Vascular endothelial growth factor receptor 1 in glioblastoma-associated microglia/macrophages

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Abstract. The anti-vascular endothelial growth factor-A (VEGF-A) monoclonal antibody (mAb) bevacizumab is an FDA-approved monotherapy for the treatment of recurrent glioblastoma (GB), a highly angiogenic and infiltrative tumour. However, bevacizumab does not increase overall survival and blockade of VEGF-A/VEGF receptor (VEGFR)-2 signal transduction is associated with severe adverse effects due to inhibition of physiological angiogenesis. Conversely, VEGFR-1 does not play a relevant role in physiological angiogenesis in the adult. VEGFR-1 is activated by both VEGF-A and placenta growth factor (PlGF), a protein involved in tumour growth and progression. In previous studies, it was demonstrated that inhibition of VEGFR-1 using a specific mAb developed in our laboratories reduced angiogenesis and GB cell chemotaxis and increased the survival of tumour-bearing mice. Failure of treatments directed toward the VEGF-A/VEGFR-2 axis could in part be due to inefficient targeting of the tumour microenvironment. In the present study, VEGFR-1 expression was investigated in GB-associated microglia/macrophages (GAMs) by analysing surgical specimens collected from 42 patients with GB. Data obtained from The Cancer Genome Atlas (TCGA) database revealed that upregulation of the VEGFR-1 ligands VEGF-A and PlGF was associated with a significant reduction in overall survival for patients with GB, highlighting the potential relevance of this receptor in the aggressiveness of GB. Immunohistochemical analysis indicated that VEGFR-1 is expressed not only in GB tissue but also in GAMs. Furthermore, the percentage of VEGFR-1-positive GAMs was significantly higher in the tumour region compared with that noted in the surrounding parenchyma. Thus, VEGFR-1 represents a potential therapeutic target for the treatment of GB, being present not only in GB and endothelial cells, but also in GAMs that are involved in tumour progression.

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Abbreviations: GAMs, glioblastoma-associated microglia/macrophages; GB, glioblastoma; Iba1, ionised calcium binding adaptor molecule 1; mAb, monoclonal antibody; MMP, matrix metalloprotease; PlGF, placenta growth factor; sVEGFR-1, soluble vascular endothelial growth factor receptor-1; TCGA, The Cancer Genome Atlas; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor

Key words: VEGFR-1, VEGF-A, glioblastoma, angiogenesis, microglia, macrophages, melanoma

Introduction

Vascular endothelial growth factor-A (VEGF-A) is a key mediator of angiogenesis via class IV tyrosine kinase receptor family of VEGF receptors (VEGFRs) (1). Three types of membrane VEGFRs have been described, VEGFR-1-3, which are encoded by different genes. The various VEGF-A isoforms facilitate angiogenesis via the activation of VEGFR-1 and VEGFR-2, whereas VEGF-C and VEGF-D bind to VEGFR-3, which promotes the formation of lymph vessels (2). It is generally agreed that VEGFR-2 is the major receptor mediating mitogenic, angiogenic and permeability effects under physiological conditions (3). In contrast, VEGFR-1 does not play a relevant role in physiological angiogenesis in the adult but does serve an important role in tumour angiogenesis. Furthermore, VEGFR-1 ligands directly stimulate signalling pathways crucial for tumour growth, progression and metastasis in cancer cells (4), and VEGFR-1 activation in a variety of tumour types, including melanoma, inhibits apoptosis, induces chemoresistance and is associated with a less favourable prognosis and recurrence (5-8). VEGFR-1 is also activated by the placenta growth factor (PlGF), a member of the VEGF family, which is an exclusive ligand of this receptor and does not interact with the other VEGFRs (9). VEGFR-1 exists as a full-length membrane protein or as several soluble forms (sVEGFR-1) deriving from alternative splicing of the corresponding pre-mRNA (10). The most abundant sVEGFR-1 is comprised of the extracellular region with a carboxyl-terminal end of 31 amino acids from intron 13. This soluble form is released into the extracellular matrix and exerts anti-angiogenic effects by sequestering VEGF-A or PlGF, thus reducing their availability for membrane receptor activation (11).

Several antitumour agents target VEGF-A signalling, including the monoclonal antibodies (mAbs) bevacizumab and ramucirumab, which block VEGF-A and VEGFR-2, respectively, the fusion protein aflibercept, which prevents VEGF-A and PlGF interaction with the membrane receptors, and a number of multi-targeted small-molecule kinase inhibitors. Despite the evidence of efficacy in the acute treatment of a number of different types of solid tumours, the approved therapeutics targeting VEGF-A/VEGFR-2 signalling lack long-term efficacy (1). In addition, long-lasting treatments are associated with unwanted side effects due to the inhibition of physiological angiogenesis, resulting in bleeding, hypertension and delayed wound healing (3,12). Therefore, it may be possible that molecules selectively inhibiting VEGFR-1 may exhibit improved safety profiles compared with agents targeting VEGF-A and/or VEGFR-2, while concurrently maintaining significant efficacy in antagonizing tumour vascularization and metastasis (4,13).

