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Molecular characterization of PDGFR-α/PDGF-A and c-KIT/SCF in gliosarcomas

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Abstract. Gliosarcomas are rare and poorly characterized malignant brain tumors that exhibit a biphasic tissue pattern with areas of gliomatous and sarcomatous differentiation. These tumors are histological variants of glioblastoma, displaying a similar genetic profile and dismal prognosis. Up-regulation of PDGFR subfamily of tyrosine kinase members, PDGFR-α and c-Kit, and their intracellular effectors RAS/RAF/MAPK has a crucial role in the cancer development. In addition, signal transduction mediated by activating mutations of *c-Kit* and *PDGFR* can be effectively blocked by specific tyrosine kinase inhibitors, such as Imatinib mesylate. The aim of this study was to characterize the molecular alterations of PDGFR signaling in gliosarcomas. Six cases were analyzed by immunohistochemistry for the expression of PDGFR-α, c-Kit and their ligands PDGF-A and SCF, respectively. The cases were further evaluated for the presence of activating mutations of *PDGFR-α* (exons 12 and 18) and *c-kit* (exons 9, 11, 13, and 17), as well as *B-RAF* (exons 11 and 15). Expression of PDGF-A was found in all cases and co-expression of PDGFR-α was observed in three cases. Four cases showed expression of SCF, and c-Kit was observed only in one case that also expressed SCF. Generally, immunoreaction predominates in the glial component. The mutational analysis of *PDGFR-α* showed the presence of an IVS17-50insT intronic insertion in two cases, one of them also with a 2472C $>$ T silent mutation; this silent mutation was also found in another case. Glioma cell line analysis of IVS17-50insT insertion showed no influence on *PDGFR-α* gene splicing. No mutations were detected in *c-kit* and *B-RAF* oncogenes. Our results indicate that activating mutations of *PDGFR-α*, *c-kit* and *B-RAF* are absent in gliosarcomas. Nevertheless, the presence of a PDGFR-a/PDGFA and c-Kit/SCF autocrine/paracrine stimulation loop in a proportion of cases, supports the potential role of specific tyrosine kinase inhibitors in the treatment of gliosarcomas.

Keywords: Gliosarcoma, PDGFR-α, PDGF-A, c-Kit, SCF, BRAF

1. Introduction

Gliosarcoma is a rare tumor of the central nervous system (CNS), characterized by the presence of a biphasic tissue pattern with alternating areas of malignant gliomatous and sarcomatous differentiation [36]. Gliosarcoma is a variant of glioblastoma, World Health Organization (WHO) grade IV, with less than 1 year median survival despite all treatment modalities [36]. Gliosarcomas disclose genetic profile similar to glioblastomas, with a high frequency of *PTEN* mutations, *p16* homozygous deletions, and also *TP53* mutations, but for the absence or low frequency of *EGFR* overexpression/amplification [1,32, 36,38]. The histogenesis of gliosarcomas has been a matter of controversy. Recently, several studies showed the presence of identical genetic alterations in both (glial and mesenchymal) components, supporting the concept of their monoclonal origin [1,6,32,38].

Receptor protein tyrosine kinases (RTKs) are central regulators of growth signaling and their con-

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stitutive activation, through genetic alterations, has been related with the oncogenesis of several neoplasms, including glioblastomas [44]. Platelet-derived growth factor receptor alpha (PDGFR-α) and c-Kit belong to the PDGFR subfamily or class III RTKs, which also includes PDGFRβ, FMS-related tyrosine kinase 3 (FLT3), and colony-stimulating factor I receptor (CSF1R) [5]. Both *PDGFR*-α and *c-kit* are located on chromosome 4p12 and have functional and structural homologies [18]. This PDGFR subfamily is characterized by an extracellular region with five immunoglobulin-like domains, a transmembrane region and an intracellular region with a regulatory juxtamembrane domain, and a catalytic tyrosine kinase domain. The binding of cognate ligands to PDGFR induces dimerization of the receptor leading to autophosphorylation of tyrosine residues and stimulation of kinase activity, with consequent recruitment of downstream intracellular cascades RAS/RAF/MAPK, PI3K/AKT and STATs, that regulate cell proliferation, differentiation, migration and survival [27]. The development of Imatinib mesylate (Gleevec®, Novartis), a selective inhibitor of tyrosine kinases, including c-abl, c-kit and PDGFR, highlights the implications of these receptors not only in the oncogenic mechanisms but also in the treatment of patients with tumors harboring alterations of these tyrosine kinases [8]. Imatinib is highly effective in the treatment of gastrointestinal stromal tumors (GISTs) with activating mutations of *c-kit*, mainly in exon 11, and is also effective in GISTs with alternative activating mutations of *PDGFR*-α [22, 23].

