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The immunohistochemical landscape of the VEGF family and its receptors in glioblastomas

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Abstract

Background: Angiogenesis is one of the hallmarks of cancer. This complex mechanism of tumor progression provides tumors cells with essential nutrients. There have been a limited number of investigations of markers of angiogenesis in Glioblastomas (GBMs), and most previous studies have focused on VEGF-A. Recent evidence suggests that there is a complex lymphatic system in central nervous system (CNS), which suggests VEGF-C and VEGF-D as interesting biomarker candidates. This study was designed to evaluate the expressions of VEGF-A, -C, -D and their co-receptors, VEGFR-1, VEGFR-2, and VEGFR-3 by immunohistochemistry (IHC) using a series of GBMs. In addition, we evaluate any putative correlations between IHC expression levels of VEGF and clinical data of patients.

Methods: Tumor samples of 70 GBM patients (64 isocitrate dehydrogenase-1 wildtype (wtIDH-1) and 6 mutant (mutIDH-1)) were assessed by IHC using tissue microarray platforms for VEGF subunits and their co-receptors. The medical records were reviewed for clinical and therapeutic data.

Results: All VEGF subunits and receptors were highly expressed in GBMs: 57 out of 62 (91.9%), 53 out of 56 (94.6%) and 55 out of 63 cases (87.3%) showed VEGF-A, VEGF-C and -D imunoexpression, respectively. Interestingly, we had found both nuclear and cytoplasmic localization of VEGF-C staining in GBM tumor cells. The frequency of immunoexpression of VEGF receptors was the following: VEGFR-1, 65 out of 66 cases (98.5%); VEGFR-2, 63 out of 64 cases (98.4%); VEGFR-3, 49 out of 50 cases (90.0%). There were no significant differences in the patient overall survival (OS) related to the VEGF staining. A weak and monotonous correlation was observed between VEGF and its cognate receptors. The pattern of VEGF IHC was found to be similar when GBM mutIDH-1 subtypes were compared to wtIDH-1.

Conclusion: Both VEGF-C and -D, together with their receptors, were found to be overexpressed in the majority GBMs, and the IHC expression levels did not correlate with OS or IDH status. To understand the significance of the interactions and increased expression of VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3 axis in GBM requires more extensive studies. Also, functional assays using a larger series of GBM is also necessary to better address the biological meaning of nuclear VEGF-C expression in tumor cells.

Keywords: Glioblastoma, Angiogenesis, Vascular endothelial growth factor, Vascular endothelial growth factor receptor, Survival

Background

Glioblastoma (GBM) remains the most common and deadliest primary malignant brain tumor in adults (Ostrom et al. 2018). Histological characteristics as tumor cell anaplasia, microvascular proliferation and necrosis are hallmarks of GBM (Louis et al. 2016). The well-known pronounced

vascular permeability and the high expression of vascular endothelial growth factor (VEGF) present in the abnormal vasculature, highlights the importance of angiogenesis in the tumor pathogenesis and therefore, constitute a rationale to target therapy (Lu-Emerson et al. 2015; Hundsberger et al. 2017).

Although great efforts have been implemented to develop new treatment strategies for GBM patients, the standard therapy is based on a multimodal approach comprising surgery, radiotherapy and chemotherapy, mostly centered on Temozolomide (TMZ) (Stupp et al.

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2005). Due to its critical role in tumor biology, VEGF signaling pathways was proposed as an attractive target in cancer therapy since the 70's (Folkman 1971). Thus far, and despite its established importance in GBM pathogenesis, only one anti-angiogenic therapy – the monoclonal anti-VEGF-A antibody, Bevacizumab is FDA approved despite its unreliable results (Friedman et al. 2009; Kreisl et al. 2009; Chinot et al. 2014; Diaz et al. 2017; Hundsberger et al. 2017; Wick et al. 2017). Indeed, as the complexity of angiogenesis has been unveiled and other VEGF family members were also implicated, alternative signaling pathways were considered as potential targets, fueling the development of new therapies, such as multitarget inhibitors – pan-vascular endothelial growth factor receptors (VEGFR) and tyrosine kinase inhibitors (TKIs). However, the benefits are unclear (Lu-Emerson et al. 2015).