In our previous studies, it was shown that the anti-VEGFR-1 mAb D16F7, which blocks receptor signal transduction without interfering with ligand binding (14), inhibited the chemotaxis and invasiveness of glioblastoma (GB) cells and patient-derived GB stem cells in response to VEGF-A and PlGF (8). This property is particularly important as this mAb did not interfere with the decoy function of sVEGFR-1, preserving its physiological anti-angiogenic activity. In addition, in an *in vivo* orthotopic model, D16F7 reduced glioma growth, tumour-associated vessel formation and increased median survival time of mice, with a high percentage of long-term survivors (13). These data suggest that VEGFR-1 represents a therapeutic target for the treatment of GB, which is the most aggressive primary brain tumour, characterised by a high rate of therapeutic failure and less favourable prognosis. Resistance to chemotherapy is frequently observed and recurrence following initial therapy is common (15,16). The anti-VEGF-A mAb bevacizumab has been approved for the treatment of recurrent GB by the US-Food and Drug Administration (FDA) but not by the European Medicines Agency (EMA). However, in this clinical setting bevacizumab does not improve overall survival (17-19). Intrinsic or acquired resistance mechanisms toward anti–VEGF-A treatments may include: i) Increased expression/activation of VEGF-A tyrosine kinase transmembrane receptors in the tumour and tumour microenvironment; ii) upregulation of different angiogenic factors including PlGF; iii) phenotypic changes of tumour cells and/or iv) upregulation of alternative angiogenic pathways (20). In addition, rescue mechanisms to metabolic changes and hypoxia, mesenchymal cell transition, M2 microglia/macrophage polarization and myeloid cell infiltration may contribute to the resistance towards anti-VEGF-A therapies (18). Therefore, both GB cells and the tumour microenvironment play a role in the failure of treatments targeting the VEGF-A/VEGFRs axis.

Regarding the GB microenvironment in particular, GB-associated microglia/macrophages (GAMs) represent the largest proportion of tumour-infiltrating cells, contributing 30-70% of the glioma mass (21). VEGFR-1 has been demonstrated to be expressed in macrophages and its activation favours the production of VEGF-A and PlGF or other angiogenic factors and matrix metalloproteases (MMPs) which enhance cancer invasiveness (22-26). However, to date, no data are available on the expression of VEGFR-1 in human GAMs.

In recent years, our studies have demonstrated the role of GAMs in different murine and human models of the GB microenvironment, examining GAM polarization, the involvement of the mTOR pathway in GAM activation, as well as the role of the chemokine receptor CCR5 in GAM migration and activation (27-31). In the present study, VEGFR-1 expression in GAMs of surgical specimens collected from 42 patients with GB was assessed and it was shown that the percentage of VEGFR-1-positive GAMs was higher in the tumour tissue than in the surrounding parenchyma.

Materials and methods

Patients and tissue specimens. A total of 42 adults (mean age 60, range 34-79; 27 males/15 females), who underwent surgery for primary GB at the Neurosurgery Department, Foundation 'Agostino Gemelli' University Hospital (Rome, Italy), between March 2005 and September 2011 were recruited for the present study. Diagnosis of GB was established by histological examination according to the WHO classification (grade IV) of central nervous system (CNS) tumours. In all cases total removal of the tumour was achieved, and tissue samples from both the tumour and the surrounding macroscopic normal brain tissue were obtained (1-2 cm away from the tumour border; larger resections were performed in tumours that grew far from eloquent areas). The characteristics of patients are presented in Table I. All patients provided written consent for use their specimens for research and the research proposal was approved by the Ethics Committee of Foundation 'Agostino Gemelli' University Hospital (Rome, Italy) (8,30,31).

Tissue preparation and immunohistochemistry. Human tumour tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.6 overnight at 4˚C. Dehydration of tissue was performed using an alcohol series of 80 and 95% ethanol for 1 h each followed by 100% ethanol overnight. Two 100% xylene washes were performed for 1 h each and then 1 h at 60˚C in Paraplast Plus (Tyco/Healthcare). After a change of Paraplast Plus, tissue was incubated in a 60˚C vacuum oven for 2 h prior to placing in molds to cool and solidify. Sections, 3- to 4-μm thick, were cut and collected on Superfrost Plus slides (Thermo Fisher Scientific, Inc.). PT Link (Dako; Agilent Technologies, Inc.) was used to deparaffinise and rehydrate the sections and for antigen retrieval. Slides were immersed in 10 mM citrate buffer, pH 6.0, for 10 min at 97˚C and then cooled and washed in PBS or TBS. Endogenous peroxidase activity was inhibited by incubating the slides with Peroxide Block (ScyTek Laboratories) for 7 min, after which, slides were washed with PBS and underwent single staining procedure, whereas slides washed with TBS underwent a double staining procedure.

For single staining, the slides were incubated with Avidin/Biotin Blocking System (Spring Bioscience Corp.) and washed 3 times in PBS. Non-specific binding was blocked by incubating tissues with Super Block Solution (ScyTek Laboratories) for 5 min. Sections were incubated for 10 min at room temperature with rabbit anti-human Flt-1/VEGFR-1 polyclonal antibody (dilution 1:50; cat. no. E2800; Spring Bioscience Corp.), or overnight at 4˚C with goat anti-human ionised calcium binding adaptor

Table I. Demographic characteristics of the GB patients.

