RESEARCH ARTICLE

Quantitative mRNA expression of genes involved in angiogenesis, coagulation and inflammation in multiforme glioblastoma tumoral tissue versus peritumoral brain tissue: lack of correlation with clinical data

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ABSTRACT. Glioblastoma multiforme is a very aggressive brain tumor. Angiogenesis, the formation of new blood vessels from pre-existing ones, is a process that plays an essential role in cancer development. The evolution of this process depends upon several proangiogenic factors as well as inhibitors of angiogenesis. Coagulation and inflammation also play an important role in tumorigenesis. Their expression is controlled by over- or under-expression of certain genes. The objective of our study was to evaluate the expression, in tissue samples, of a set of six genes involved in tumoral angiogenesis. The study concerned a group of 14 patients with pathologically-confirmed glioblastoma multiforme. Samples of tumoral and peritumoral brain tissue were taken during surgery. We used RT-PCR to evaluate the expression of six genes involved in angiogenesis: VEGF, PDGF, EPCR, TNF, ICAM-1 and CTGF. Gene expression was calculated by comparing the values obtained for the tumoral tissue with those obtained for the peritumoral tissue. Increased transcription of VEGF, PDGF and ICAM-1 genes were observed in glioblastoma tumoral tissues compared with peritumoral brain tissues. Correlations were observed between transcription of the CTGF and TNF genes (rho = 0.54, p-value = 0.05) and PDGF and ICAM-1 genes (rho = 0.54, p-value = 0.05). Under-expression of CTGF, EPCR and TNF genes was observed in glioblastoma tumoral tissue in comparison with peritumoral brain tissue. The association between gene expression and histopathological results (endothelial hyperplasia, coagulation necrosis and ischemic necrosis) was evaluated. No statistically significant associations were observed between gene expression and survival rates.

Key words: glioblastoma multiforme, angiogenesis, inflammation, coagulation, RT-PCR, histopathology

Despite the availability of multimodal treatment [1], glioblastoma multiforme (GBM) remains the brain tumor with the worst prognosis, having an average, overall survival rate of 12-15 months [2]. The development of malignant tumors is governed by complex interactions between angiogenesis, coagulation and inflammation [3]. GBM has one of the highest angiogenesis rates of all solid tumors [2]. It is also one of the tumors associated with the highest rate of thrombotic events [3]. The main histopathological characteristics of GBM are the presence of atypical nuclei, mitosis, endothelial hyperplasia, coagulation necrosis and ischemic necrosis [4]. Angiogenic vessels in GBM have a typical morphology; they are disorganized and leaky, with increased permeability disrupting the blood-brain barrier [5]. Vascular endothelial growth factor (VEGF) has been shown to play an important role in the development of GBM [6, 7], and is activated by hypoxia [8]. Platelet-derived growth factor (PDGF) stimulates proliferation and migration of endothelial cells [9]. It has also been shown to stimulate the proliferation of glial and smooth muscle cells [10]. VEGF and PDGF play a determinant role in the development of GBM, representing an important target for treatment agents [11]. Intercellular adhesion molecule-1 (ICAM-1) is a surface glycoprotein, overexpressed on the membrane of endothelial cells [12]. It represents the ligand for LFA-1, an integrin that activates the inflammatory response mediated by cytotoxic T cells. Downregulation of ICAM-1 leads to a decreased antitumoral response [13]. The expression of the molecule is regulated by various factors, including TNF-α [14]. Tumor necrosis factor (TNF-α) is a proinflammatory cytokine that acts through complex and poorly elucidated mechanisms in

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the tumorigenesis and angiogenesis of GBM [15]. Connective tissue growth factor (CTGF) is a protein included in the CCN family [16]. It is thought to stimulate extracellular matrix production, integrin expression, endothelial cell growth, adhesion and migration [17]. It modulates angiogenesis by inhibition and/or stimulation of MMP and TIMP activity [18], and it was also observed that it interacts with the VEGF-A molecule [19].

Endothelial receptor of C protein (EPCR) plays an important role in the activation of protein C [20]. Activated C protein (APC) is a molecule with anti-apoptotic, anticoagulant, cytoprotective and anti-inflammatory functions [21] that is activated by thrombomodulin and EPCR [20]. These molecules were seen to be associated with the development of pathological characteristics of GBM [22]. Many studies have tried to reveal the pathways underlying the tumorigenesis and angiogenesis of GBM, by studying the various molecules involved in these processes.

The purpose of this study was to evaluate the expression of six genes involved in angiogenesis, coagulation and inflammation pathways, in a group of patients with glioblastoma multiforme, and investigate any possible associations with clinical and pathological features, and outcome. The genes we evaluated were: vascular endothelial growth factor (VEGF) gene, platelet-derived growth factor (PDGF) gene, tumor necrosis factor alpha (TNF-α) gene, intercellular adhesion molecule-1 (ICAM-1) gene, connective tissue growth factor (CTGF) gene and endothelial receptor of C protein (EPCR) gene.