The complexities and biological obstacles curb the use of angiogenesis as a potential therapeutic target in GBM patients. This might be partially explained by the intricate interaction of distinct signaling pathways and the higher number of involved molecules. Primarily, seven VEGF subunits are well distinguished, VEGF-A, -B, -C, -D, -E, -F and placental growth factor (PGF) – which can bind to their common receptors VEGFR-1, -2, and -3 and trigger an elaborate process of angiogenesis and lymphangiogenesis. These processes are invariably present in embryological, physiological and pathological conditions, such as tumorigenesis and tumor progression (Roy et al. 2006).

For instance, VEGF-A – the prototype member of the VEGF family – mediates VEGFR-1 and VEGFR-2 activation and predominantly regulates the process of angiogenesis in the central nervous system (CNS) (Carmeliet and Jain 2011). VEGF-C is a precursor protein that, in its cleaved form, has high affinity for both VEGFR-2 and VEGFR-3 that, when upregulated, may be associated with more unfavorable prognosis in GBM patients (Grau et al. 2007; Kessler et al. 2015; Zhao et al. 2016). Interestingly, VEGF-C, was initially described as primarily involved in the formation and maintenance of lymphatic vessels (Veikkola et al. 2001), which were classically thought to be absent from the CNS (Antila et al. 2017). However, increasing evidence indicates that VEGF-C is also expressed in the brain and may regulate several biological processes in physiological and pathological settings (Jenny et al. 2006; Grau et al. 2007; Shin et al. 2008; Zhao et al. 2016). From structural and functional perspective, VEGF-D is closely related to VEGF-C (Achen et al. 1998; Stacker and Achen 2018) and binds to the same receptors, i.e., VEGFR-2 and -3 (Roy et al. 2006) and thus, exerting an overlap role by inducing angiogenesis and lymphangiogenesis (Fagiani et al. 2016).

Emerging data has pointed out an increasing importance of VEGF-D, along with -C, as an alternative pro-angiogenic pathway in cancer (Grau et al. 2011; Yang

et al. 2015; Stacker and Achen 2018), although their role in CNS tumors is barely understood (Debinski et al. 2001; Moffat et al. 2006; Grau et al. 2007; Ramani et al. 2012). Also, the precise implications of VEGF-B in cancer cells are not fully known (Roy et al. 2006; Lal et al. 2018) and VEGF-E and VEGF-F have not been reported in mammals (Roy et al. 2006). Additionally, only few studies have described the patterns of expression of angiogenic factors in human GBM, most of them just focusing on VEGF-A (Xu et al. 2013; Chen et al. 2015; Kessler et al. 2015; Baumgarten et al. 2016), VEGF-C and VEGF-D.

Therefore, this study aimed to evaluate the expression of VEGF-A, -C, -D and its receptors, VEGFR-1, -2, and -3 in a series of GBMs as well as whether any correlation exists between immunostaining and clinical features. Also, it was investigated the potential prognostic value of distinct VEGF family members.

Methods

We retrospectively review consecutive adult patients (≥ 18 years old) with newly diagnosed GBMs according to the current World Health Organization Classification of Tumors of Central Nervous System (WHO, 2016) (Louis et al. 2016). These patients underwent to neurosurgical procedures, aiming maximal safe resection whenever possible, between January 2003 and December 2011, from two Brazilian hospital databases: Hospital Israelita Albert Einstein (HIAE) and Hospital São Paulo - Universidade Federal de São Paulo (HSP-UNIFESP). All tumor specimens were overnight fixed in buffered formalin and subsequently embedded in paraffin blocks.

Tumor samples from 97 patients were available and carefully reviewed by a reference neuropathologist (LN). Samples with predominantly necrotic tissue or small sample sizes as those from patients with inconsistent clinical and/or therapeutic data were excluded. Thus, the study group comprised tumor tissue from 70 GBM samples (HSP-UNIFESP, $n = 57$; HIAE, $n = 13$).