Patient	Age (years)	Sex	Tumour location	Primary (P) vs. Recurrent (R)	VEGFR-1-positive cells (%)	
					Tumour	Parenchyma
1	67	M	NA[a]	P	79	44
2	70	M	Temporal	P	48	56
3	34	M	Temporal	P	57	0
4	72	F	Frontal	R	52	20
5	62	F	Frontal	P	100	0
6	66	M	NA	P	100	20
7	76	M	Frontal	P	100	64
8	79	M	Frontal	P	100	8
9	37	M	Temporal	P	100	84
10	71	M	Occipital	P	38	42
11	70	M	Temporal	P	0	0
12	52	F	NA	P	40	44
13	48	M	NA	R	43	62
14	46	F	Temporal	P	46	34
15	44	F	Parietal	P	38	5
16	71	F	NA	P	100	35
17	67	F	Frontal	P	36	38
18	49	M	Frontal	P	100	42
19	47	F	Tempo-Parietal	R	81	75
20	50	M	Temporal	P	100	72
21	62	M	NA	P	61	65
22	71	F	Temporal	P	32	20
23	75	M	Parietal	P	100	32
24	73	F	NA	P	71	20
25	42	M	Temporal	P	100	54
26	64	M	NA	R	50	22
27	66	F	Frontal	R	29	55
28	69	M	Occipital	P	100	100
29	66	F	NA	P	100	86
30	38	M	Temporal	P	100	8
31	74	M	NA	P	66	44
32	76	M	Temporal	P	100	88
33	73	M	Temporal	P	39	26
34	51	F	NA	P	100	44
35	58	M	Frontal	P	34	44
36	70	M	Fronto-temporal	P	66	62
37	50	F	Occipital	P	100	80
38	62	F	Frontal	P	16	40
39	51	M	Temporal	P	100	40
40	51	M	NA	P	16	56
41	NA	M	NA	R	100	72
42	61	M	Frontal	P	100	40
Total			Mean ± SEM		69.9±4.8	43.9±4.0[a,b]

[a]Statistical analysis was performed using Wilcoxon signed rank test; [b]P<0.0001. F, female; M, male; NA, not available.

molecule 1 (Iba1) polyclonal antibody (dilution 1:250; cat. no. NB100-1028; Novus Biologicals). Sections were washed extensively with PBS and subsequently treated with Ultra Tek horseradish peroxidase anti-polyvalent kit

(ScyTek Laboratories). Finally, after 3 washes with PBS, the sections were treated with the chromogen 3,3'-diaminobenzidine (ScyTek Laboratories), counterstained with haematoxylin and mounted.

For double staining, non-specific binding was blocked incubating tissues with Background Punisher (Biocare-Medical Pacheco) for 10 min. Sections were incubated for 10 min at room temperature with rabbit anti-human Flt-1/VEGFR-1 polyclonal antibody (dilution 1:50; Spring Bioscience Corp.) and overnight at 4˚C with goat anti-human Iba1 polyclonal antibody (dilution 1:250; Novus Biologicals). Thereafter, sections were washed extensively in TBS and subsequently incubated with the MACH 2 rabbit horseradish peroxidase-polymer (Biocare-Medical) for Flt-1/VEGFR-1 and with Ultratek horseradish peroxidase kit (ScyTek Laboratories) for Iba1. Finally, after 3 washes in TBS, sections were treated with 3,3'-diaminobenzidine (Biocare-Medical) as the chromogen for Iba1 and with Vina Green (Biocare-Medical) as the chromogen for Flt-1/VEGFR-1 and then counterstained with haematoxylin and mounted.

Immunostaining analysis. Quantitative analyses were performed by counting under the microscope (Optech Optical Technology) the number of VEGFR-1$^+$, Iba1$^+$, or VEGFR-1$^+$ and Iba1$^+$ double-positive cells in 50 cells. Two blinded examiners evaluated three different areas of the same slides and counted 50 cells that included the number of positive cells for each antibody, the number of positive cells for both antibodies and the number of negative cells. The average of 6 counts was reported as percentage of positive cells.

Immortalised human microglia cell line. The immortalised human microglia-SV40 (IMhu) cell line was purchased from Applied Biological Materials Inc. The IMhu cells were grown in Prigrow III media containing 10% foetal calf serum and antibiotics in PriCoat T25 flasks (all from Applied Biological Materials Inc.) and seeded at a density of $4x10^4$ cells per cm^2. Cells were split when they reached ~80% confluence. For an extensive characterization of the IMhu cell line and its culture conditions refer to Chiavari *et al* (32).

Reverse transcription-quantitative (RT-q)PCR. Total cellular RNA was prepared using an RNeasy Midi kit from Qiagen, Inc., according to the manufacturer's protocol. Total RNA (3 μg per sample) was subjected to reverse transcription using SuperScript III enzyme (Invitrogen; Thermo Fisher Scientific, Inc.) at 50˚C for 60 min. Quantification of membrane VEGFR-1 levels was performed by RT-qPCR using a dual-labelled fluorogenic probe method and an ABI Prism 7000 sequence detector (PerkinElmer, Inc.), as previously described (33). The $2^{-\Delta\Delta Cq}$ relative quantification method was utilised to calculate relative mRNA expression levels. The sequences of the primers were as follows: VEGFR-1, forward 5'-ACCGAATGCCACCTCCATG-3' and reverse 5'-AGGCCTTGGGTTTGCTGTC-3'. The level of VEGFR-1 transcript was normalised to that of 18S RNA (TaqMan® Gene Expression Assay, Applied Biosystems, Inc.) and referred to the values obtained for the VEGFR-1 negative human melanoma M14 cell line, to which an arbitrary value of 1 was assigned.

