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Lymphangiogenic VEGF-C and VEGFR-3 expression in genetically characterised gastrointestinal stromal tumours

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Summary. This study aimed to assess the distribution of VEGF-C and VEGFR-3 expression in gastrointestinal stromal tumours (GISTs), and to analyse the value of lymphatic vessel density (LVD) in a tumour that is believed to preferentially metastasize through blood vessel conduits. A panel of immunohistochemical antibodies was used to evaluate 51 cases of genetically characterised GISTs: VEGF-C, VEGFR-3, D2-40 (for LVD assessment) and CD31 (for blood vessel density – BDV – assessment). The results were correlated with the clinical-pathological data. The large majority of cases (86.2%; 44/51) showed a mutation of the *KIT* gene, most of them (72.5%; 37/51) revealing mutations in exon 11. VEGFR-3 was predominantly expressed in *KIT* mutated GISTs (p=0.019). High LVD was correlated with the absence of metastasis (p=0.010) and high BVD showed a positive correlation with the occurrence of metastasis (p=0.049). The strong expression of VEGF-C and VEGFR-3 in GIST's cells was not correlated with the clinical parameters of aggressiveness, nor with high LVD.

Key words: Gastrointestinal stromal tumours, KIT, D2-40, VEGF-C, VEGFR-3

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Introduction

Gastrointestinal stromal tumours (GISTs) have a low frequency that ranges from 10 to 20 cases per million yearly, and the estimated prevalence is 129 per million (Blackstein et al., 2006; Corless and Heinrich, 2008; Stamatakos et al., 2009). They are rarely found in patients younger than 40 years of age, although there have been some reports from paediatric populations (Chourmouzi et al., 2009). Cajal's cells are recognised as the precursor cells of GISTs, but presently, there is some evidence that these tumours may also originate from multipotential mesenchymal stem cells (Corless and Heinrich, 2008; Stamatakos et al., 2009).

Morphologically, GISTs are generally composed of spindle cells; epithelioid mixed patterns are also identified (Miettinen et al., 2005; Zhang et al., 2010). CD117 positive reaction is used to characterise GISTs and rule out other tumours with similar morphology, such as sarcomas, mesotheliomas, melanomas and poorly differentiated carcinomas (Stamatakos et al., 2009). A commercial immunohistochemical antibody is used to identify specific epitopes of GISTs (Miettinen et al., 2006). CD117 protein is the product of *c-kit* (*KIT*) proto-oncogene, which is located in the 4q12 chromosome region (Mushtaq et al., 2009). GISTs frequently exhibit oncogenic mutations in the *KIT* gene and, less commonly, in the *PDGFRA* gene (Gomes et al., 2008; Martinho et al., 2009), which is a related gene that also encodes a type III tyrosine-kinase receptor. Likewise, *PDGFRA* mutation is found in circa 5% of

GISTs and commonly originates in exon 18 (4-7%) and exon 12 (2-6%) (Badalamenti et al., 2007; Gajiwala et al., 2009; Blay, 2010). Nearly 10-15% of cases lack mutations in both *KIT* and *PDGFRA* (wild-type GISTs) (Agaimy et al., 2009; Gronchi et al., 2010), and a subset of GISTs displays *KIT* and *PDGFRA* wild-type activating mutations of the oncogene *BRAF* (Agaimy et al., 2009; Martinho et al., 2009).

Patients with exon 11 mutations have a better response rate to imatinib than patients with exon 9 mutations or wild-type *KIT* (Badalamenti et al., 2007). The role of the mutational status in patients' response to imatinib treatment is also observed by the resistance to imatinib in association with gain of secondary *KIT* mutations, mainly in exons 13 and 17, hampering the therapeutic efficacy. Sunitinib and, more recently, nilotinib, have been used to replace imatinib in the cases of imatinib-resistant mutants (Gajiwala et al., 2009; Gramza et al., 2009; Blay, 2010).