Tissue microarray (TMA) construction

At least two different and of the most representatives areas of each tumor were selected for analysis. Cylindrical cores of 2-mm were removed and used in the construction of tissue microarray (TMA) blocks, as previously described (Saggoro et al. 2014). Eleven TMA blocks were constructed using a Beecher tissue array instrument™ (Beecher Instruments, Silver Spring, MD, USA) in accordance to the manufacturer's instructions. For each immunostaining, 4 μ m slices were cut from TMA blocks by using a Leica microtome and placed on glass charged slides. Slides were cut consecutively to minimize the influence of tissue heterogeneity when comparing the expression of the different VEGF family members within each patient tumor sample. As control, a normal prostate tissue prostate section was added in each TMA block.

Immunohistochemistry assays and evaluation

The immunohistochemical (IHC) procedures were performed on 4- μ m-thick TMA sections and mounted on charged slides. Briefly, for immunostaining, the slides were deparaffinized, and rehydrated through a graded ethanol series. The antigen retrieval was done using Diva Decloaker solution (Biocare Med™, Concord, CA, USA), for 40 min at 98 °C in a steamer chamber. The slides were incubated with the pre-diluted antibody overnight at room temperature and subsequently washed with Tris-buffered saline with Tween 20, as shown in Table 1.

The immunohistochemistry test was chosen as the preferable evaluation method because of its practical approach, cost-effectiveness, widely availability, replicability, and has a proven usefulness in previous studies (Debinski et al. 2001; Jenny et al. 2006; Grau et al. 2007).

The IHC stains of VEGF and its cognate receptors were blindly evaluated at $\times 200$ magnification using a DX-51 Olympus microscope, according to the following semi-quantitative grading score: *negative* (0), absence or up to 10% IHC staining of the core area; *score 1+*, focal expression in > 10% to up to 25% of the core area; *score 2+*, intermediate expression in > 25% to up to 75% of the core; *score 3+*, diffuse expression in more than 75% of core area. At least a quarter of the core area had to be evaluable for scoring and faint immunostains were not considered. For each case (patient), the definitive IHC score was determined by the mean IHC stain obtained from the cores evaluated (up to 3 cores per case). The intensity of IHC stains was not considered. Finally, for each VEGF member the cellular location of IHC staining was noted, i.e., membrane, cytoplasmic and/or nuclei staining as well as the neoplastic and non-neoplastic endothelial cells.

The isocitrate dehydrogenase-1 (IDH-1) status was evaluated by IHC using the anti-IDH-1 mutant antibody for R132H (H09 clone, Dianova™, Hamburg, Germany) at 1:20, according to manufacturer's recommendations.

Clinical profile and outcomes

The medical records of 70 patients were reviewed for the following data: gender, age at diagnosis, Karnofsky Performance Status (KPS), date of the first symptom, date of death or last evaluation, date and extent of resection and radiation therapy (RT) and chemotherapy regimens.

Table 1 Summary of antibodies' clones and titration

Antibody	Clone	Dilution
VEGF-A	VG1	1:50
VEGF-C	MM0006-2E65	1:50
VEGF-D	Polyclonal	1:20
VEGF-R1	Polyclonal	1:20
VEGF-R2	1(1B6)	1:100
VEGF-R3	MM003.7563	1:20

Neurosurgery was performed to attain the maximum safe and feasible resection according to the guidelines of both institutions, which have suitably equipped surgical centers. The extension of tumor resection was defined based on immediate (48 h) postoperative imaging (CT or MR), as radical resection (absence of residual contrast enhancement), partial resection or biopsy. Patients underwent 3D localized external beam RT delivered to the contrast-enhancing lesion shown at CT/T1-weighted images or T2/fluid attenuated inversion recovery sequence MR. The RT dose was prescribed according to the guidelines of the International Commission of Radiological Units fields, once daily at 2 Gy per fraction, 5 days a week, for a total of 60 Gy. The treatment protocols and personnel varied over time and between centers. Chemotherapy regimens also varied between centers. At HIAE, all patients were treated with concomitant and adjuvant Temozolomide according to the European Organization for Research and Treatment of Cancer–National Cancer Institute of Canada protocol (Stupp et al. 2005). At HSP-UNIFESP until 2008, patients received 200 mg/m² carmustine (bis-chloroethylnitrosourea [BCNU]) at 6-week intervals starting 6 weeks after RT. Since 2009, TMZ has been available and patients could be treated with the EORTC-NCIC protocol. The patients who underwent chemotherapy treatment according to the EORTC-NCIC protocol were categorized as “RT concurrent with chemotherapy.” The patients who received BCNU were defined as having “RT and sequencing chemotherapy.” The patients who did not receive RT and/or chemotherapy were defined as having “best supportive care”.