PDGFR-α and its best-characterized ligand (PDGF-A) are major proliferation factors for mesenchymal, smooth muscle and glial cells, and play an important role in normal development, especially of the CNS [39]. In glioblastomas, the overexpression of *PDGFR*-α and PDGF-A is consistent with an oncogenic autocrine loop [17,19,24,35,48]. Amplification of *PDGFR*-α has been considered an infrequent event, observed in approximately 10% of glioblastomas [48]. However, a recent study reports a frequency of 29% of glioblastomas with *PDGFR*-α amplification [28]. Expression of c-kit and its ligand, stem cell factor (SCF), is important for hematopoiesis, gametogenesis, melanogenesis and development of interstitial cells of Cajal [2]. Overexpression of c-kit/SCF is thought to play a role in neoplasms either through mutations of *c-kit* (e.g. GISTs) or by autocrine/paracrine growth stimulation (e.g. small cell lung cancer) [18]. Expression of both c-kit/SCF in glioma cell lines has been reported, but so far, there are no reports in human gliomas [20, 42]. The few studies concerning c-kit in glioblastomas, showed absence or low frequency of c-kit overexpression and absence of *c-kit* mutations [28,33,41,46].

Deregulation of cell proliferation in neoplasms can also be accomplished by up-regulation of RAS/RAF/MAPK intracellular signaling pathway [16]. Mutations of *RAS* oncogene are found in around 30% of human tumors [16]; however, *RAS* alterations have been rarely identified in gliomas [7,13]. Recently, our group and others reported the presence of *B-RAF* activating mutations in a small fraction (3–11%) of glioblastomas [3, 13,29]. B-RAF is a member of the RAF family of serine/threonine cytoplasmatic kinases. The primary consequence of *B-RAF* mutation is constitutive kinase activation, with consequent stimulation of downstream targets, independently of RTK and RAS status [45].

In the present work we intended to evaluate the role of PDGFR-α/PDGF-A, c-kit/SCF and B-RAF, in the pathogenesis of gliosarcomas. We performed the evaluation of PDGFR-α, PDGF-A, c-Kit, and SCF immunohistochemical expression in the gliomatous and sarcomatous components of 6 *bona-fide* gliosarcomas. In addition, we studied the molecular alterations of hotspot regions of *c-kit* (exons 9, 11, 13 and 17), *PDGFR*-α (exons 12 and 18) and *B-RAF* (exons 11 and 15) oncogenes, in order to clarify the potential use of kinase inhibitor-based therapies in gliosarcomas.

2. Material and methods

2.1. Patients and tumor samples

Six formalin-fixed, paraffin-embedded cases of gliosarcoma were retrieved from the pathology archives of the Department of Pathology, Hospital S. João, Porto, Portugal. All cases have been previously characterized [38]. The clinical pathologic information was obtained from the surgical pathologic reports: two men and four women with a mean age of 62 years (age range of 55–72 years); the most frequent (5/6) cerebral tumor location was the temporal region.

2.2. Cell lines

Human glioma-derived A-172, SW 1088 and U-87 MG tumor cell lines were kindly provided by Prof. M. Mareel, Laboratory of Experimental Cancerology, University Hospital, Ghent, Belgium. Cells were maintained in Eagle's Minimum Essential Medium (MEM)

supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and streptomycin (Gibco, Paisley, UK) in a humidified incubator at 37°C in 5% CO_2 atmosphere. Routinely, the medium was replaced every 2–3 days.