Immunofluorescence. Millicell EZ slide 8-well glass (EMD Millipore) were used to seed the T98G and IMhu cells ($5x10^4$ cells/well). Cells were fixed with 4% paraformaldehyde in PBS with calcium and magnesium for 20 min at room temperature. After three washes in PBS, cells were blocked with BSA and incubated overnight in the presence of the primary anti-VEGFR-1 antibody (Spring Bioscience Corp.; dilution 1:50). After three washes in PBS with gentle agitation, the cells were incubated with a secondary donkey anti-rabbit antibody (dilution 1:1,000; cat. no. A16028; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h and mounted with Vectashield with DAPI (Vector Laboratories, Inc.).

Statistical analysis. Statistical comparison of the differences between pairs of groups was performed using a Student's t-test or a Wilcoxon signed-rank test. Statistical significance was determined at α=0.05 level. $P<0.05$ was considered to indicate a statistically significant difference. The non-parametric log-rank test was performed to compare the survival distributions of the Kaplan-Meier curve.

Results

Relevance of VEGFR-1 ligands expression on the survival of patients with GB. The role of the VEGFR-1 ligands VEGF-A and PlGF on survival of patients with GB was assessed using data generated by the TCGA Research Network (https://www.cancer.gov/tcga). The results showed that 345 of 690 patients (dataset, TCGA lower grade glioma and glioblastoma) exhibited upregulated expression of VEGF-A, and this was significantly associated with a ~66% reduction in overall survival (Fig. 1A). High levels of the VEGFR-1 specific ligand PlGF were detected in 347 of 690 patients and were significantly associated with reduced survival (~60%; Fig. 1B). These data support the involvement of VEGFR-1 expression in the aggressiveness of GB.

Differences in VEGFR-1 expression between tumour and brain parenchyma in human GB specimens. To analyse the distribution of VEGFR-1 expression in GB, tissue specimens of the tumour and matching surrounding parenchyma of patients with GB were collected following surgical removal of the tumours from 42 patients. In agreement with our previous study (8), the majority of GB tissue samples (>90%) showed >25% of total cells positive for VEGFR-1 staining (Table I). There was no significant association of VEGFR-1 expression with age or primary and recurrent GB.

The tumour tissue presented a significantly higher number of VEGFR-1-stained cells compared with the surrounding parenchyma (Fig. 2). In fact, in the parenchyma ~40% of the cells were positive for VEGFR-1 expression; whereas in the tumour, the percentage of cells expressing VEGFR-1 was ~70% (Figs. 2 and S1). These data suggest that VEGFR-1 is expressed in both GB cells and cells of the microenvironment.

VEGFR-1 expression is increased in GB-associated microglia. As VEGFR-1 staining was observed also in cells of the tumour-associated microenvironment, and microglia are the principal resident immune cells in the CNS, the expression of VEGFR-1 was analysed in GAMs. To determine whether

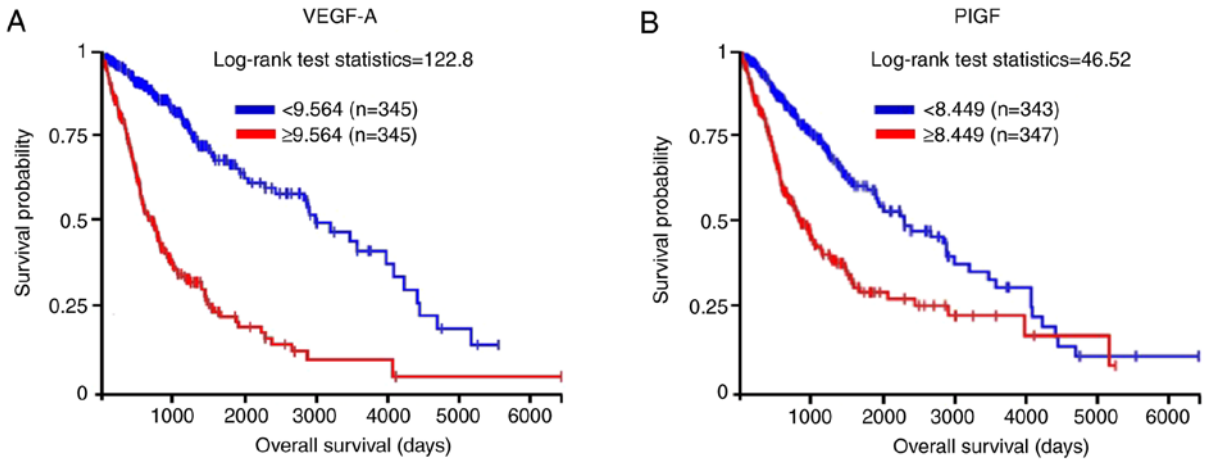

Figure 1. Association between VEGF-A or PlGF expression in tumour tissues with survival of GB patients. Data from TCGA for GB samples were examined for a possible correlation between overall survival and gene expression levels of VEGF-A (A) and PlGF (B). A total of 690 patients were divided into two groups representing: Low expression, blue lines, n=345 for VEGF-A and n=343 for PlGF; and high expression, red lines, n=345 for VEGF-A and n=347 for PlGF. Kaplan Meier curves were generated to determine the association between expression and survival using a log-rank test. P<0.0001 in both cases.