PATIENTS AND METHODS

Patients. The study group consisted of 14 patients, with pathologically-confirmed glioblastoma, according to the WHO classification [17]. All patients underwent MRI before surgery. At 72h post-surgery, an MRI or TC was done, in order to evaluate the efficiency of the tumor resection. Patients were informed about the study objectives and gave their written informed consent to be included in the study. The confidentiality of the information was also ensured. All study procedures complied with EU norms and the Declaration of Helsinki.

Sample collection. Samples of tumoral and peritumoral tissue were taken during surgery and were stored in liquid nitrogen until gene expression was evaluated using qRT-PCR.

Determination of gene expression

Total RNA isolation. Total RNA was isolated with TriReagent (Sigma-Aldrich), and further analyzed for quantity and quality with ND-1000 and Agilent Lab-on-a-chip Bioanalyzer 2100 (Agilent Technology). All the RNAs presented an RNA integrity number (RIN) between 7.5 and 10.

cDNA synthesis. The Random Hexamer Priming method for the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnosis, Germany) was used for cDNA synthesis. Dilutions were performed for each sample so as to obtain a final concentration of 1000ng for each sample.

qRT-PCR. The LightCycler TaqMan Master kit was used for RT-PCR analyses according to the protocol provided by the manufacturer using the LightCycler 480 Detector System (Roche). Template cDNA (diluted 1:10) was added to TaqMan Universal Master Mix with specific primers and probes for each gene:

– β-actin: CCAACCGCGAAGATGA/CCAGAGGCGTACAGGGATAG,
– VEGF (vascular endothelial growth factor): CCACTTCgTgATgATTCgC/TACCTCCACCATgCCAAgT,
– PDGF (platelet-derived growth factor): TgATCTCCAACgCCTgCT/TCATgTTCAggTCCAACTCg,
– CTGF (connective tissue growth factor): CTCCTgCAggCTAgAgAAgC/gATgCACTTTTgCCCTTCTT,
– EPCR(endothelial protein C receptor): gTAgCCAAgACgCCTCAgAT/gATAggggTCgCggAAgTA,
– ICAM-1 (intercellular adhesion molecule-1): CCTTCCTCACCgTgTACTgg/AgCgTAgggTAAggTTCTTgC,
– TNF-α (tumor necrosis factor-alpha): CagCCTCTTCTCCTTCCTgAT/GCCAGAgggCTGATTAAgAgA.

The primers and UPL probes (Roche) used in the qRT-PCR evaluations were specific for every gene. For all the genes, we used 1 μM of both primers and 0.2 μM from the UPL (Universal Probe Library). Changes in the expression of target genes were measured relative to the mean critical threshold (Ct) values of the 18S housekeeping gene. The mRNA levels were analyzed using the $\Delta\Delta C_t$ method, which is based on fold-change calculations with normalization for all the genes.

Statistical analysis

Gene expression was calculated using the $\Delta\Delta C_t$ method. The gene ratio was calculated for each gene and represents the average of the values for gene expression.

Quantitative data were described using median and quartiles. Evaluation of normality was performed using QQ-plot curves and the Shapiro-Wilk test.

We evaluated the association between the quantitative expression of the six genes and the pathological characteristics of GBM (coagulation necrosis, ischemic necrosis and endothelial hyperplasia), using the Exact 2-Sample Permutation Test.

Bivariate correlation was performed using Spearman's correlation test, in order to evaluate the association between expression of the genes, as quantitative variables.

We considered a -fold regulation value <1 as underexpression and >1 as overexpression, for the PDGF, CTGF, TNF-α, ICAM-1 and EPCR genes. For the VEGF gene, the -fold regulation value was <3 for underexpression and >3 for overexpression, as all the expression values were higher than 1. We assessed the data for pathology (necrosis of coagulation, ischemic necrosis and endothelial hyperplasia) with gene expression (overexpressed/underexpressed). As these are qualitative variables, the relation between them was assessed using contingency tables with absolute values and percentages, Fisher's exact test, and relative risk and risk difference indicators.

Survival probability was described using the Kaplan-Meyer method, in order to analyze whether the expression of the genes might influence the disease-free survival rate (DFS) or the overall survival rate (OS) of the patients. The expression of the genes was reported to a cut-off point (cut-off point = 3 for VEGF gene, cut-off point = 1 for the other genes). The comparison between survival curves of subgroups with over- or underexpression of the genes was performed using the log-rank test. A Cox

proportional-hazard regression analysis was performed, in order to determine the hazard ratio, with a confidence interval of 95%.