Lymphatic and blood vessel invasion by cancer cells are directly related with metastasis occurrence, and the evaluation of vascular density is a useful parameter to predict poor prognosis (Tammela and Alitalo, 2010). Augmented lymphatic vessel density (LVD) is usually associated with an aggressive behavior in the majority of cancers that frequently use lymphatics, due to the fragile structure of these vessels (Afonso et al., 2009). GISTs preferentially invade and metastasize through blood vessel conduits (Shinomura et al., 2005), although recently the expression of lymphatic markers, such as podoplanin / D2-40 (Kimura and Kimura, 2005; Yu et al., 2007; Agaimy and Carney, 2010), was reported in GISTs. Podoplanin is a transmembrane glycoprotein expressed in a variety of human cell types, including lymphatic endothelium. D2-40 is a commercially available monoclonal antibody directed against human podoplanin (Kalof and Cooper, 2009). GISTs' lymph nodal invasion has been reported in children and young adults with the Carney triad (Agaimy and Carney, 2010). Specific reasons for the high rate of tumoral lymphatic spread in these paediatric GISTs patients are unknown.

GISTs treatment was improved by the anti-tyrosine kinase and anti-angiogenic drugs (McAuliffe et al., 2007), but no evidence of lymphangiogenic associated-proteins was reported in these tumours.

The goal of this study was to characterise, in molecularly characterized GISTs, the expressions of the most potent lymphangiogenic protein and its receptor, vascular endothelial growth factor (VEGF) -C and VEGFR-3 (respectively), to assess tumoral LVD and blood vessel density (BVD), and to correlate these parameters with the clinical-pathological data.

Materials and methods

All of the patients with clinically diagnosed GISTs were examined and surgically treated at Barretos Cancer Hospital, State of São Paulo, Brazil, between 2000 and 2008. Clinical-pathological data were retrospectively retrieved from the files of the hospital medical records. Cases with history of previous cancer treatment were excluded. The general information included tumoral necrosis, ascites, distant metastases, time elapsed until recurrence, survival rates and cause of death (when death occurred). Tumours were classified in accordance with the WHO criteria and the parameters analysed in each case included age, gender, primary tumour site, tumour size, histological type, mitotic index and risk group (Miettinen et al., 2000; Fletcher et al., 2002).

Follow-up data were available for all patients, collected through direct interviews with patients or their relatives and by reviewing the patients' records.

Tumoral persistence was defined as the presence of a palpable mass seen at clinical follow-ups within the first three months. Recurrence was defined as tumour detection at a clinical follow-up after three months of tumour absence.

DNA isolation

Tumoral areas containing at least 85% of tumour tissue were macro-dissected into a microfuge tube using a sterile needle (Neolus, 25G, 0.5 mm) and DNA isolation was performed using Qiagen's QIAamp® DNA Micro Kit, as formerly described (Gomes et al., 2008; Martinho et al., 2009).

Mutation analysis on KIT, PDGFRA and BRAF genes

Evaluation of the hotspot regions affecting *KIT* (exons 9, 11, 13 and 17), *PDGFRA* (exons 12, 14 and 18) and *BRAF* (exons 11 and 15) genes was conducted by means of PCR followed by direct sequencing for exons 9 and 11 of the *KIT* gene, and by means of PCR-SSCP for the remaining regions, as previously described (Gomes et al., 2008; Martinho et al., 2009). Samples showing a mobility shift in the PCR-SSCP analysis that differed from the normal pattern were directly sequenced (Stabvida, Investigation and Services in Biological Sciences Lda, Oeiras, Portugal). Additionally, analyses of cryptic *KIT* exon 11 duplications were made using specific PCR, followed by direct sequencing, as previously reported (Martinho et al., 2009). All positive cases were confirmed twice with a new and independent PCR amplification, followed by direct sequencing.

Immunohistochemical reactions

CD117 immunohistochemistry

Immunohistochemistry protocol was as previously described (Gomes et al., 2008; Martinho et al., 2009). Briefly, the reaction was performed using the streptavidin-biotin-peroxidase complex, and a specific antibody raised against CD117 (dilution 1:500, clone A 4502, DAKO, Carpinteria, Denmark). Sections were semi-quantitatively scored as follows: (-), 0% presence of immunoreactive cells; (+), <5% presence of

immunoreactive cells; (++), 5-50% presence of immunoreactive cells; and (+++), >50% presence of immunoreactive cells. Samples with scores (-) and (+) were considered negative, and those with scores (++) and (+++) were considered positive.