Statistical analyses

Data were described using absolute and relative frequencies for categorical data. Quantitative data were described using median and range, due to skewness. Overall survival (OS) was calculated from date of surgery until death or last follow-up and the cut-off date was November 30th 2018.

Survival curves were constructed according to the Kaplan-Meier method and comparison between groups by using log-rank test to explore relationships between well-recognized prognostic factors (age, KPS, extent of tumor resection, adjuvant treatments) and survival time in the univariate analysis. A conditional stepwise proportional hazard analysis (Cox-regression model) was used to identify independent predictors of survival. The level of significance was 0.05 ($p < 0.05$). [$p \leq 0.05$].

For statistical analyses, only cases with consistent IHC expression (scores 2+ and 3+) were considered “*positive or high expression*”, i.e., cases with at least > 25% of IHC staining in the core area at definitive score. Cases showing < 25% IHC staining were considered as “*negative or low expression*”. Plausible associations among the IHC score of multiple VEGFs and their receptors were assessed using Spearman rank correlation coefficient.

The statistical analysis was performed using the statistical software R (R Core Team, 2017) added to the Car graphic package (Fox and Weisberg 2011) and survival (Therneau 2015).

Results

Clinicopathological features and patient outcomes

The median age of the patients at surgery was 59.5 years (range, 18–78 years), with 44 males (62.8%, male/female ratio of 1.7:1) (Table 2). The median follow-up time of all survivors was 8.21 months (range, 1.2–95.8 months) and the estimated OS was 7.25 months (range, 5.33–14.08 months).

VEGF-A, -C, -D and VEGFR-1, -2, -3 IHC expression and patient outcomes

The overwhelming majority of GBM cases showed consistent VEGF expression, regardless of its subunit or receptor, as shown in Table 3.

Even though several TMA cores were lost, of which the IHC score was not performed, mostly in regards to VEGFR-3 (28.6%) and VEGF-C (20.0%), the majority of GBM cases showed “high expression” for all VEGF subunits and their cognate receptors (> 25%, 2+ or 3+), as shown Table 3. Interestingly, VEGF-C showed conspicuous nuclear immunostaining (repeated three times) (Fig. 1). The remaining VEGF subunits and receptors showed cytoplasmic staining (data not shown).

Table 2 Demographics, clinical and treatment data of GBM patients (univariate analyses)

	N (%)	HR	CI 95%	P
Gender				
Female	26 (37.1)	1		
Male	44 (62.9)	1.26 (0.75–2.11)	0.39	
Age (yr)				
< 50	15 (21.4)	1		
≥ 50	55 (78.6)	1.61 (0.89–2.92)	0.12	
KPS (%)				
< 70	20 (28.6)	1		
≥ 70	43 (61.4)	0.51 (0.29–0.89)	0.02	
NA	7 (10.0)			
Neurosurgery (extent of resection)				
Biopsy	4 (5.7)	1		
Partial	41 (58.6)	0.42 (0.13–1.41)	0.16	
Radical	25 (35.7)	0.24 (0.17–0.57)	0.02	
Adjuvant treatment				
Best supportive care	21 (30.0)	1		
Only RT	16 (22.9)	0.52 (0.26–1.05)	0.07	
RT and sequencing chemotherapy	20 (28.6)	0.32 (0.17–0.62)	0.001	
RT concurrent with chemotherapy	13 (18.6)	0.30 (0.14–0.63)	0.002	

NA Not available

Table 3 Number of cases that expressed (“positive cases”, 1+ to 3+) and those with “high expression” (> 25%, 2+ or 3+) for each VEGF subunit and cognate receptor^a