We used the R environment for Statistics and Graphics, version 1.12.1, with the graphic medium Rcmdr, version 1.6-2. For the permutation tests, we used the R coin package. For Fisher's Exact test and survival analysis we used Epiinfo, version 3.5.1. For all tests, we have considered a p-value lower than 0.05 as statistically significant.

RESULTS

Between July 2009 and August 2010, 14 patients were included in the study, of which 57% were males. The average age was 55.5 years. The genes that displayed overexpression were VEGF (gene ratio = 6.55), PDGF (gene ratio = 1.55) and ICAM-1 (gene ratio = 2.03). The underexpressed genes were EPCR (gene ratio = 1), TNF-α (gene ratio = 0.81) and CTGF (gene ratio = 0.66) (*table 1*).

Correlations between expression of the genes are shown in *table 3* . An association was found between the TNF-α and CTGF genes (rho = 0.54, p-value = 0.05) and the PDGF and ICAM-1 genes (rho = 0.54, p-value = 0.05).

Associations between histopathological results and gene expression

Median values for gene expression in patients, compared with histopathological results are shown in *table 2*.

Quantitative expression of the genes was not statistically significantly associated with histopathological results (p-value>0.05) (*table 4*).

The values for the relative risk indicator, risk difference and p-value for the association between gene expression and histopathological results are found in *tables 5*, *6* and *7*.

Patients with overexpression of CTGF and EPCR genes showed a decreased risk of developing coagulation necrosis (RR<1). For TNF-α, the value for RR was undefined. The overexpression of ICAM-1, PDGF and VEGF genes might increase the risk of development of coagulation necrosis (RR>1). The results were not statistically significant (p-value>0.05) (*table 5*).

Patients with overexpression of PDGF and ICAM-1 genes showed a decreased risk of developing ischemic necrosis (RR<1). For EPCR, the value of RR was undefined. The overexpression of CTGF, TNF-α and VEGF genes might increase the development of ischemic necrosis (RR>1). The results were not statistically significant (p-value>0.05) (*table 6*).

Patients with overexpression of the CTGF gene showed a decreased risk of developing endothelial hyperplasia (RR<1). The overexpression of EPCR, ICAM-1, PDGF, TNF-alpha and VEGF genes might increase the development of endothelial hyperplasia (RR>1). The results were not statistically significant (p-value>0.05) (*table 7*).

Associations between gene expression and survival rates

The underexpression of the PDGF gene was associated with a longer DFS, between one and 16 months, and a longer overall survival rate after one month, according to Kaplan Meyer curves. Patients with overexpression of EPCR showed a tendency towards a higher DFS, between one and 12 months, and a higher OS after three months, according to the Kaplan Meyer curves. Overexpression of ICAM-1 was associated with a tendency towards a higher overall survival rate after three months. Patients with underexpression of TNF-α gene showed a higher DFS rate after one month and a higher OS rate after three months *(tables 8* and *9)*. The results were not statistically significant (p-value>0.05). For the CTGF and VEGF genes, the curves showed no differences for the OS and DFS rates (*figures 1* and *2*).

Table 1
Gene expression in patients

Sample number	VEGF	PDGF	ICAM-1	CTGF	EPCR	TNF-α
1	3.61	2.77	1.38	0.74	0.43	0.42
2	1.79	0.87	1.52	1.02	1.01	0.38
3	2.79	1.36	2.38	0.66	1.58	0.61
4	12.47	1.57	1.89	1.04	0.43	1.43
5	23.59	1.79	10.22	0.16	0.96	0.23
6	5.82	1.26	1.18	0.89	0.7	0.93
7	3.27	1.34	1.09	0.8	1.25	3.12
8	3.89	2.14	3.05	0.48	0.84	0.74
9	2.6	0.27	0.95	0.45	0.59	0.28
10	1.29	0.9	1.18	0.89	1.18	0.92
11	25.28	1.31	1.23	0.75	1.2	0.82
12	1.68	3.18	1.45	0.15	2.78	0.11
13	2.47	1	0.35	1.05	0.36	0.44
14	1.18	1.31	0.62	0.26	0.78	0.91
Average (gene ratio)	6.55	1.55	2.03	0.66	1	0.81

Table 2
Median values of gene expression in patients and histopathological results

Gene	Coagulation necrosis		Ischemic necrosis	
	Present (10 samples)	**Absent (four samples)**	**Present (four samples)**	**Absent (10 samples)**
VEGF	3.44 [1.707–5.337]	2.69 [2.567–8.412]	8.04 [3.357–15.250]	2.63 [1.707–3.735]
PDGF	1.45 [1.272–2.052]	1.15 [0.817–1.322]	1.68 [1.245–2.035]	1.31 [1.065–1.355]
ICAM-1	1.41 [1.18–1.797]	1.09 [0.80–1.517]	1.63 [1.272–3.972]	1.20 [1.112–1.502]
CTGF	0.77 [0.315–0.890]	0.70 [0.607–0.825]	0.59 [0.377–0.815]	0.77 [0.525–0.890]
EPCR	0.90 [0.720–1.137]	0.89 [0.532–1.295]	0.51 [0.430–0.682]	1.09 [0.795–1.237]
TNF-α	0.91 [0.38–0.93]	0.52 [0.40–0.662]	0.35 [0.267–0.672]	0.82 [0.44–0.92]