VEGF-C, VEGFR-3, D2-40 and CD31 immunohistochemistry

Table 1 was adopted from Afonso et al. (2009) and summarizes the principal information of the immunohistochemical reaction for VEGF-C, VEGFR-3, D2-40 and CD31 antibodies. For all procedures, the positive reactions were highlighted with diaminobenzidine and substrate chromogen system, DakoCytomation® (DAKO Corporation, Carpinteria, CA, USA) incubated for 10 minutes at room temperature. The positive expressions of VEGF-C and VEGFR-3 were semi-quantitatively assessed using ?200 amplification. The positive reactions were assessed in hotspot areas where GISTs cells and proliferating vascular structures were present and stained. The following grading system was used: negative (-), absence of expression; slightly positive staining (+), expression in up to 10% of cells; moderately positive (++), expression in over 10% up to 50% of cells; strongly positive (+++), expression in over 50% of cells.

VEGF-C and VEGFR-3 strongly stained the cytoplasm of malignant cells and infrequently stained the endothelial cells. For this reason, only the positive staining of malignant cells was considered for evaluation.

Evaluation of vascular densities

The immunohistochemical evaluation of positive reactions for D2-40 (for LVD assessment) and CD31 (for BVD assessment) was performed as postulated by Weidner et al. (1991), with slight modifications. The number of vessels was quantified at x 200 magnification. A median of 10 hotspot fields was defined as vessel density. The examination of each hotspot corresponds to a number of vessels confined to an area of 0.15 mm^2. Both D2-40 and CD31 immunohistochemical positive reactions were independently counted in blood and lymphatic vessels from intratumoral and peritumoral areas. Intratumoral area was defined as the stromal tissue within two or more neoplastic aggregates, and peritumoral area was defined as the stroma tissue surrounding this neoplastic mass (Afonso et al., 2009). D2-40 positivity in tumor cells was classified as negative (negative or weak immunoreaction) and positive (moderate to strong immunoreaction).

Statistical analysis

The available clinical-pathological and immunohistochemistry data were analysed by means of the SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). Associations between categorical variables were investigated using Fisher's exact test. For LVD and BVD analysis, the cut-off values that were better correlated with tumour aggressiveness were determined by ROC (receiver operating characteristic curves) analysis. Cumulative survival probabilities were calculated using the Kaplan-Meier method. Differences between survival rates were tested by the log-rank test. P-values lower than 0.05 were considered to be significant.

Results

Twenty six male (51.0%) and 25 female (49.0%) patients were analysed. Ages were divided into ≤55

Table 1. Summary of the methodological protocols used for VEGF-C, VEGFR-3, D2-40 and CD31 immunohistochemical procedures.

Antibodies	VEGF-C	VEGFR-3	D2-40	CD31
Antigen Recovery	600 W, 15 min	700 W, 15 min	600 W, 15 min	600 W, 10 min
Primary Antibody	Clone Z-CVF3, Zymed® Laboratories (CA, USA) Dilution: 1:70 Incubation: ON, 4ºC	Clone C234, Santa Cruz Biotechnology®, Inc. (Santa Cruz, CA, USA) Dilution: 1:200 Incubation: 60 min, RT	Clone D2-40, DakoCytomation® (DAKO Corporation, Carpinteria, CA, USA) Dilution: 1:100 Incubation: ON, 4ºC	Clone JC70A, DakoCytomation® (Denmark) Dilution: 1:100 Incubation: 60 min, RT
Secondary Antibody	RTU Biotinylated Universal Antibody, Vector® (Vector Laboratories Inc.,CA, USA) Incubation: 30 min, RT	Goat Anti-Polyvalent, Labvision® (LabVision Corporation, Fremont, CA) Incubation: 10 min, RT	RTU Biotinylated Universal Antibody, Vector® (Vector Laboratories Inc.,CA, USA) Incubation: 30 min, RT	Goat Anti-Polyvalent, Labvision® (LabVision Corporation, Fremont, CA) Incubation: 10 min, RT
Streptavidin Peroxidase / Avidin-Biotin Complex	Vectastain RTU Elite ABC reagent, Vector® Incubation: 45 min, 37ºC	Large volume Streptavidin Peroxidase, Labvision® Incubation: 10 min, RT	Vectastain RTU Elite ABC reagent, Vector® Incubation: 45 min, 37ºC	Large volume Streptavidin Peroxidase, Labvision® Incubation: 10 min, RT
Positive Control	Colon carcinoma	Breast carcinoma	Tonsil	Breast carcinoma

W: watt; min: minutes; ON: overnight; RT: room temperature; RTU: ready-to-use; ABC: avidin-biotin complex.

years old (24, or 47.1%) or >55 years old (27, or 52.9%). The primary sites of GISTs were stomach (27, 52.9%), small intestine (11, 21.6%), colon and rectum (7, 13.7%), mesentery (2, 3.9%), retroperitoneum (3, 5.9%) and rectovaginal area (1, 2.0%).

