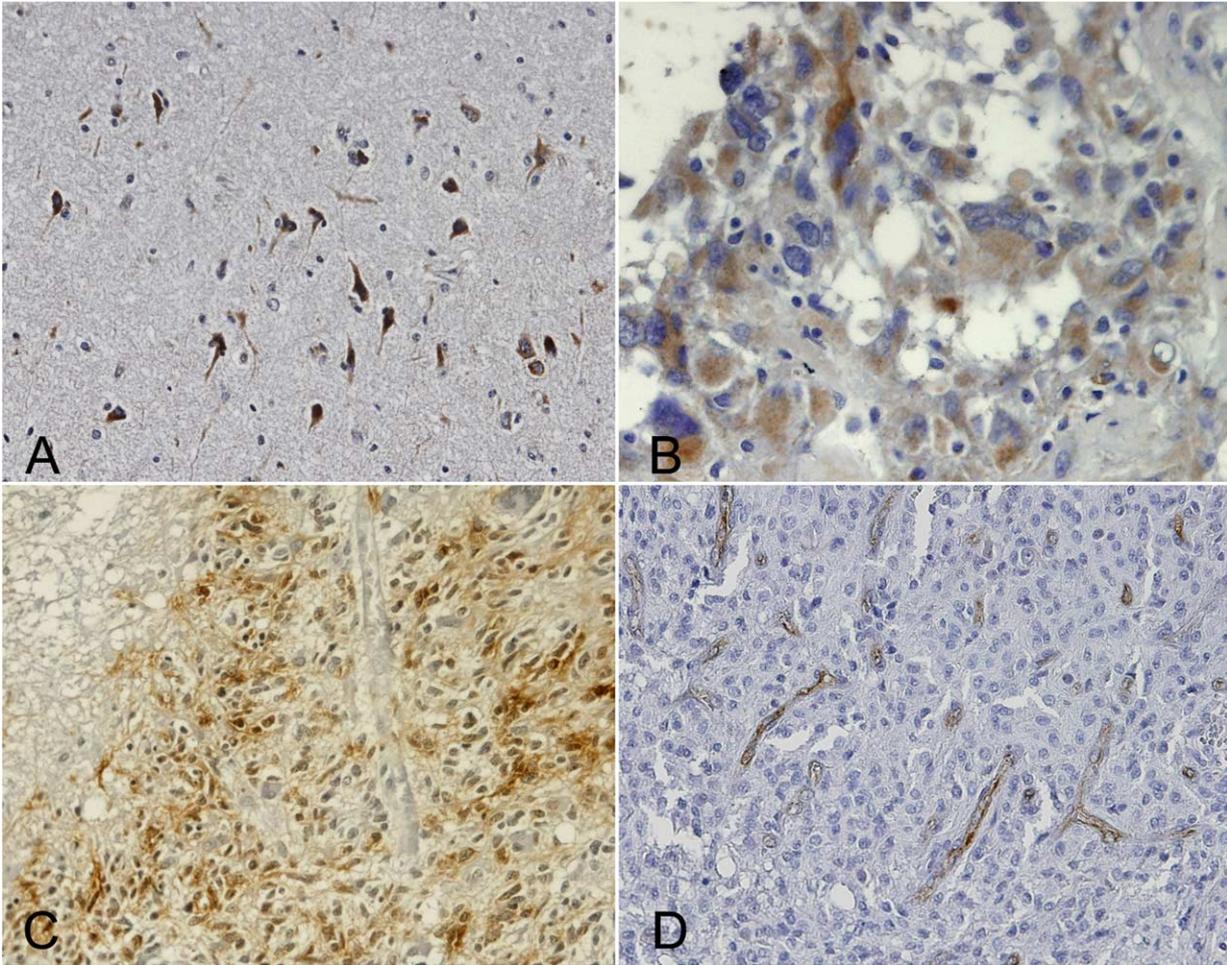


Fig. 1. Immunohistochemistry analysis of CD117 expression in: (A) peri-tumoral neurons (200 \times); (B) glioblastoma neoplastic cells (200 \times); (C) anaplastic oligoastrocytoma neoplastic cells (200 \times); (D) glioblastoma endothelial cells (200 \times).

CISH results we observed the presence of gene amplification in both components (Table 1). Overall, the percentage of cases with *KIT* gene amplification was 33.3% (8/24). In three cases, it was possible to evaluate the topographic distribution of CD117 expression and *KIT* amplification by CISH. This analysis demonstrated that regions harbouring *KIT* gene amplification also harboured CD117 expression, whereas regions with no *KIT* copy number aberrations did not display positivity for anti-CD117 antibody.