Values are expressed as median [quartile 1-quartile 3]

Table 3
Spearman's correlations for gene expression

	VEGF	***PDGF***	***ICAM-1***	***CTGF***	***EPCR***	***TNF-α***
VEGF						
Rho		0.33	0.42	0.09	-0.15	0.15
p-value		0.27	0.14	0.77	0.62	0.62
PDGF						
Rho			0.54	-0.45	0.22	
p-value			0.05	0.12	0.46	
ICAM-1						
Rho				-0.20	0.32	
p-value				0.52	0.28	
CTGF						
Rho				-	-	-
p-value						
EPCR						
Rho				-0.44		
p-value				0.13		
TNF-α						
Rho		-0.13	-0.27	0.54	-0.06	
p-value		0.66	0.36	0.05	0.83	

Table 4
Association between histopathological results and gene expression

Genes	Coagulation necrosis		Ischemic necrosis		Endothelial hyperplasia	
	Z	**p-value**	**Z**	**p-value**	**Z**	**p-value**
VEGF	-0.50	0.68	1.17	0.24	1.44	0.18
PDGF	1.60	0.11	0.29	0.78	0.38	0.72
ICAM-1	0.77	0.50	1.51	0.12	0.97	0.46
CTGF	-0.44	0.67	-0.51	0.62	0.26	0.79
EPCR	0.28	0.81	-1.53	0.12	-0.22	0.87
TNF-α	0.85	0.48	-0.69	0.59	0.53	0.75

DISCUSSION

The genes that showed overexpression were VEGF, PDGF and ICAM-1

Increased levels of VEGF and MVD were associated with lower survival rates and a higher rate of malignant progression in low grade astrocytomas [23].

The presence of ischemic and coagulation necrosis in GBM was associated with hypoxia and a procoagulative status that might facilitate the transition from astrocytoma to GBM [22]. Cells surrounding the areas of necrosis displayed an overexpression of VEGF and HIF-1 [24]. This would also lead to endothelial hyperplasia and tumor progression [25]. Our results also suggested the involvement of VEGF in the mechanisms of necrosis (RR>1), but the results were not statistically significant.

Table 5
Association between gene expression and coagulation necrosis

Gene	Coagulation necrosis		
	Relative risk (RR)	**Risk difference(RD)**	**p-value**
VEGF	1.5 [95% CI(0.73-3.04)]	28.57 [CI(-16.32; 73.47)]	0.27
PDGF	1.09 [95% CI(0.45-2.62)]	6.06 [CI(-53.42; 65.54)]	0.67
ICAM-1	1.8 [95% CI(0.66-4.9)]	40.0 [CI(-12.4; 92.4)]	0.17
CTGF	0.8 [95% CI(0.09- 6.54)]	-5 [CI(-54.14; 44.14)]	0.67
EPCR	0.8 [95% CI(0.23- 2.76)]	-10 [CI(-67.64; 47.64)]	0.59
TNF-α	Undefined	20.0 [CI(-4.79; 44.79)]	0.49

Table 6
Association between gene expression and ischemic necrosis

Genes	Ischemic necrosis		
	Relative risk (RR)	**Risk difference (RD)**	**p-value**
VEGF	1.87 [95% CI(0.72-4.83)]	35.0 [CI(-17.17; 87.17)]	0.27
PDGF	0.93 [95% CI(0.49-1.78)]	-5.0 [CI(-54.14; 44.14)]	0.67
ICAM-1	0.93 [95% CI(0.49-1.78)]	-5.0 [CI(-54.14; 44.14)]	0.67
CTGF	1.25 [95% CI(0.15-10.22)]	5.0 [CI(-44.14; 54.14)]	0.67
EPCR	Undefined	-60[CI(-90.36; -29.63)]	0.06
TNF-α	2.5 [95% CI(0.2-30.99)]	15.0 [CI(-31.33; 61.33)]	0.5