The term metastasis was used to categorise distant neoplastic invasion. No lymph node invasion was observed in this casuistic. Actually, in GIST surgery, lymphadenectomy is not usually performed. Distant liver metastasis was found in 16 cases, peritoneum invasion was found in 13 patients and lung invasion was found in just one case.

Table 2. Molecular analysis of gastrointestinal stromal tumors.

Case	*KIT*	*PDGFRA*	*BRAF*
1	Exon 11 (p.Trp557_Val559delinsCys)	ND	ND
2	Exon 11 (p.Trp557_Val559delinsCys)	ND	ND
3	Exon 11 (p.Trp557_Lys558del)	ND	ND
4	Exon 11 (p.Val560Glu)	ND	ND
5	Exon 11 (p.Trp557_Lys558del)	ND	ND
6	Exon 11 (p.Leu576Pro)	ND	ND
7	Exon 11 (p.Trp557_Val559delinsCys)	ND	ND
8	Exon 11 (p.Val559Asp)	ND	ND
9	Exon 11 (p.Tyr553_Lys558del)	ND	ND
10	Exon 11 (p.Trp557_Lys558del)	ND	ND
11	Exon 11 (p.Gln556_Glu561delinsPro)	ND	ND
12	Exon 11 (p.Pro585Thr)	ND	ND
13	Exon 11 (p.Val559Asp)	ND	ND
14	Exon 11 (p.Val559Asp)	ND	ND
15	Exon 11 p.Trp557_Glu561del)	ND	ND
16	Exon 11 (p.Glu554_Ile571del)	ND	ND
17	Exon 11 (p.Val560Asp)	ND	ND
18	Exon 11 (p.Asp579Asn)	ND	ND
19	Exon 11 (p.Lys558delinsAsnPro)	ND	ND
20	Exon 11 (p.Lys550_Trp557delinsIleLeu)	ND	ND
21	Exon 11 (p.Trp568_Gln575delinsSer)	ND	ND
22	Exon 11 (p.Trp557_Lys558del)	ND	ND
23	Exon 11 (p.Val559Asp)	ND	ND
24	Exon 11 (p.Trp557_Val559delinsCys)	ND	ND
25	Exon 11 (p.Trp557_Val559delinsCys)	ND	ND
26	Exon 11 (p.Trp557_Lys558del)	ND	ND
27	Exon 11 (p.Trp557Arg)	ND	ND
28	Exon 11 (p.Pro573_Arg586dup)	ND	ND
29	Exon 11 (p.Leu576Pro)	ND	ND
30	Exon 11 p.Trp557_Lys558del)	ND	ND
31	Exon 11 (p.Trp557_Lys558del)	ND	ND
32	Exon 11 (p.Trp557_Lys558del)	ND	ND
33	Exon 11 (p.Lys558_Val559delinsAsn)	ND	ND
34	Exon 11 (p.Trp557del)	ND	ND
35	Exon 9 (p.Ala502_Tyr503dup; p.Val473Met)	ND	ND
36	Exon 9 (p.Ala502_Tyr503dup)	ND	ND
37	Exon 9 (p.Ala502_Tyr503dup)	ND	ND
38	Exon 9 (p.Ala502_Tyr503dup)	ND	ND
39	Exon 9 (p.Ala502_Tyr503dup)	ND	ND
40	Exon 17 (p.Asp820Tyr)	ND	ND
41	Exon 17 (p.Asp820Tyr)	ND	ND
42	Wild Type	Exon 18 (p.Asp842Val; p.Asp842Glu)	ND
43	Wild Type	Exon 18 (p.Asp842Val)	ND
44	Wild Type	Exon 18 (p.Asp842Val)	ND
45	Wild Type	Exon 18 (p.Asp842_Ile843delinsVal)	ND
46	Wild Type	Wild Type	Wild Type
47	Wild Type	Wild Type	Wild Type
48	Wild Type	Wild Type	Wild Type
49	Wild Type	Wild Type	Wild Type
50	Wild Type	Wild Type	Wild Type
51	Wild Type	Wild Type	Wild Type

ND: not done

Mutation analysis of KIT, PDGFRA and BRAF oncogenes

The mutational status of GISTs showed *KIT* gene alterations in 86.2% (44/51) of the cases, most of them (72.5%, 37/51) revealing mutations in exon 11, followed by mutations in exon 9 (9.8%, 5/51) and exon 17 (3.9%, 2/51) (Table 2). PDGFRA mutations were recognized in 7.8% (4/51) of the cases, exclusively observed in exon 18 (Table 2). None of the cases showed mutations of the *BRAF* oncogenes; 11.0% of the cases (6/51) were wild-type (Table 2).