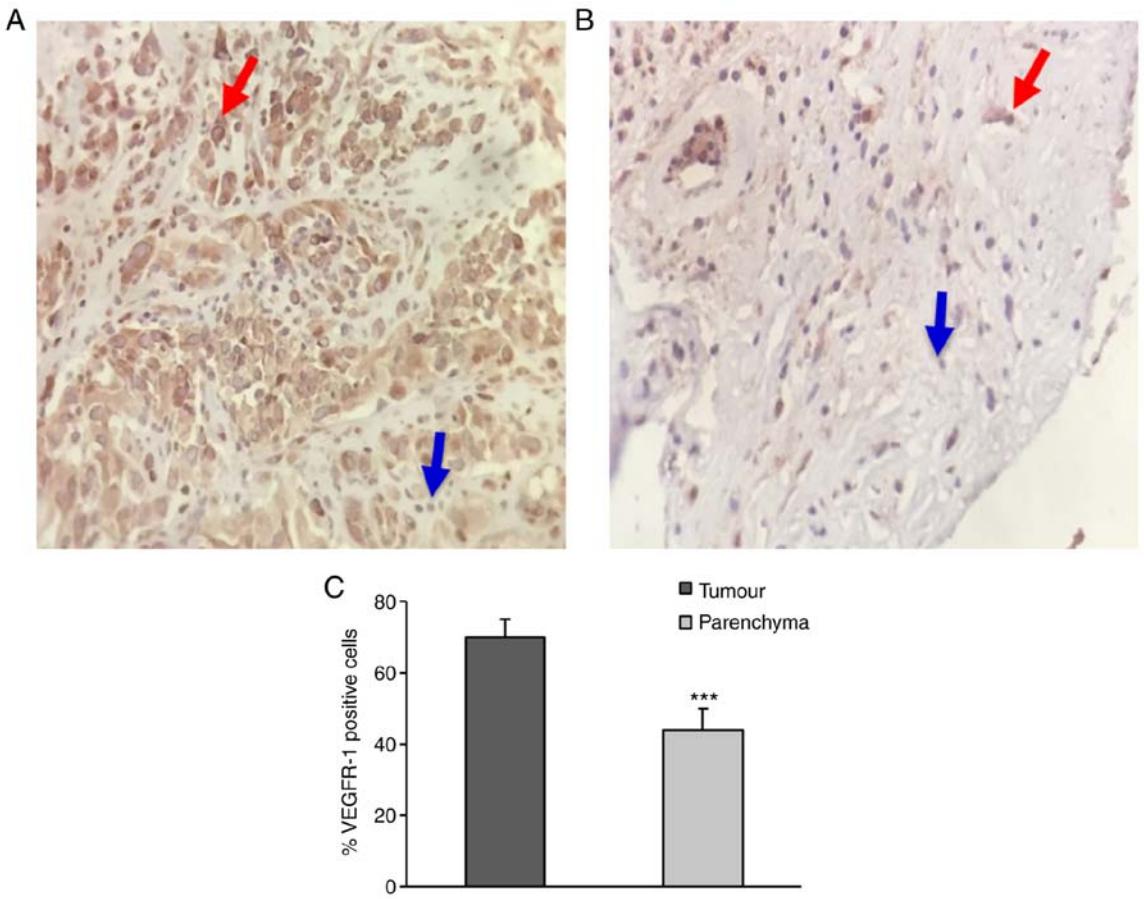

Figure 2. VEGFR-1 staining in GB specimens. Representative images of tumour (A) and parenchyma (B) fields stained with the anti-VEGFR-1 antibody. Magnification, x40. Red arrows and blue arrows point to VEGFR-1-positive and -negative cells, respectively. (C) Histogram representing the percentage of VEGFR-1-positive cells in each GB specimen, taking into account the tumour or the parenchyma region. Data are expressed as mean ± standard error of the mean (SEM) of samples collected from 42 patients. ***P<0.001.

VEGFR-1 was differentially expressed in the microglia infiltrating the tumours compared with those present in the peripheral parenchyma, 23 samples were selected with similar number of cells positive for Iba1, a microglia-macrophage biomarker, in the parenchyma and the tumour (36.9 vs. 35.6%, respectively), but with different levels of VEGFR-1 expression (Fig. 3A-D). Using double staining for VEGFR-1 and Iba1 in these samples, it was shown that the percentage of microglia-macrophages expressing VEGFR-1 present in the tumour (Fig. 3E) was significantly higher compared with