Table 7
Association between gene expression and endothelial hyperplasia

Genes	Endothelial hyperplasia		
	Relative risk (RR)	**Risk Difference (RD)**	**p-value**
VEGF	1.77 [95% CI(0.61-5.12)]	29.16 [CI(-21.31; 79.64)]	0.29
PDGF	1.60 [95% CI(0.93-2.73)]	37.5 [CI(3.95; 71.04)]	0.15
ICAM-1	1.11 [95% CI(0.64-1.9)]	8.33 [CI(-33.97; 50.63)]	0.61
CTGF	0.66 [95% CI(0.07-5.74)]	-8.33 [CI(-50.63; 33.97)]	0.61
EPCR	1.33 [95% CI(0.4-4.42)]	12.5 [CI(-39.71; 64.71)]	0.52
TNF-α	1.33 [95% CI(0.1-17.27)]	4.16 [CI(-33.44; 41.77)]	0.69

Table 8
Analysis of disease-free survival rate, according to gene expression

Genes	Median DFS		Log rank	p-value	HR
	Overexpression	**Underexpression**			
VEGF	12	11	1.07	0.30	0.48 [95% CI(0.11-2.06)]
PDGF	11	15	0.01	0.89	1.11 [95% CI(0.22-5.58)]
ICAM-1	12	11	0.62	0.42	0.52 [95% CI(0.09-2.86)]
CTGF	11	12	0.06	0.80	1.21 [95% CI(0.24-6.11)]
EPCR	12	11	0.05	0.81	1.17 [95% CI(0.29-4.77)]
TNF-α	6.5	11.5	1.80	0.17	2.82 [95% CI(0.54-14.65)]

PDGF, which has been shown to play an important role in the progression of low grade astrocytomas to GBM [26], was absent in normal brain tissue. One of the ways through which PDGF contributes to tumorigenesis might be the inhibition of tumor suppressor genes [27]. In our study, the underexpression of PDGF was associated with a tendency to a longer DFS, between one and 16 months, and a longer overall survival rate after one month, according to Kaplan Meyer curves. The results were not statistically significant (p-value$>$0.05).

PDGF has also been shown in previous studies to stimulate endothelial hyperplasia [28]. Our study also suggested that

Table 9
Analysis of overall survival rate, according to gene expression

Genes	Median OS		Log rank	p-value	HR
	Overexpression	Underexpression			
VEGF	14	11	0.01	0.91	0.91[95% CI(0.18-4.64)]
PDGF	11	15	0.41	0.51	1.96[95% CI(0.22-17.20)]
ICAM-1	14	11	1.23	0.26	0.39[95% CI(0.06-2.38)]
CTGF	11	13	0.05	0.81	0.78[95% CI(0.09-6.76)]
EPCR	13	12	0.40	0.52	0.59[95% CI(0.10-3.24)]
TNF-α	8.5	12.5	1.66	0.19	2.78[95% CI(0.50-15.21)]

the overexpression of PDGF, VEGF and TNF-alpha might stimulate the proliferation of endothelial cells, owing to their mitogenic activity, although the results were not statistically significant (RR>1, p-value>0.05).

ICAM-1 is absent in normal fetal brain tissue and low grade gliomas. In glioblastoma, it has shown a high level of expression. This molecule was found to be upregulated by IL-l, TNF-α and IFN-γ [14]. ICAM-1 plays an important role in the interaction between cytotoxic T lymphocytes and tumor cells. By binding LFA-1 (lymphocyte function-associated antigen-1), ICAM-1 molecules determine the activation of T cells. The downregulation of ICAM-1 will effect a decrease in the immune response of the host, though a lack of cytotoxic T cell activity. A low expression of the ICAM-1 gene is associated with a poor prognosis in melanoma, breast, colorectal and ovarian carcinoma [13]. ICAM-1 presented overexpression in patients with GBM, but not in patients with brain metastases or healthy patients [29]. Previous studies have demonstrated a decrease in intercellular adhesion in GBM. ICAM-1 and PECAM-1 molecules might contribute to the infiltrative potential of GBM [30].

In our study, patients with overexpression of the ICAM-1 gene showed a tendency towards a higher, overall survival rate, although the results were not statistically significant.

We have found an association between the PDGF and ICAM-1 genes (rho = 0.54, p-value = 0.05). Previous studies have not shown any association between these genes. A possible common mechanism of action for the PDGF and ICAM-1 genes might be the NF-κB pathway. PDGF has been shown to control the expression of NF-κB, which stimulates proliferation in glioblastoma cell lines [31]. NF-κB also induces the expression of ICAM-1, which might explain the association between the expression of the two genes [32]. IL-1 was shown to induce the expression of ICAM-1, in a PDGFR-, Src- and PI3K/Akt- dependent manner [33].