VEGF-C and VEGFR-3 expression

Table 3 shows the distribution of VEGF-C (Fig. 1) and VEGFR-3 (Fig. 2) expression in malignant cell according to the mutational status of GISTs. The proteins' positive reactions were constantly strong and predominantly expressed in malignant cells' cytoplasm. Essentially, VEGF-C expression did not exhibit significant differences among the molecular characterization of GISTs. Conversely, VEGFR-3 appeared to be more importantly expressed in *KIT* and *PDGFRA* mutated than in non-mutated cases (p=0.019). Expression of VEGF-C and VEGFR-3 in lymphatic vessels was not quantified due to the scarcity of positive reactions. Additionally, Table 4 depicts the correlation between both VEGF-C and VEGFR-3 expression, and clinical-pathological parameters. No significant

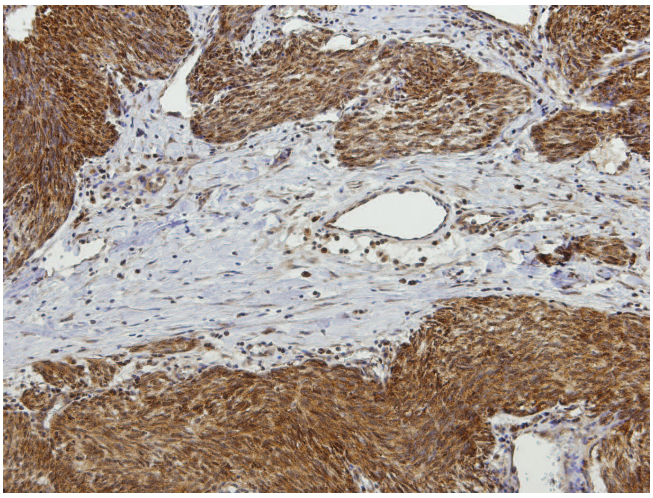

Fig. 1. Strong VEGF-C positive reaction in spindled GIST malignant cells. x 200

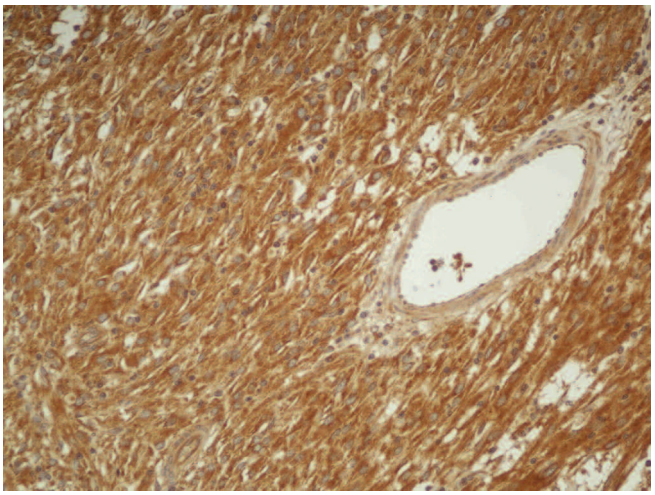

Fig. 2. Strong VEGFR-3 positive reaction in GIST malignant cells. x 200

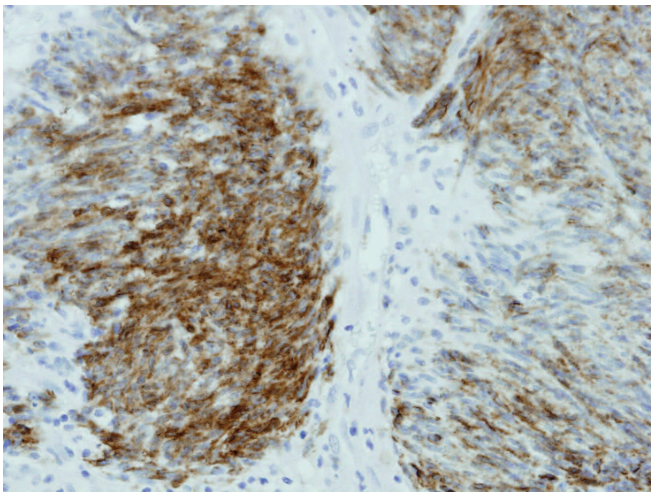

Fig. 3. D2-40 positive reaction in GIST malignant cells. x 200

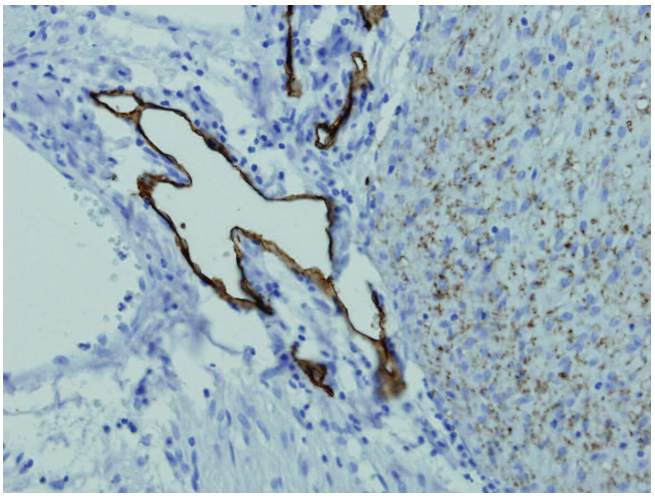

Fig. 4. Lymphatic vessels density in GIST. Endothelial cells from lymphatic vessels are stained with D2-40 antibody. x 200

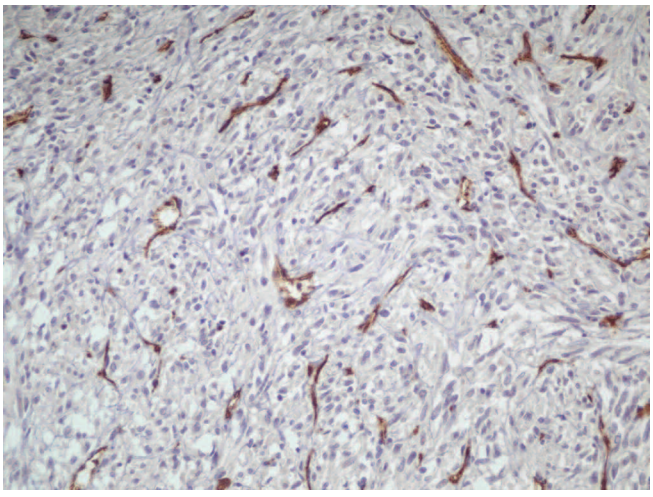