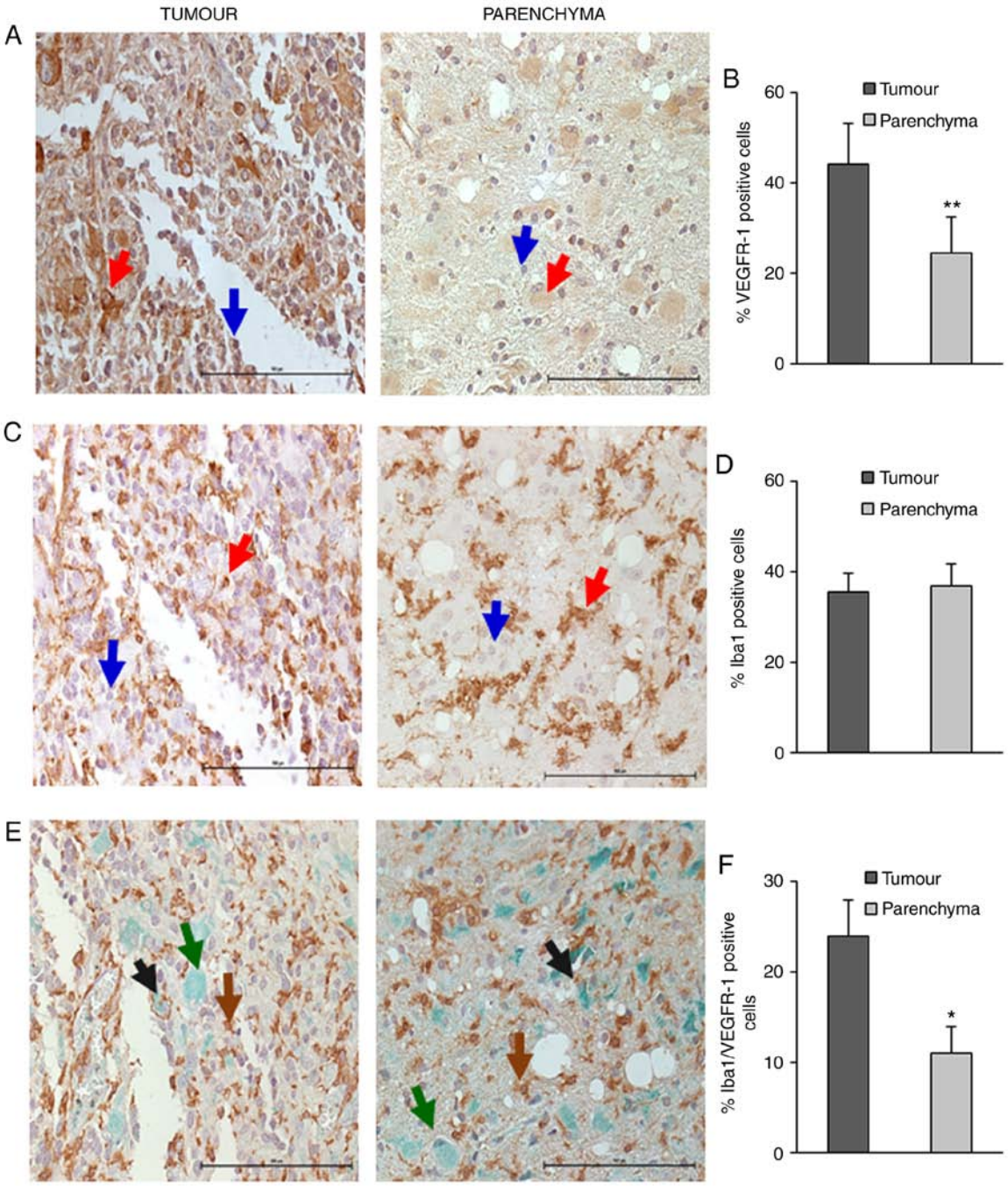

Figure 3. Iba1 and VEGFR-1 staining of microglia present in the parenchyma or tumour regions in GB specimens. Representative images of tumour regions stained for VEGFR-1 (A), Iba1 (C) and both proteins (E), and of parenchyma regions stained for VEGFR-1 (B), Iba1 (D) and both proteins (F). Magnification, x40. Red and blue arrows point to positive and negative cells, respectively. Brown, green and black arrows point to VEGFR-1, Iba1 and double-positive cells, respectively. Histograms indicate the percentages of VEGFR-1, Iba1 and Iba1/VEGFR-1 positive cells in the tumour and parenchyma regions. Data are expressed as mean ± SEM of 23 samples. Scale bar, 100 μm. **P<0.01; *P<0.05.

the parenchyma (Fig. 3F). Taking into account only the microglia-macrophage cell population in the tumour, 24% of the Iba1-positive cells also expressed VEGFR-1, whereas in the parenchyma the percentage of double-positive cells was 11% (Table SI). When considering all the cells, ~9 and 4% of the cells were Iba1/VEGFR-1 double-positive in the tumour and the surrounding tissue, respectively (data not shown).