	Positive cases N	High expression N
VEGF-A	57/62 (91.9%)	49/57 (85.9%)
VEGF-C	53/56 (94.6%)	48/53 (90.5%)
VEGF-D	55/63 (87.3%)	49/55 (89.0%)
VEGF-R1	65/66 (98.5%)	55/65 (84.6%)
VEGF-R2	63/64 (98.4%)	56/63 (88.8%)
VEGF-R3	49/50 (98.0%)	46/49 (93.8%)

^a > 25% of immunostaining in the core area

Overall, there were no significant differences in OS regardless the VEGF staining score on tumor cores (Table 4).

Additionally, other univariate analyses was performed using patients grouped according a binary IHC category: “no or low expression” (up to 1+) and “high expression” (2+ or 3+) for each VEGF subunit and its cognate receptors. The model was created to statistically strengthen groups and overcome the hurdles as a result of a small sample size due to the missing TMA cores. However, no significant differences were found between OS and these IHC categories (Table 5).

Finally, regarding the IDH-1 mutation 6 out 70 patients harbor the canonical R132H mutation that is detected by IHC (8.6%). However, the IHC expression of VEGF subunits and cognate receptors did not differ from those with *wt*IDH-1, which constitutes the overwhelming majority of casuistry.

Evaluation of the relationship between distinct VEGF subunits and receptors

Table 6 shows the Spearman rank correlation coefficients among the IHC expression of VEGFs. The VEGF and its cognate receptors showed a weak and monotonous correlation pattern. A moderate correlation was only found for the VEGF-A and VEGFR-2 (Spearman = 0.478), as well as for VEGF-D and VEGFR-2 (Spearman = 0.456).

Discussion

In the current study, VEGF-C and -D along with its receptors VEGFR-2 and -3 were overexpressed in the majority of GBM tumor samples, ranging from 87.3% (VEGF-D) to 98.5% of the cases (VEGFR-1). However, there were no significant differences in the OS according to the VEGFs and their receptors and the immunostaining on tumor cells. This finding was expected since the VEGFs were overexpressed and our casuistry was overwhelmed by cases of GBMs with *wt*IDH-1 (only 6 out of 70 showed the IHD-1 R132H mutation).

Our results emphasized the hurdles for establishing angiogenic prognostic and predictive biomarkers in CNS tumors, which is in line of unclear benefits no matter the anti-