Fig. 5. Endothelial cells from blood vessels stained with CD31 antibody. x 200

Table 3. Association between GIST genetic status and VEGF-C and VEGFR-3 immunohistochemical positive reactions.

	VEGF-C			VEGFR-3		
	Total	Positive (%)	p*	Total	Positive (%)	p*
GIST †						
With KIT/PDGFRA mutation	41	22 (53.7)	0.476	39	39 (100)	0.093
Wild-type	1	0 (0.0)		4	3 (75.0)	
Mutation						
Wild-type	1	0 (0.0)	0.391	4	3 (75.0)	0.019
KIT exon 9	5	2 (40.0)		5	5 (100)	
KIT exon 11	30	15 (50.0)		29	29 (100)	
KIT exon 17	2	2 (100)		1	1 (100)	
PDGFRA exon 18	4	3 (75.0)		4	4 (100)	

*: $p < 0.05$; †: Fisher exact test.

association was observed, although VEGFR-3 expression showed a tendency to be associated with higher risk of malignancy (considering Fletcher classification) (p=0.059).

Lymphatic and blood vessel density

D2-40 immunoreactivity was observed in both malignant (Fig. 3) and endothelial cells from lymphatic vessels (Fig. 4). CD31 immunoreactivity was only assessed in blood vessels (Fig. 5).

The cut-off values (determined by ROC analysis) for LVD (assessed by D2-40 immunoreactivity) and BVD (assessed by CD31 immunoreactivity) were 3.2 and 7.4, respectively (considering intratumoral and peritumoral vascular counting). Peritumoral D2-40 positive reactions

Table 4. Association between GIST clinical-pathological parameters and VEGF-C and VEGFR-3 immunohistochemical positive reactions.