Previous studies have demonstrated overexpression of CTGF in patients with GBM. The CTGF molecule is a member of the CCN family. The nomenclature of this family of genes is based on Cyr 61(cysteine-rich protein or CCN1), CTGF (connective tissue growth factor or CCN2) and nephroblastoma-overexpressed protein (Nov or CCN3). This molecule was shown to mediate adhesion and migration of endothelial cells through the integrin(v)beta-3 pathway, to promote endothelial cell survival, and to induce angiogenesis *in vivo* [34]. CTGF was shown to stimulate tumor growth progression and drug resistance in GBM by increasing the expression of the antiapoptotic protein Bcl-2 [35]. Integrin avß3 was shown to increase Bcl-2 and inhibit expression of the Bax gene, thus contributing to resistance to apoptosis and promoting invasion in GBM cells [36]. Our study did not show statistically significant differences between the survival rates of the patients with overexpression and those with underexpression of the CTGF gene. Patients with GBM have an altered coagulation status and a high risk for pulmonary embolism and deep vein thrombosis [37].

High levels of EPCR have been identified in breast cancer cells. This molecule is involved in the pathogenesis of some types of neoplasia and also in the development of coagulopathy in cancer. EPCR is known as a negative regulator of coagulation, together with protein C and thrombomodulin [38]. Protein C plays a cytoprotective, anti-inflammatory and anticoagulative role [39]. Thrombin and thrombomodulin are not present in the brain. It is supposed that the activation of protein C takes places on the luminal side of the blood-brain barrier. A previous study showed that EPCR plays a major role in the transport of APC (activated protein C), in a dose-dependent manner [40]. The ligation of EPCR to APC in human renal proximal tubule cells inhibits the synthesis of TNF-α and downregulates the expression of extracellular matrix proteins [41]. APC protein and its receptor, EPCR, decreased the rate of lung metastasis in malignant melanoma, by inhibiting tumoral cell adhesion and transmigration, and by decreasing the expression of P-selectin and TNF-α [50]. Intravascular thrombosis might influence the transition from astrocytoma to GBM [2]. Our study showed that high levels of EPCR reduce the risk of tumor progression by decreasing the risk of coagulation necrosis and endothelial hyperplasia, which are two of the main characteristics that differentiate astrocytoma from glioblastoma (RR>1).

TNF-α influences tumor development and angiogenesis in a diverse manner. Low levels of this molecule were shown to stimulate angiogenesis, while high levels have an inhibitory effect. It was also shown to mediate VEGF, IL-8 and bFGF expression [42].

A previous study demonstrated a relationship between the low levels of TNF-α, and the aggressiveness of gliomas. This might be due to the fact that APCs would not be activated and so CD4+ and CD8+ cells would not infiltrate the tumor, which is associated with poor prognosis [43].

Bone marrow-derived human mesenchymal stem cells treated with TNF-α, achieved astroglial cell morphology. Proliferation was not affected. TNF-α was shown to upregulate the expression of certain genes important in the growth and functioning of neural cells, including SOX-2,