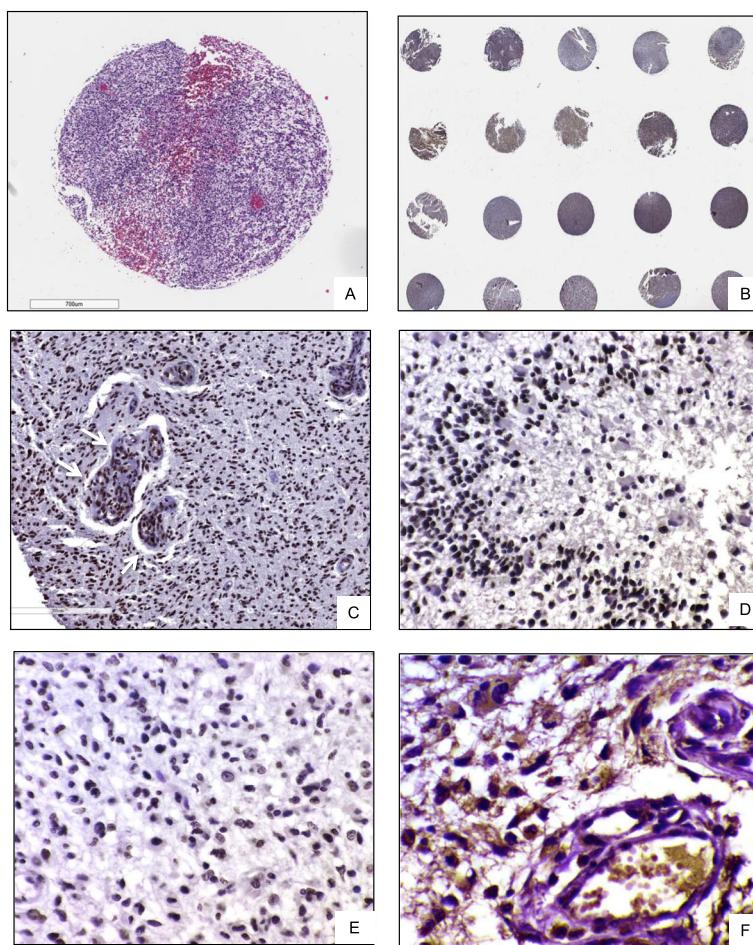


Fig. 1 Morphological and immunohistochemical features of VEGF-C and -D in GBM tissue microarray (TMA) sections. **A**) Cylindrical core of 2-mm from a representative GBM area (H&E, $\times 100$). **B**) TMA design in a 4-um tissue section used for VEGF-C immunostaining (lower magnification). **C** to **E**) VEGF-C staining. Note the nuclear IHC expression of VEGF-C in tumor cells and in neoplastic endothelium of glomeruloid vessels (1C, arrows). **F**) VEGF-D expression in cytoplasm of GBM tumor cells and endothelial cells ($\times 400$)

Table 4 Univariate analysis of overall survival according to the VEGF and VEGFR immunohistochemical score in GBMs

	N = 70	Staining score				
		HR (CI 95%)				
	Total Stained/Lost (Lost %)	Total Positive N (%)	Absent (-)	Focal (+)	Intermediate (++)	Diffuse (+++)
VEGF-A	62 / 8 (11.4%) ^a	57/62 (91.9%)	1 5 (7.1%)	0.96 (0.29–3.20) 8 (11.4%)	0.78 (0.26–2.32) 20 (28.6%)	0.86 (0.30–2.47) 29 (41.4%)
VEGF-C	56 /14 (20%) ^a	53/56 (94.6)	1 3 (4.3%)	1.149 (0.35–6.34) 5 (7.1%)	1.19 (0.31–4.59) 9 (12.9%)	1.11 (0.34–3.63) 39 (55.7%)
VEGF-D	63 /7 (10.0%) ^a	55/63 (87.3)	1 8 (11.4%)	4.27 (1.30–13.95) 6 (8.6%)	1.45 (0.52–4.06) 11 (15.7%)	1.07 (0.45–2.57) 38 (54.3%)
VEGF-R1	66/4 (5.7%) ^a	65/66 (98.5)	1 1 (1.4%)	2.14 (0.27–16.98) 10 (14.3%)	1.33 (0.18–10.0) 23 (32.9%)	0.94 (0.13–6.96) 32 (45.7%)
VEGF-R2	64/6 (8.6%) ^a	63/64 (98.4)	1 1 (1.4%)	1.04 (0.13–8.61) 7 (10.0%)	1.24 (0.16–9.94) 10 (14.3%)	1.29 (0.18–9.42) 46 (65.7%)
VEGF-R3	50/20 (28.6%) ^a	49/50 (98.0)	1 1 (1.4%)	0.81 (0.07–9.01) 3 (4.3%)	1.27 (0.17–9.56) 20 (28.6%)	1.07 (0.14–8.03) 26 (37.1%)

^a Percentage of missing TMA cases

Table 5 Univariate model for analysis of the immunohistochemical expression according a binary category (absent/low vs high expression) for each VEGF subunit and receptors in GBMs

	Category	N (%)	Median OS (CI 95%)*	HR	(CI 95%)	P
VEGF-A (62)	Absent/Low	5 (8.1)	4.77 (2.63; –)	1		
	High expression	57 (91.9)	6.43 (4.69; 12.90)	0.84	(0.30–2.35)	0.74
VEGF-C (56)	Absent/Low	3 (5.4)	6.22 (2.04; –)	1		
	High expression	53 (94.6)	5.72 (4.01; 12.70)	1.15	(0.35–3.72)	0.82
VEGF-D (63)	Absent/Low	8 (12.7)	10.08 (3.52; –)	1		
	High expression	55 (87.3)	6.02 (4.64; 12.70)	1.22	(0.52–2.88)	0.64
VEGFR-1 (66)	Absent/Low	1 (1.5)	14.97 (–; –)	1		
	High expression	65 (98.5)	6.66 (5.20; 12.80)	1.19	(0.16–8.64)	0.87
VEGFR-2 (64)	Absent/Low	1 (1.6)	14.97 (–; –)	1		
	High expression	63 (98.4)	6.02 (4.64; 12.80)	1.25	(0.17–9.10)	0.83
VEGFR-3 (50)	Absent/Low	1 (2)	14.97 (–; –)	1		
	High expression	49 (98)	5.89 (4.57; 14.10)	1.12	(0.15–8.27)	0.91