2.3. PDGF-A, PDGFR-α, SCF and c-kit immunohistochemistry

Immunohistochemistry procedure was performed according to streptavidin–biotin–peroxidase complex principle, using rabbit polyclonal anti-human antibodies raised against PDGF-A (dilution 1 : 80; clone N-30, Santa Cruz Biotechnology, Santa Cruz, CA), PDGFR-α (dilution 1 : 175; RB-9027-P, LabVision Corp, Fremont, CA), c-kit (dilution 1 : 50; clone A 4502, DAKO Corporation, Carpinteria, CA), and mouse monoclonal antibody raised against SCF (dilution 1 : 200; clone G-3, Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, deparaffinized and rehydrated sections used to study PDGF-A expression were pre-treated by microwaving in 10 mM citrate buffer (pH 6.0) three times for 5 minutes at 600 W. The sections used in PDGFR-α expression study were submitted to heat-induced epitope antigen retrieval with 10 mM citrate buffer (pH 6.0) for 20 minutes; for SCF immunoexpression analysis the same antigen retrieval procedure was used with EDTA (pH 8.0). No antigen retrieval was used for c-kit immunoexpression. After incubation with primary antibody at room temperature, PDGF-A, and PDGFR-α for 30 minutes or 2 hours for SCF and c-kit, the secondary biotinylated goat anti-polyvalent antibody was applied for 15 minutes followed by incubation with streptavidin–peroxidase complex. The immune reaction was visualized by DAB as a chromogen (Ultravision Detection System Anti-polyvalent, HRP/DAB; Lab Vision, Fremont, CA). Appropriated positive and negative controls were included in each run: GIST samples were included as SCF and c-kit positive controls (tumor cells) as well as cells that were internal c-kit negative controls (smooth muscle cells of muscularis mucosa and muscularis propria). For PDGF-A and PDGFR-α, cutaneous–mucosa transition of the anal region, namely medium dimension vessels with a muscular layer were used as positive controls. For negative controls, primary antibodies were omitted and also primary antibodies were replaced by another irrelevant antibody (CEA, rabbit anti-human, DAKO Corporation, Carpinteria, CA) produced from the same species that produced the primary antibody. All sections were counterstained with Gill-2 haematoxylin.

The immunohistochemical reactions were analyzed in blind fashion protocol, and immunoreactivity was assessed as described previously [10]. Only tumor cell with an intense cell–membrane-bound and/or intracytoplasmatic immunoreactivity were evaluated as positive. A very faint or nuclear staining was not considered positive. Sections were scored semiquantitatively, in both glial and mesenchymal components as follows: (−), 0% of immunoreactive cells; (+), <5% of immunoreactive cells; (++), 5–50% of immunoreactive cells; and (+ + +), >50% of immunoreactive cells. Samples with scores (−) and (+) were considered negative, and those with scores (++) and (+ + +) were considered positive.

2.4. DNA extraction

Tumor DNA was obtained from paraffin sections as previously described [3]. Briefly, serially 10 μm thick unstained sections were obtained, with one 4 μm H&E section for identification and selection of the sarcomatous and gliomatous components, and the selected portions excised using sterile blades. In four cases (GS1, GS2, GS5 and GS6), we were able to dissect both components separately. Paraffin was removed by incubation in xylene, followed by ethanol washing, acetone precipitation, drying and then digestion with approximately 100 μl of lyses buffer (500 mM Tris-HCl [pH 8.5], 1 mM EDTA [pH 8.0]) containing proteinase K (Roche, Mannheim, Germany) at a final concentration of 0.5 mg/ml, at 55°C for 48 hours. After proteinase K denaturation, DNA samples were stored at −20°C for subsequent molecular analysis.

For human cell lines, DNA was extracted according to salting out procedure, with some modifications. Briefly, cells pellets were dissolved in Sodium/EDTA buffer and digested with proteinase K (Roche, Mannheim, Germany) at a final concentration of 0.5 mg/ml, at 55°C. After overnight incubation, saturated NaCl and chloroform were added to the suspension allowing proteins and DNA separation. DNA precipitation was performed with isopropanol. Finally, DNA was washed with 70% ethanol and dissolved in Tris/EDTA buffer. The concentrations were determined by spectrophotometry and aliquot DNA stored at −20°C until use.

2.5. Screening for c-kit, PDGFR-α, and B-RAF mutations

2.5.1. PCR amplification

Screening of c-kit mutations (exons 9, 11, 13 and 17) was carried out by PCR, followed by direct sequencing

and pre-screening of *PDGFR-α* (exons 12 and 18), and *B-RAF* mutations (exons 11 and 15) was done by PCR – single-strand conformational polymorphism (PCR-SSCP) followed by direct DNA sequencing of samples that showed a mobility shift in the PCR-SSCP analysis. Briefly, PCR was carried out with 2 μl of DNA solution, 0.2–0.6 μM of both sense and antisense primers, 0.2 mM of dNTPs, $MgCl_2$ (1.5–1.75 mM), 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 U of Platinum *Taq* polymerase (Invitrogen, Life Technologies Inc., UK) in a final volume of 25 μl. Thirty-seven cycles of denaturation (94°C), annealing (58–60°C) and extension (72°C) for 45 s each were carried out in a thermocycler (BioRad, Hercules, CA, USA). Primer sequences were previously described [3,11,23].