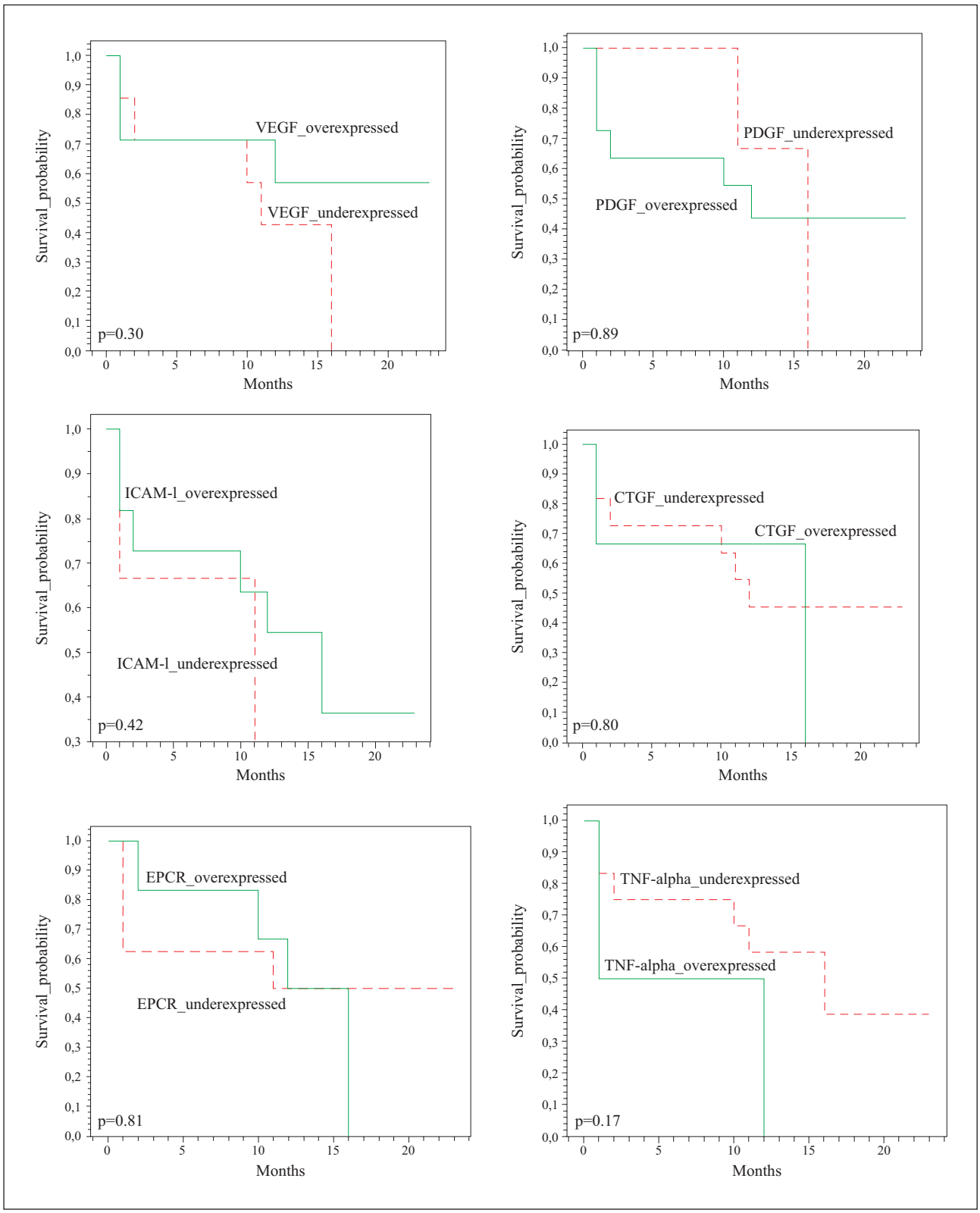

Figure 1
Relationship between disease-free survival rate (months) and gene expression.

LIF, BMP-2, and GFAP [44]. In glioma cells, the oxidative stress induced by TNF-α was shown to influence the Akt pathway and to affect actin cytoskeletal organization [45]. It also increased susceptibility of glioblastoma cells to NK cell cytolysis [46].

A previous study found no significant associations between plasma levels of TNF-α and VEGF, and patient survival [3]. In our study, patients with an underexpressed TNF-α gene showed a tendency towards higher DFS and OS rates.

We found no association between the TNF-α and ICAM-1 genes. The complex mechanisms of action of the TNF-α gene on the tumorigenesis and angiogenesis of GBM remains a mystery that needs to be solved, as the results obtained up until now are controversial.