Statistical correlation between presence of *KIT* gene amplification and clinico-pathological features was not assessed due to the small sample size of *KIT* amplified cases (glioblastoma, $n = 6$; anaplastic oligoastrocytomas, $n = 2$).

In addition, mutation and gene amplification analysis of *KIT* were performed in 15 randomly selected

negative glioma cases [(-) or (+)] for CD117 overexpression. All cases analysed, including 2 oligodendroglioma, 3 anaplastic oligodendroglioma, 1 anaplastic oligoastrocytoma, and 9 glioblastomas, displayed wild-type *KIT* and lacked *KIT* amplification.

4. Discussion

In the present study, *KIT* (CD117) overexpression was observed in up to 16% of cases and was preferentially expressed in tumours with astrocytic differentiation. The frequency of CD117 overexpression in gliomas reported in the literature is variable, ranging from 2% to 83.3% in glioblastomas, the best characterised entity [7,14,29,35]. These inconsistent results may be due to technical issues, such as different anti-

Table 2
Clinical-pathological analysis of CD117 expression in gliomas

Parameter	N	CD117 negative	CD117 positive	p value
Age, mean \pm SD	179	49.9 \pm 16.2	48.8 \pm 16.6	0.7256
Gender				0.0343
Male	97	78	19	
Female	82	75	7	
Cellular lineage				0.0029
Astrocytic	105	83	22	
Oligodendroglial	64	62	2	
Mixed	10	7	3	
Histological type (WHO grade)				0.0668
Pilocytic astrocytoma (WHO I)	4	3	1	
Diffuse astrocytoma (WHO II)	19	15	5	
Anaplastic astrocytoma (WHO III)	5	4	1	
Glioblastoma (WHO IV)	77	62	15	
Oligodendroglioma (WHO II)	29	29	0	
Anaplastic oligodendroglioma (WHO III)	35	33	2	
Oligoastrocytoma (WHO II)	1	1	0	
Anaplastic oligoastrocytoma (WHO III)	9	6	3	
Malignancy grade (WHO)				0.3290
Low-grade (I, II)	54	48	6	
High-grade (III, IV)	125	104	21	
Follow-up – mean months \pm SD				0.9788
Glioblastomas (WHO IV)	45	8.7 \pm 1.2	9.2 \pm 1.3	

SD: Standard deviation.

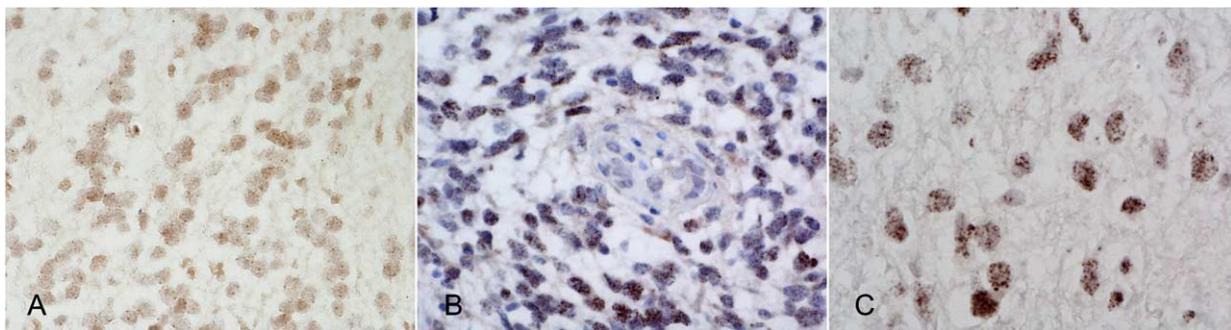


Fig. 2. CISH analysis of *KIT* amplification status in CD117 expressing gliomas. (A) Oligodendroglioma without *KIT* amplification (600 \times , no H.E. counterstaining). (B) Glioblastoma with *KIT* amplification (600 \times , H.E. counterstaining). (C) Anaplastic oligoastrocytoma with *KIT* amplification (1000 \times , no H.E. counterstaining).

bodies, and antigen retrieval procedures. In this study, the methodology used for CD117 immunostaining is considered the most reliable [16], and we observed CD117 overexpression in 20.8% of glioblastomas. No association between CD117 overexpression and patient's clinical pathological features was observed. Interestingly, CD117 overexpression was observed not only in tumour cells, but also in endothelial cells, a finding that has been recently reported in gliomas,

suggesting that *KIT* signalling may also play an important role in glioma angiogenesis, and may have potential for therapeutic management [27,36,38].