To confirm VEGFR-1 expression in human microglia, the expression of this receptor was assessed in an immortalised human microglial cell line (IMhu). This cell line was shown to express considerable levels of the receptor compared with other GB cell lines. RT-qPCR analysis showed that VEGFR-1 expression was 4 times higher in IMhu cells compared with that found in the T98G, U87MG and A123 GB cell lines. The U373 line expressed very low levels of VEGFR-1 (Fig. 4A). To study the localisation of VEGFR-1, immunofluorescence experiments were performed using T98G and IMhu cells. As shown in Fig. 4B, both cell lines expressed VEGFR-1 on the cell surface, and a minor amount of expression was also observed at the cytoplasmic level. In addition, qualitative analysis performed by a researcher in a

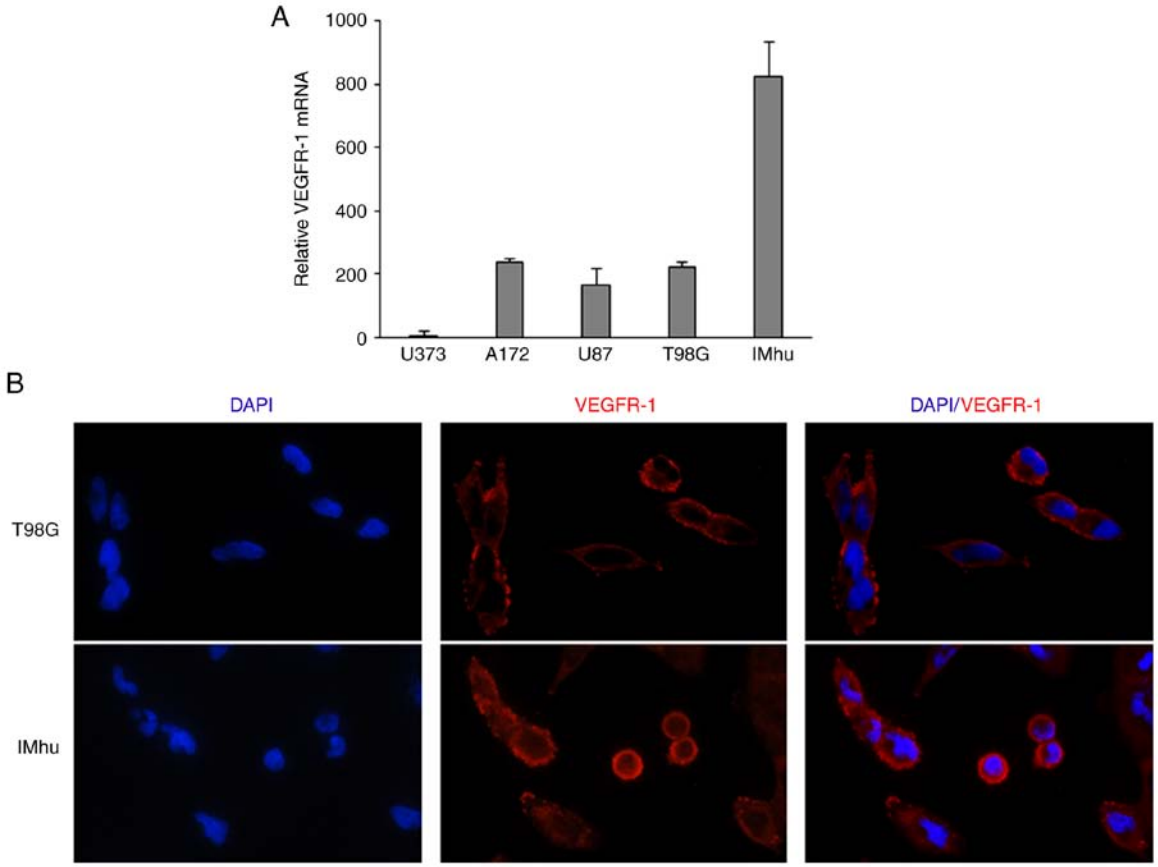

Figure 4. VEGFR-1 expression in human immortalised microglia and GB cells. (A) mRNA expression levels of VEGFR-1 expression in IMhu microglia cells and GB U373, A172, U87 and T98G cell lines. Data are expressed as mean ± standard deviation of three independent repeats. (B) Representative immunofluorescence images of staining for VEGFR-1 in T98G and IMhu cells. DAPI nuclear staining, VEGFR-1 staining and merged images are shown. Magnification, x40.

blinded manner confirmed that the expression of VEGFR-1 was higher in IMhu compared with that in the T98G cell line.

Discussion

In our previous studies, it was demonstrated that in GB tissues a high percentage of cells express VEGFR-1 (8). In the present study, by analysing brain tissue specimens collected from patients with GB, it was demonstrated for the first time that: i) The number of VEGFR-1-positive cells was significantly higher in the tumour tissue compared with that noted in the surrounding parenchyma; ii) VEGFR-1-positive cells included a considerable quantity of GAMs; and iii) the number of VEGFR-1-positive microglia-macrophages was significantly higher in the tumour area compared with the parenchymal region.

The data in the present study support the notion that VEGFR-1 and its ligands are involved in the pathology of GB. High expression levels of VEGF-A and of the selective VEGFR-1 ligand PlGF are inversely associated with overall survival. Furthermore, in GB tissue sections a high percentage of cells, including the tumour and microenvironment cells, were found to express VEGFR-1. A reliable analysis of the effect of membrane-bound VEGFR-1 expression on survival could not be performed, as the probe utilised in the consulted database did not discriminate between the membrane and soluble forms of the receptor, which possess opposite effects on angiogenesis and tumour progression.