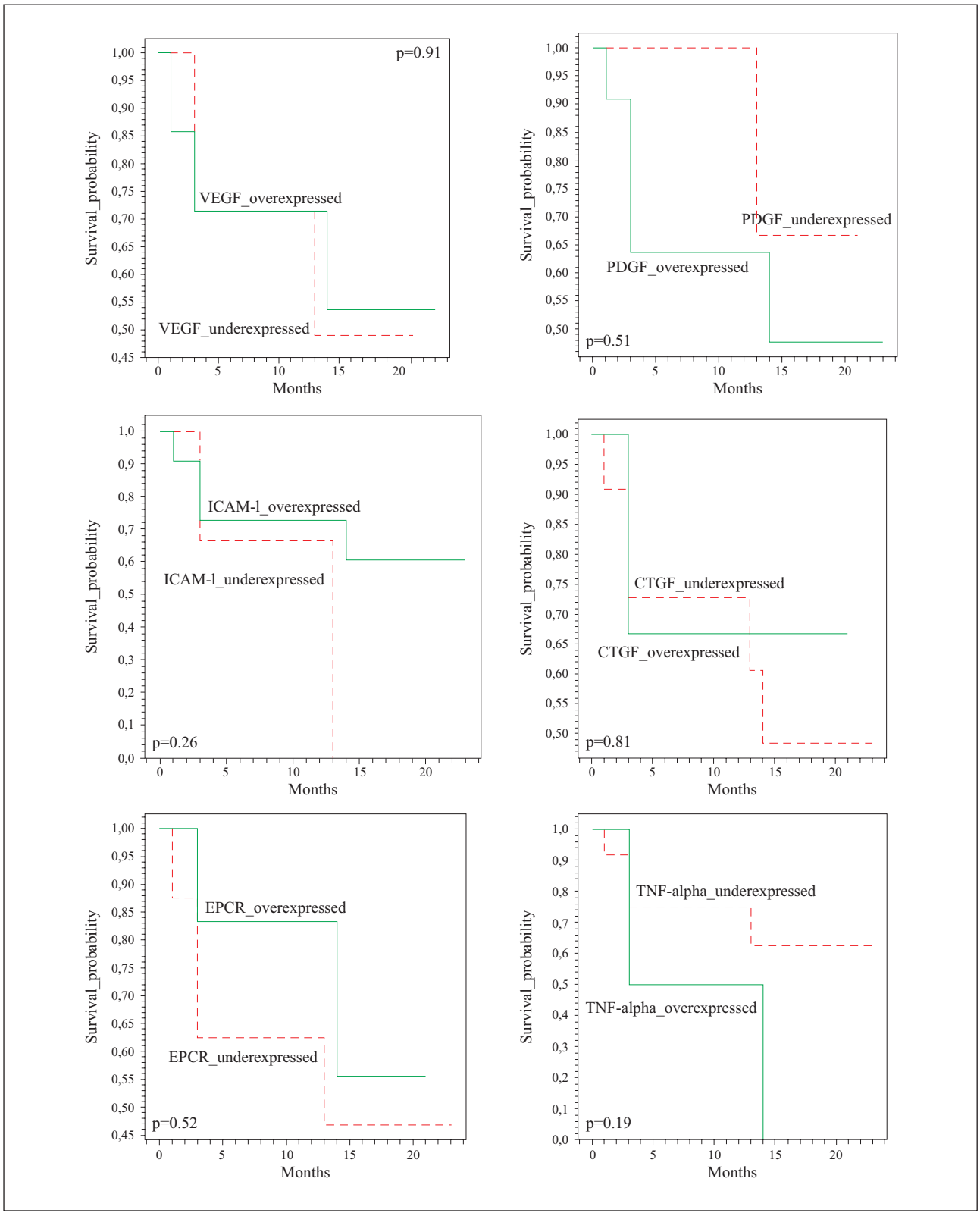

Figure 2
Relationship between overall survival rate (months) and gene expression.

We have found an association between the TNF-α and CTGF genes (rho = 0.54, p-value = 0.05). Previous studies have not shown any association between these groups of genes. The relationship between the TNF-α and CTGF genes might be explained by various pathways, of which one could be the MMP pathway. TNF-α modulates the activity of MMP on glioblastoma cell lines in various ways. It can promote invasion, by enhancing the activity of some MMP, or it can inhibit it by decreasing MMP activity [47]. Overexpression of the MMP-9 molecule was also associated with lower survival rates in GBM [48]. MMP-2 is known to play an important role in the development of

glioma cell lines by inhibition of apoptosis. [15]. TNF-α stimulates the expression of the CX3CL1 molecule, which upregulates MMP-9. Overexpression of CX3CL1 was associated with lower survival rates in GBM [49]. The effect of CTGF on MMP in glioblastoma has not yet been demonstrated. In gastric cancer cells, CTGF upregulates the expression of MMP-2 and MMP-9 [50].

A common mechanism of action might be mediated via TGF-beta. This molecule was shown to be involved in the pathogenesis of glioblastoma. It stimulates tumor growth, angiogenesis and immunosuppression [51, 52]. TNF- α was shown to promote the expression of TGF-β in lung fibroblasts [53], while TGF-β was shown to stimulate the expression of CTGF in smooth muscle cells of rats [54].

CRP was shown to upregulate the expression of TNF-α in a mouse model of diabetic kidney and also to stimulate the TGF-β/Smad/CTGF pathway [55].

CONCLUSIONS

Our study showed no statistically significant difference between the DFS and OS rates in patients with overexpression and those with underexpression of the VEGF and CTGF genes. The underexpression of PDGF and TNF-α showed a tendency towards higher DFS and OS rates, but the results were not statistically significant. Patients with overexpression of ICAM-1 and overexpression of the EPCR gene showed a tendency to higher overall survival rates. We found an association between the TNF-α and CTGF genes, and between the PDGF and ICAM-1 genes.

In our study, overexpression of ICAM-1, PDGF and VEGF genes was clinically associated with an increased risk of the development of coagulation necrosis (RR>1) and overexpression of CTGF and EPCR genes with a tendency towards a decreased risk of the development of coagulation necrosis (RR<1). Overexpression of CTGF, TNF-α and VEGF was clinically associated with an increased risk of developing ischemic necrosis (RR>1), while overexpression of the PDGF and ICAM-1 genes was associated with a tendency towards a decreased risk of developing ischemic necrosis (RR<1). Overexpression of EPCR, ICAM-1, PDGF, TNF-α and VEGF genes could be relevant, from a clinical point of view, as regards a higher risk of the development of endothelial hyperplasia (RR>1), while the overexpression of CTGF might indicate a lower risk (RR<1). The results are not statistically significant (p-value>0.05).

The lack of statistical significance of our results might be due to the small number of patients. Some of our data have clinical significance, which suggests that further studies, on a larger group of patients, are needed in order to confirm our results. Determination of serum marker levels in the same patients, and associations with gene expression would also be necessary in order to validate them as markers of prognosis.

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