	VEGF-C			VEGFR-3		
	Total	Positive (%)	p*	Total	Positive (%)	p*
Ascites†						
Presence	9	5 (55.6)	1.000	9	9 (100)	1.000
Absence	33	17 (51.5)		34	33 (97.1)	
Fletcher Risk of Malignancy						
Very Low/ Low	6	3 (50.0)	0.927	6	5 (83.3)	0.059
Moderate	12	7 (58.3)		12	12 (100)	
High	21	11 (52.4)		21	21 (100)	
Loco-regional Recidive §†						
Presence	7	5 (71.4)	0.407	6	6 (100)	1.000
Absence	18	9 (50.0)		20	19 (95.0)	
Cancer Persistence						
Yes	17	8 (47.1)	0.569	17	17 (100)	1.000
No	25	14 (56.0)		26	25 (96.2)	
Metastasis						
Presence	19	11 (57.9)	0.516	20	20 (100)	1.000
Absence	23	11 (47.8)		23	22 (95.7)	
Tumor size (cm)						
<10	21	11 (52.4)	0.882	23	22 (95.7)	1.000
≥10	18	9 (50.0)		17	17 (100)	
Mitotic index (per field)†						
≤5	28	15 (53.6)	0.956	28	27 (96.4)	1.000
>5	11	6 (54.5)		11	11 (100)	
Necrosis†						
Presence	30	16 (53.3)	0.845	30	29 (96.7)	1.000
Absence	12	6 (50.0)		13	13 (100)	

*: p<0.05; †: Fisher exact test; §: the cases with no persistent disease were also analyzed.

Table 5. Association between GIST mutation status and D2-40 (for lymphatic vessel density assessment) and CD31 (for blood vessel density assessment) immunohistochemical positive reactions.

	Lymphatic Vessels density (D2-40)			Blood Vessels Density (CD31)		
	Total	>3.2 (%)	p*	Total	>7.4 (%)	p*
GIST †						
With KIT/PDGFRA mutation	40	21 (52.5)	0.607	40	26 (65.0)	0.651
Wild-type	5	1 (33.3)		5	4 (80.0)	
Mutation						
Wild-type	5	1 (33.3)	0.936	5	4 (80.0)	0.398
KIT exon 9	4	2 (40.0)		4	3 (75.0)	
KIT exon 11	31	16 (55.2)		31	18 (58.1)	
KIT exon 17	1	1 (50.0)		1	1 (100)	
PDGFRA exon 18	4	2 (50.0)		4	4 (100)	

*: p<0.05; †: Fisher exact test.

Table 6. Association between GIST clinical-pathological parameters and D2-40 (for lymphatic vessel density assessment) and CD31 (for blood vessel density assessment) immunohistochemical positive reactions.

	Lymphatic Vessels density (D2-40)			Blood Vessels Density (CD31)		
	Total	>3.2 (%)	p*	Total	>7.4 (%)	p*
Ascites†						
Presence	9	4 (44.4)	0.454	9	5 (55.6)	0.454
Absence	34	18 (52.9)		36	25 (69.4)	
Fletcher Risk of Malignancy						
Very Low/ Low	6	5 (83.3)	0.200	9	6 (66.7)	0.117
Moderate	12	7 (58.3)		11	11 (91.7)	
High	21	9 (42.9)		21	12 (57.1)	
Loco-regional Recidive §†						
Presence	7	5 (71.4)	1.000	7	6 (85.7)	1.000
Absence	19	12 (63.2)		22	18 (81.8)	
Cancer Persistence						
Yes	17	-- (--)	--	18	11 (61.1)	0.519
No	26	-- (--)		27	19 (70.4)	
Metastasis						
Presence	20	6 (30.0)	0.010	24	20 (83.3)	0.049
Absence	23	16 (69.6)		18	10 (55.6)	
Tumor size (cm)						
<10	23	13 (56.5)	0.337	28	19 (67.9)	1.000
≥10	17	7 (41.2)		12	8 (66.7)	
Mitotic index (per field)†						
≤5	27	15 (55.6)	0.748	14	11 (78.6)	0.321
>5	12	6 (50.0)		31	19 (61.3)	
Necrosis†						
Presence	13	8 (61.5)	0.370	9	5 (55.6)	0.454
Absence	30	14 (46.7)		36	25 (69.4)	

*: p< 0.05; †: Fisher exact test; §: the cases with no persistent disease were also analyzed.

were rarely observed. High LVD (>3.2) did not correlate with the molecular genetic status (Table 5). Regarding the clinical-pathological parameters, high LVD was correlated with the absence of metastasis (p=0.010) (Table 6); in contrast, BVD was associated with the occurrence of GISTs' metastases (p=0.049).