VEGF-A has been extensively studied in regard to its role in a number of different types of cancer, including GB, with particular attention to VEGF-A/VEGFR-2-mediated pathways. In 2009 bevacizumab as a single-agent therapeutic was granted provisional approval under the FDA's accelerated approval program for the treatment of recurrent GB, based on the observation of durable objective responses in two phase II clinical trials (34,35). In 2017, bevacizumab received full approval based on the results of a phase III clinical trial on the combination of bevacizumab with lomustine. An increase of 2.7 months in progression-free survival (PFS) was observed in the cohort treated with the drug combination compared with chemotherapy alone (36). However, EMA considered these data insufficient and refused the marketing authorization of bevacizumab to treat GB. In line with the EMA decision, in 2014, a phase III trial in newly diagnosed GB, testing bevacizumab in addition to the standard therapy (radiotherapy-temozolomide followed by maintenance temozolomide), did not show significantly improved survival (37). Another phase III trial in the same clinical setting reported a higher PFS in the bevacizumab cohort compared with the control cohort, but with no difference in overall survival. Furthermore, in contrast to other phase III clinical trials, a decline in health-related quality of life and a greater deterioration in neurocognitive

function were more frequently observed in patients receiving bevacizumab compared with those receiving the placebo (38).

The causes of bevacizumab failure/resistance are not yet completely understood but are often associated with changes in the tumour microenvironment. In preclinical models and in clinical specimens from patients with GB whose tumours progressed during bevacizumab treatment, an increase in the presence of GAMs was reported, which was also correlated with a less favourable survival (39-41). In addition, resistance to bevacizumab was associated with decreased expression of macrophage migration inhibitory factor, which drives polarization of antitumour M1 macrophages, and with an increase of pro-tumoural M2 macrophages (42). These data provide a rationale for combining anti-angiogenic therapies with strategies which target M2 macrophages or promote polarization of macrophage-microglia toward the M1 phenotype.

GAMs are known to stimulate angiogenesis and invasion in response to various cytokines or growth factors, including basic fibroblast growth factor, MMP9 and VEGF-A and this has previously been reviewed elsewhere (21). Under pathological conditions, GAMs are a mixture of antitumour (M1) and pro-tumoural (M2) phenotypes. Our laboratory previously demonstrated a characterization of the GB microenvironment showing that a significant proportion of cells expressing the M2 and M1 markers, CD163 or arginase 1 and the inducible nitric oxide synthase, respectively, are present in the GB tissue (30). In addition, bone marrow-derived cells, including $CD163^+$ M2 GAMs, have been associated with tumour progression, angiogenesis and treatment failure (43,44).

In a murine model, VEGFR-1 was found to be preferentially expressed in M2 tumour-associated macrophages (TAMs) (45). Moreover, VEGFR-1 activation by PlGF was able to stimulate angiogenesis initiated by TAM polarised toward the pro-tumoural M2 phenotype (46). It should be noted that the expression of VEGFR-1 and VEGF-A might not necessarily correlate. In fact, in the tumour microenvironment other cell types different from cancer cells can produce the VEGFR-1 ligands VEGF-A and PlGF. Therefore, we focused our attention on VEGFR-1 expression in tumours as well as in microglial cells. The present study demonstrated that VEGFR-1 is expressed in a quarter of GAMs and the percentage of positive cells was significantly higher in the tumour compared with the parenchyma. These results reinforce the rationale for targeting VEGFR-1 in GB, as blockade of this receptor may exert antitumour activity via various direct and indirect mechanisms involving tumour, endothelial cells and GAMs. Thus, VEGFR-1 inhibition may impair: i) GB cell invasiveness and vasculogenic mimicry (formation of vascular channels similar to those produced by endothelial cells); ii) tumour-associated angiogenesis, and iii) activation of GAMs with the pro-tumoural M2 phenotype which further stimulates formation of new vessels and brain parenchyma infiltration by GB cells. A critical issue associated with VEGFR-1 targeting is that molecules directed toward this receptor should inhibit signal transduction through the membrane protein whilst maintaining the antitumour/antiangiogenic activity of the soluble form, which is able to sequester VEGF-A and PlGF released in the extracellular matrix. In fact, a low sVEGFR-1/VEGF-A ratio in GB has been associated with higher aggressiveness compared with astrocytoma (47). These properties are recapitulated in the anti-VEGFR-1 mAb D16F7 developed by our laboratory previously, since this mAb inhibits the membrane receptor activation without affecting ligand binding, and it does not interfere with the decoy function of the soluble receptor. Therefore, the manipulation of GAMs-glioblastoma crosstalk through the VEGFR-1 axis may represent a suitable and promising therapeutic strategy for the treatment of GB.

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Availability of data and materials

The analysed datasets generated during the study are available from TCGA database in Xena browser (https://xenabrowser.net).

Authors' contributions

LL, PML, GG and PN designed the experiments and drafted the manuscript. All authors critically reviewed and revised the article. LL, GMPC, MC, FR and PML performed the experiments and analysed the results. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All patients signed an informed consent form, and the experimental protocol was approved by the Ethics Committee of Foundation 'Agostino Gemelli' University Hospital (Rome, Italy).

Patient consent for publication

Not applicable.

Competing interests

The authors have declared no competing interests.

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