angiogenic drug prescribed for unselected CNS tumor patient population (Winkler et al. 2018). Hopes persist to rest on the discovery of predictive biomarkers on tumor tissue, blood and/or radiographic parameters, which could merit the broad use of angiogenesis inhibitors, particularly for GBM patients (Lu-Emerson et al. 2015; Winkler et al. 2018). On the other hand, VEGF-C, -D, VEGFR-2 and -3 were not fashionable research targets due to their established role in primarily promoting the growth and remodeling of lymphatic vessels, which were considered absent in CNS, until recently. However, a considerable amount of evidence suggests the existence of a complex lymphatic system in CNS, mainly within the meninges, which drains cerebrospinal fluid into the deep cervical lymph nodes (Aspelund et al. 2014, 2015; Louveau et al. 2015; Weller et al. 2016; Antila et al. 2017). The development and modeling of that specific lymphatic system are closely related to the interactions between VEGF-C and VEGFR-3 (Antila et al. 2017). The emerging data brought upfront the exploration of VEGF-C and its receptors – VEGFR-2 and -3 – as its partially homologous VEGF, the VEGF-D (Achen et al. 1998; Grau et al. 2011; Michaelsen et al. 2018). To the best of our knowledge, this study represents the first to focus on the immunohistochemical expression of the expanded axis of proangiogenic factors simultaneously – VEGF-C, -D and VEGFR-2, -3 – and that theoretically relate to lymphangiogenesis in GBM. However, a nuclear VEGF-C staining was found in

GBM tumor cells, as was similarly previously described (Cai et al. 2012). Whether this nuclear VEGF-C staining means an abnormal cell reprogramming or reflects the functional status of VEGF-C in the tumor cell is unknown. This finding deserves further investigations.

The accumulating data from experimental models and resected human tumor samples further described the expression of various VEGFs and their cognate receptors in glial tumors (Machein and Plate 2000; Huang et al. 2005). The first description of VEGF-D immunoreactivity in GBM was reported by Debinski et al. (2001) who used GBM cell lines and ten tissue sections. They demonstrated that nearly all the GBM cells produce VEGF-D what may be partially implicated in oncogenic transformation and appeared to be an attractive target for novel treatment strategies. Following these results, Jenny et al. (2006) observed VEGF-C, -D and VEGFR-3 expression in normal brain tissue and in most brain tumors, as such glioblastomas and hemangioblastomas. The expression of VEGF-C and -D has been demonstrated to be diverse in GBM tissue samples, since the VEGF-C is found to be overexpressed compared to VEGF-D (Grau et al. 2011; Michaelsen et al. 2018).

Consistently, our results indicate that VEGF-C is proportionally overexpressed (94.6%), but is without any significant influence in patient prognosis. In contrast, low expressions of the VEGF-D (HR of 4.27 (CI 95%, 1.30 – 13.95) impaired the patient' OS significantly ($p = 0.016$). Weickhardt et al. 2015 related that the anti-angiogenic therapy (i.e. Bevacizumab) might exhibited superior efficacy when VEGF-D is low expressed in other clinical scenario. On the other hand, despite its wide variability, previous reports suggested that VEGF-D, when upregulated, is associated with higher tumor grade of malignancy (Grau et al. 2011; Jiang et al. 2018). It is important to note that when the VEGF-D evaluations were grouped in a binary category ("absence/low expression" and "high expression") no significant OS

Table 6 Spearman rank correlation coefficients among VEGF subunits and their receptors