In order to elucidate the molecular mechanisms responsible for CD117 overexpression in gliomas, we screened the extracellular, juxtamembrane, and the two intracellular kinase domains of *KIT* gene for genetic alterations. No activating mutations were identified. The present results are in agreement with those of previ-

ously reported studies, where no *KIT* mutations were found in glial tumours [19,29,32,35]. Apart from gene activating mutations, CD117 overexpression may also be driven by *KIT* gene amplification. Therefore, we employed two distinct methodologies, chromogenic *in situ* hybridization (CISH) and quantitative real-time PCR (qRT-PCR), to investigate the prevalence of *KIT* gene amplification. We identified *bona fide* *KIT* amplification in 33.3% (8/24) of CD117 overexpressing gliomas. No *KIT* amplification was identified in the three low-grade astrocytic tumours and in two anaplastic oligodendrogliomas evaluated. In addition, no *KIT* gene mutation or amplification was detected in any of the fifteen CD117 negative gliomas.

Recently, using fluorescent *in situ* hybridization (FISH) and/or chromogenic *in situ* hybridization (CISH), the presence of *KIT* amplification was reported in a series of gliomas from Finland [19,29]. The authors observed *KIT* amplification in approximately half of glioblastomas (47%, 20/43) [19] and also in low-grade astrocytomas and oligoastrocytomas, however at a lower frequency [29]. Several reasons may account for the distinct frequencies of *KIT* gene amplification reported in those studies and in our series. The threshold levels used as the definition of *KIT* gene amplification were of >3 *KIT* signals/centromeric signals in 10% of the cells for FISH analysis [19] and >6 signals per nucleus for CISH analysis [29]. Furthermore, the tumour populations were distinct. In fact, using similar methods and thresholds, the authors [19] detected in the same cohort a very high frequency of *PDGFR- α* amplification (29%), an event commonly reported to be infrequent (<10%) in glioblastomas [24,37,40]. Surprisingly, only 2 out of 20 *KIT* amplified glioblastomas [19] and 4 out of 27 *KIT* amplified gliomas [29] showed strong *KIT* (CD117) immunorexpression [19,29].

Targeted therapies, using RTK inhibitors, such as Imatinib, are a great improvement in cancer treatment [12]. Imatinib mesylate is a small-molecule selective inhibitor of BCR-ABL, *KIT* and *PDGFR* tyrosine kinases, by competing with ATP for its binding site, preventing further phosphorylation of signalling molecules downstream the receptor, responsible for abnormal viability and proliferation signals in these cells [12]. In systemic mastocytosis and GIST, Imatinib acts by inhibiting *KIT*, which is frequently altered in these two malignancies [10,11]. Furthermore, in GIST patients intolerant to Imatinib, Sunitinib (another RTK inhibitor) has proven to be quite successful, overcoming Imatinib resistance [6]. Importantly, *KIT* mutation

status is the major predictive factor of Imatinib-based GISTs response [6].

It remains to be determined whether *KIT* amplification leads to *KIT* receptor activation, and importantly, if patients harbouring *KIT* amplified tumors would be responsive to therapy with Imatinib and/or other RTK inhibitors. However, there are several lines of evidence to suggest that oncogene amplification in other malignancies constitutes a biological marker for response to directed target therapy, such as *HER2* in breast cancer, and response to both monoclonal antibodies against *HER2* and *HER2* tyrosine kinase inhibitors [42]. Therefore, further studies are warranted to elucidate the role of *KIT* amplification in glioma response to RTK inhibitors, mainly Imatinib and Sunitinib based therapy.

In conclusion, this is a comprehensive study on *KIT* characterization in human gliomas. We demonstrated that *KIT* gene amplification is present in a fraction of glioblastomas and anaplastic oligoastrocytomas, possibly being one of the mechanisms underlying *KIT* overexpression in these tumours. Furthermore, these results raise the possibility that a subgroup of patients with malignant glioma may benefit from therapy with anti-*KIT* RTK inhibitors.

Acknowledgments

ALG is recipient of a fellowship grant from FCT (SFRH/BI/15257/2004). This work was partially supported by NOVARTIS Oncology, Portugal, and Breakthrough Breast Cancer, UK.

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