2.5.2. PCR-SSCP

PCR products of *PDGFR-α* and *B-RAF exons* were mixed with an equivalent volume of the denaturing loading buffer (98% formamide, 0.05% xylene cyanol and bromophenol blue). After denaturating at 95°C for 10 minutes and quenched on ice, 15 μl of the mixture were loaded onto 0.8X MDE gel (Cambrex, Rockland, USA). Gels were run at 180 V, 4°C for 16–20 h, silver stained and dried at 80°C for 2 h.

2.5.3. Direct sequencing

The PCR products were first purified using a MicroSpin™ S-400 HR Columns (Amersham Biosciences, Piscataway, NJ, USA). Purified PCR products were submitted to a cycle sequencing reaction using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.6. Detection of alternative RNA splicing by reverse transcriptase–PCR

Total RNA from human glioma-derived cell lines was extracted using the Trizol® (Invitrogen, Life Technologies Inc., UK) method according to the manufacturer's instructions. Briefly, cell pellets were immersed in Trizol and homogenized. Proteins were removed with chloroform and the RNA pellets were washed once with isopropyl alcohol, and once with 75% ethanol. The total RNA pellets were reconstituted in RNase free water (Ambion, Cambridge, UK).

Reverse transcriptase (RT)–PCR was performed according to manufacturer, using a set of primers designed to amplify a region overlapping exon 17 to exon 19: 5′-TATCAAGTTGCCCGAGGAAT-3′ (forward) and 5′-GATCTCCCAGAGCAGAATGC-3′ (reverse). First-strand cDNA synthesis was initiated by incubating 100 ng total RNA with 5 μg/μl oligo(deoxythymidine) primers and 1 mM dNTPs, for 5 min at 65°C. The following reaction mixture, consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 40 U RNaseOUT™ Recombinant RNase Inhibitor and 1 U Superscript™ II RT (Invitrogen, Life Technologies Inc., UK) in 20 μl total volume, was added to each RNA/primer mixture prepared previously and incubated at 42°C for 50 min. The synthesis was terminated by an incubation at 70°C for 15 min and the addition of 2 U *E. coli* RNase H. Amplification of the target cDNA was performed with 2 μl RT cDNA products, 0.2 μM each primer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 U of *Taq* DNA polymerase (Fermentas, Lithuania) in a final volume of 25 μl. Thirty-five cycles of denaturation (94°C, 15 s), annealing (56°C, 30 s) and extension (72°C, 1 min) were carried out in a thermocycler (BioRad, Hercules, CA, USA).

3. Results

Immunohistochemical results of PDGF-A, PDGFR-α, SCF and c-kit expression in gliomatous and sarcomatous components of gliosarcomas are summarized on Table 1. The staining of the positive cases was predominantly cytoplasmatic, without any case with exclusive membranal staining. Controls for both positive and negative immunohistochemistry reaction gave appropriated results. Positive expression of PDGF-A was found in all cases: three with similar immunoexpression in both (glial and mesenchymal) components (Fig. 1A); two with expression in the glial component, and one in the mesenchymal component. Positive co-expression of PDGFR-α (Fig. 1B) and PDGF-A was observed in the glial component of three cases. SCF was positive in the glial component of four cases, one of them also in the mesenchymal component (Fig. 1C). Simultaneous SCF and c-kit positive immunoexpression was identified in both components of one case (GS 4) (Fig. 1D). Overall, PDGF-A, PDGFR-α, SCF and c-kit expression was heterogeneous, but predominate in the glial component of gliosarcomas.