Discussion

The results herein reported corroborate, in part, the statement that GISTs' metastasis occurs preferentially through blood vessels (Miettinen et al., 2005). Our data did not show any appreciable parameter to correlate the lymphatic vessel net with the clinical-pathological variables of tumour aggressiveness. However, we observed that VEGF-C, which is believed to be essentially a powerful cancer lymphangiogenic factor, and its preferential receptor VEGFR-3, are consistently expressed by GIST cells. Intriguingly, the expression of VEGFR-3 was significantly correlated with the presence of *KIT* mutations, but did not correlate with the clinical variables, which leads us to suppose that GIST cancer cells are able to up-regulate VEGF-C and VEGFR-3 expression, although these molecules do not influence lymphatic vessel proliferation in these tumours.

In the present study, we found a statistical correlation between KIT/PDGFRA mutations and VEGFR3 expression. Despite the absence of published data on such correlating, some studies found a positive association between VEGFR3 expression and activating mutations of oncogenes downstream of the KIT and PDGRA pathway, such as KRAS, suggesting that constitutive activating of receptor tyrosine kinase signaling can lead to VEGFR3 overexpression (Schimanski et al., 2010).

Our results have shown that GIST malignant cells remarkably express both lymphangiogenic molecules, which suggests that these molecules can be involved with the regulation of other structures, likely blood vessels. GISTs are highly vascularised tumours and metastasis generally occurs through the blood vessel net. Many cancer cells express VEGFR-3, but the function of this receptor is imprecise. Paradoxically, VEGF-C/VEGFR-3 activity can be associated with the development of angiogenic sprouts, mainly in association with COX2 upregulation (Chien et al., 2009). The widespread distribution of VEGF-C/VEGFR-3 in GISTs observed in this series encourages us to

hypothesise that VEGF-C/VEGFR-3 favour the improvement of blood vessel densities. Favouring this angiogenic hypothesis, we observed high blood vessel densities in intra and peritumoral area. Additionally, high BVD was related to metastatic status. Cases with high level of BVD assessed with CD31 monoclonal antibody and VEGF upregulation were identified as poor prognostic GISTs (Imamura et al., 2007).

Blood vessel density, however, is not necessarily guaranteed to ascertain tumour aggressiveness. Paradoxically, a high BVD could be related to a lesser metastatic potential if the new blood vessels are predominantly weak, distorted and exhibit defects of vascular assembly (Afonso et al., 2009). In our casuistic, conversely, BVD was associated with metastasis occurrence, which reinforces the premise that BVD correlates with GISTs' aggressiveness.

The expression of D2-40 in GISTs has been recently investigated, but no informative assessment is yet available to improve knowledge of GIST behaviour (Kimura and Kimura, 2005; Yu et al., 2007; Agaimy and Carney, 2010). In Carney triad patients, intra-tumoral lymphangiogenesis was not confirmed to be responsible for GISTs' lymphatic spread, usually observed in this group; also, the biological meaning of D2-40 expression in GIST cells is not fully understood (Agaimy and Carney, 2010). We observed a remarkable D2-40 positive immunoreaction in GIST cells, but we failed to understand the implication of this finding in these tumours. Kuroda et al. (2008) suggested that D2-40 expression may be an available marker of GISTs, but is definitely not associated with the degree of risk of GISTs' aggressiveness.

Our results were corroborated, in part, by Mahendra and colleagues (2008) who observed that, except in a few types of sarcomas (epithelioid, leiomyosarcoma, rhabdomyosarcoma and synovial sarcoma) lymphatics are usually absent in most of the malignant soft tissue tumours, which is consistent with the infrequent finding of sarcoma metastasis to lymph nodes.

Altogether, our findings have detailed for the first time in literature, to the best of our knowledge, the distribution of the lymphangiogenic promoter VEGF-C and its receptor VEGFR-3 in GISTs. The strong expression of both markers was not necessarily correlated with the clinical parameters of aggressiveness, but BVD was importantly correlated with metastasis. One can speculate that both lymphangiogenic markers may be more closely associated with angiogenesis than lymphangiogenesis in GISTs. Further studies should be planned to investigate this hypothesis.

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