Comparison between the IHC expression	Spearman
VEGFA × VEGFR1	0,150
VEGFA × VEGFR2	0,478
VEGFC × VEGFR3	0,378
VEGFD × VEGFR3	0,372

influence was found (HR of 1.22 (CI 95% 0.52–2.88), $p = 0.64$). We should emphasize that caution must be taken when interpreting this study's VEGF-D findings, which might be partially explained by the restricted number of tumor samples with weak VEGF-D IHC expression (8.6%). In the same way, it was not found any significant differences on OS for the expression of others VEGF factors or cognate receptors. This finding might be partially explained by the relatively small number of patients in our series.

Substantial data support the correlation of the expressions of VEGF receptors, such as VEGFR-3 and the tumor grading (Grau et al. 2007, 2008). Baumgarten et al. (2016) have shown an overexpression of VEGFR-1, -2 and, -3 in GBM. Moreover, the expression of VEGFR-3 trends to turn positive as the pathologic grade of malignancy increases (Jiang et al. 2018). These previous findings were compatible with our results, as it was found overall higher immunoexpression of VEGFRs (VEGFR-1, 92.9%; -2, 90%; -3, 70%). However, in the present study it was found only a weak and monotonous correlation pattern between VEGF and its cognate receptors.

Overall, it is still unknown whether VEGFs and their receptors expression profiles are plausible biological markers to predict response and/or patient prognosis. Nevertheless, the are indications that VEGF-C, -D and VEGFR-2, -3 in particular may have implications on alternative signaling pathway of primary and acquired resistance to anti-VEGF therapy (Moffat et al. 2006; Grau et al. 2011; Li et al. 2014; Michaelsen et al. 2018). Our results did not support this hypothesis since no recurrent or progressive GBM tumor samples were investigated. However, the overexpression of the entire axis of VEGF-C, -D, VEGFR-2 and -3 might be interpreted as an investigational target to clarify that theory, considering the recent data that suggests that there is a complex lymphatic system in the CNS (Aspelund et al. 2014, 2015; Louveau et al. 2015; Choy and Rahul Jandial 2016; Antila et al. 2017) and its development and modeling may be closely related to the interactions between VEGF-C and VEGFR-3 (Antila et al. 2017).

Conclusions

VEGF-C and -D and its receptors were overexpressed in the overwhelming majority GBMs but their expressions did not correlate with patient's OS. The overexpression of the axis of VEGF-C, VEGF-D, VEGFR-2 and VEGFR-3 might be interpreted as a potential target for further studies, particularly the interactions between VEGF-C and VEGFR-3 as well the mild nuclear VEGF-C expression. Whether this nuclear staining means an abnormal cell reprogramming or reflects the functional status of VEGF-C in the GBM cell is unknown. Additional studies with more extensive series of GBM are still necessary to

better evaluate the roles of VEGF-C, VEGF- D and their cognate receptors, VEGFR-2 and VEGFR-3, in GBMs.

Abbreviations

GBM: Glioblastoma; VEGF: Vascular endothelial growth factor; TMZ: Temozolomide; VEGFR: Endothelial growth factor receptors; TKIs: Tyrosine kinase inhibitors; PGF: Placental growth factor; CNS: Central nervous system; HIAE: Hospital Israelita Albert Einstein; HSP-UNIFESP: Hospital São Paulo - Universidade Federal de São Paulo; TMA: Tissue microarray; IHC: Immunohistochemical; KPS: Karnofsky Performance Status; CT: Computer tomography; MR: Magnetic resonance imaging; RT: Radiotherapy; BCNU: Carmustine (bis-chloroethylnitrosourea); OS: Overall survival

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Authors' contributions

LVML: acquisition of clinical data, interpretation of data, and drafted the manuscript. LN: perform pathological assays and interpretation, and drafted the manuscript; DCF: drafted the manuscript; LOK: drafted and manuscript revision; JNS: acquisition of pathological data and interpretation; SMFM: design of the study, acquisition of clinical data and interpretation, and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The ethics review board of both institutions approved this study under the reference #33741714.4.0000.0071. This work does not represent a clinical trial and was therefore not registered as such.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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