The mutational analysis of *PDGFR-α* revealed the presence of an IVS17-50insT insertion at intron 17 (Fig. 2A) in two gliosarcomas (GS 3 and GS 4), and

Table 1
Immunohistochemistry and mutation features in gliosarcomas

Case	Area	Immunohistochemistry*				Mutational status		
		PDGF-A	PDGFR-α	SCF	c-kit	*PDGFR-α*	*c-kit*	*B-RAF*
GS1	G	++	++	+	−	normal	nd	normal
	S	++	+	−	−	normal	nd	normal
GS2	G	++	++	++	−	normal	nd	normal
	S	++	+	−	−	normal	nd	normal
GS3	G	+	+	+	−	IVS17-50insT		
	S	++	−	−	−	2472C > T	nd	normal
GS4	G	+++	+	++	++	IVS17-50insT	normal	normal
	S	+++	−	++	++			
GS5	G	+++	++	++	−	2472C > T	nd	normal
	S	+	−	+	−	2472C > T	nd	normal
GS6	G	+++	−	++	−	normal	nd	normal
	S	+	−	−	−	normal	nd	normal

G, gliomatous component; S, sarcomatous component; nd, not determined.
*Scores as described in Material and methods.

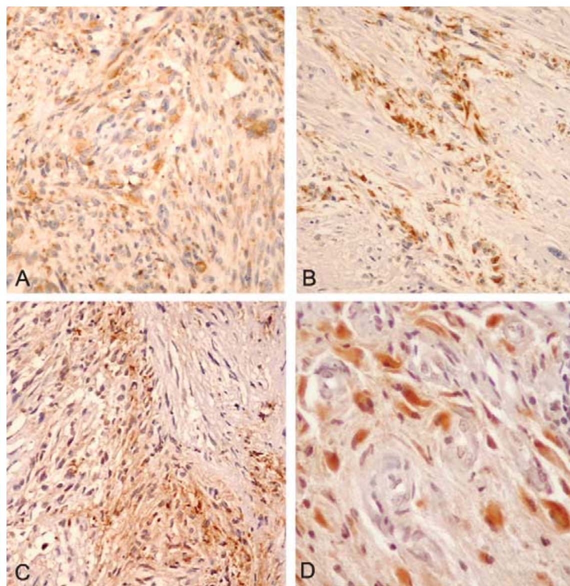

Fig. 1. Immunohistochemical expression of PDGF-A (A); PDGFR-α (B); SCF (C) and c-kit (D) in gliosarcomas (amplification: A–C, 200×; D, 400×).

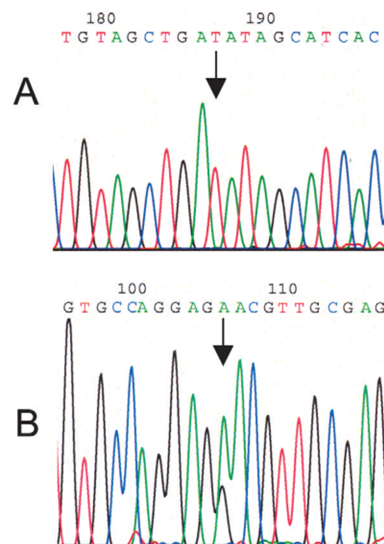

Fig. 2. Sequencing analysis of *PDGFR-α*: (A) insertion of a thymine (arrow) at position minus 50 of intron 17 (sense strand); and (B) cytosine to thymine substitution (arrow) at nucleotide 2472, resulting in silent mutation (antisense strand).

a base substitution 2472C > T (Fig. 2B), leading to a silent mutation Val824Val on exon 18 in two cases (Table 1). One case (GS 3) exhibited both aforementioned alterations (Table 1). There was no correlation between mutation status and PDGFR-α expression. The *c-kit* mutation analysis of the gliosarcoma that exhibit c-kit overexpression (GS 4) did not reveal any mutation. No mutations were identified on *B-RAF* gene (Table 1).

The mutational analysis of *B-RAF*, *c-kit* and *PDGFR-α* genes in glioma-derived cell lines showed the absence of *B-RAF* and *c-kit* mutations and the presence of the IVS17-50insT insertion at intron 17 of *PDGFR-α* in all cell lines. SW 1088 cell line was heterozygous for the inserted thymine, whereas A-172 and U-87 MG cell lines were homozygous for the insertion (data not shown). Although the intronic position of the insertion suggests no biological consequence, we investigated the possibility of splicing deregulation introduced by

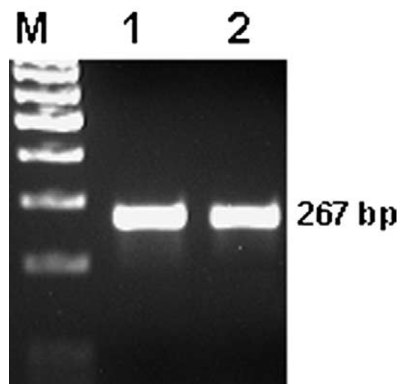

Fig. 3. Agarose gel showing no differences in fragment length between genotypes. M, molecular size marker; lane 1, SW 1088 cell line heterozygous for the inserted intronic thymine; lane 2, A-172 cell line homozygous for the inserted intronic thymine.

the IVS17-50insT insertion. cDNA analysis of amplified exon 17 to 19 was evaluated either in heterozygous and homozygous cell lines and no differences in fragment length were detected (Fig. 3).

4. Discussion

Imatinib was initially designed to inhibit the BCR/ABL fusion kinase protein in chronic myeloid leukemia and was later discovered that it is also effective against GISTs harboring *c-kit* and *PDGFR-*α mutations, as well as dermatofibrosarcoma protuberans with translocation involving *PDGFB* and chronic myelomonocytic leukemia with translocation involving *PDGFR-*β gene [14,34,37,43]. The molecular characterization of PDGFR subfamily in gliosarcomas can be important for the identification of patients that could benefit from Imatinib based therapy. The development of strategies that block PDGFR subfamily downstream signaling components, such as B-RAF, warrant their analysis in gliosarcomas [16].

The present study analyzed the immunoexpression of PDGFR-α, PDGF-A, c-kit and SCF, in addition to the presence of *PDGFR-*α, *c-kit* and *B-RAF* activating mutations in both components of 6 well-characterized gliosarcomas. Immunohistochemical reactions showed the expression of PDGF-A mainly in the gliomatous component, and half of the cases also expressed its receptor PDGFR-α, suggesting the existence of an autocrine/paracrine stimulation loop. These results are in agreement with the well-documented description of both receptor and ligand overexpression in glioblastomas [48]. Overexpression of PDGF-A was already reported to be present in the glial component of gliosarcomas [12]. The mutational analysis of *PDGFR-*α showed the presence of a silent mutation in exon 18, previously identified by our group in breast carcinomas [9], and a base insertion on intron 17. This later alteration was also found in breast phyllodes tumors and in normal population, and is consistent with a polymorphism [10]. In addition, our cDNA analysis showed that *PDGFR-*α RNA splicing is not affected by this insertion. None of the gliosarcomas studied disclosed *PDGFR-*α mutations, at variance with reports on GISTs, and similar with reports on glioblastomas [21,41]. Therefore, other molecular mechanisms should be responsible for PDGFR-α overexpression in gliosarcomas. Noteworthy, a small fraction of gliosarcomas (3%) were found to display *PDGFR-*α gene amplification [1].

In our series, c-kit overexpression was identified in one gliosarcoma. Despite the variety of c-kit antibodies and antigen retrieval methodologies, the antibody and immunohistochemical method used in our study are considered as the most sensitive and specific for c-kit expression evaluation [26,47]. The mutational analysis of this case did not reveal any activating mutation. These results are in agreement with recent studies of c-kit in a wide range of neoplasms, including glioblastomas, where intense immunostaining was rare and none exhibited *c-kit* mutations [28,41, 46]. Recently, Joensuu et al. found a significant proportion of glioblastomas (47%) with *c-kit* gene amplification [28]. We found SCF immunoexpression in half of the cases, and co-expression of c-kit in one gliosarcoma. Simultaneous expression of c-kit and SCF has been reported in several tumors, suggesting the role of a c-kit/SCF autocrine/paracrine activation loop in tumorigenesis [4,15,25,40]. Furthermore, studies of uveal melanoma and small cell lung cancer cell lines exhibiting c-kit/SCF autocrine loop, even in the absence of activating *c-kit* mutations, showed cell growth inhibition upon Imatinib exposure [30,31].

The absence of activating mutations of *B-RAF* in gliosarcomas, supports its minor role in gliomagenesis [3,29].

In summary, our results on PDGFR subfamily alterations of six gliosarcomas are similar to those reported in glioblastomas. *PDGFR-*α*, c-kit and B-RAF* activating mutations were not found in gliosarcomas. Further studies are needed to determine whether gene amplification mechanism is responsible for PDGFR-α and c-kit overexpression in gliosarcomas. The presence of a PDGFR-α/*PDGFA* and c-Kit/SCF co-expression, sup-

ports the role of their autocrine/paracrine stimulation loop in gliosarcoma development, and raises the possible relevance of specific tyrosine kinase inhibitors in the treatment of gliosarcomas.

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