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Expression of Vascular Endothelial Growth Factor A and Its Type 1 Receptor in Supratentorial Neoplasm

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Abstract

Background: Vascular endothelial growth factor (VEGF) is one of the primary angiogenesis regulators in solid cancers. Brain solid tumors are life-threatening diseases in which angiogenesis is an important phase of tumor development and progression. In the present study, VEGF-A and VEGF receptor (VEGF-R1) gene expression was evaluated in CNS brain tumors.

Methods: VEGF-A and VEGF-R1 expression was quantified using real-time PCR on fresh biopsies of 38 supratentorial brain tumors compared to 30 non-tumoral tissues. Then, the correlations were investigated with clinic-pathological and demographic factors of the patients.

Results: PCR product sequencing confirmed the validity of qRT-PCR. Although VEGF-A and VEGF-R1 expression showed increasing trends with the progression of cell proliferation in different stages of astrocytoma, VEGF-R1 did not meet the 95% confidence interval in other brain tumors. An increasing trend in VEGF-A expression and a declining trend in VEGF-R1 expression from Stage I to II were observed in meningioma. VEGF-A and VEGF-R1 expression had no significant correlation with age and gender. Although peritumoral brain edema (PTBE) in astrocytoma was significantly associated with tumor stages, VEGF-A and VEGF-R1 were not correlated with PTBE in meningioma and metastasis.

Conclusions: VEGF-A is a valuable factor for the prognosis of PTBE and malignancy in astrocytoma and is helpful in monitoring treatment approaches.

Keywords: Angiogenesis, Brain edema, Brain neoplasm, Peritumoral brain, VEGF, VEGFR1.

Introduction

Vascular endothelial growth factor (VEGF), a potent mitogenic and angiogenic di sulphide-linked homodimer, is one of the central angiogenesis regulators and an essential factor in promoting migration, proliferation, and tube formation of endothelial cells (1, 2). These molecules bind to endothelial cells through their tyrosine-kinase receptors (VEGFR-1) flt-1, FLK-1/KDR (VEGFR-2), which increase the permeability of endothelial cells up to 1000 times more than histamine (3). VEGF contributes to tumorigenesis, neovascularization, and edema development and progression (3, 4). Some previous studies have shown a significant correlation between increased VEGF mRNA expression and tumor vascularity in glioma, meningioma, and metastatic tumors (5-7). Moreover, increased angiogenesis associated with severe brain edema has been reported in astrocytoma before

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progression of malignancy (6). In patients with meningioma, there is a close relationship between peritumoral brain edema (PTBE) and VEGF and VEGFR expression (5, 8- 9). In clinical levels, variable results have been reported on VEGF expression in meningioma. Of note, the presence of edema cannot conclusively confirm the higher stages of malignancy in these tumors (3, 10). The extent of PTBE and VEGF expression in glioma is associated with the malignancy stage (11), meaning that glioma in the lower stage has low VEGF expression. VEGF expression will increase more than 50 times when PTBE shifts toward a more malignant tumor phenotype (3). Furthermore, a significant correlation has been observed between VEGF and VEGFR expression in the pathological stage of astrocytoma, which is more evident in the case of VEGF expression (5). On the other hand, the expression of VEGF receptors is not necessarily related to VEGF expression and is different in several forms of tumors (5). Huang et al. reported a different result pattern of VEGF and VEGFR expression in various brain tumors, which are not necessarily parallel to other studies' findings (6). In general, better understanding of the role of VEGF expression in brain tumors and its impact on tumor mobility toward different stages of malignancy and vascularity helps researchers introduce more useful therapeutic approaches based on inhibition of VEGF or VEGFR signaling.

Therefore, in this study, VEGF-A and VEGF-R1 expression was evaluated in meningioma, astrocytoma, and metastatic levels of brain tumors because of their importance in tumor angiogenesis.

Materials and Methods

Subjects

A cross-sectional study was carried out from April to December 2010 on 38 newly diagnosed patients with supratentorial tumors in the neurosurgery ward at the Ghaem and Kaamyab Hospitals, the Mashhad University of Medical Sciences, Mashhad, Iran. All the patients were visited by two experienced neurologists and referred for three-dimensional MRIs to approve supratentorial tumors. Patients with a history of previous treatment or any other malignancy were excluded from the study. After removing the tumor, two separate specimens were kept from each patient. One specimen was transferred to the pathology unit for confirmation. The demographic and medical history data for each patient were recorded, and tumors were classified into three different forms; astrocytoma, meningioma, and metastatic, based on the World Health Organization's parameters and previously reported results.

RNA extraction and cDNA synthesis

RNA samples were extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality was checked on 1% agarose gel. Then, 1 µL of the extracted RNA for each sample individually was used for making cDNA. Reverse transcription reaction was performed with the Revert AidTM H minus first strand cDNA synthesis kit (Fermentas, Germany) at 42 °C for 60 min followed by RT inactivation at 70 °C for 5 min. Ultimately, cDNAs were kept at minus 70 °C.

Primer design

Primers for VEGF, VEGF-R, and beta 2 microglobulin (β2M), as a reference gene, were designed at exon junctions using Beacon Designer. Then, the designed sequences were assayed with the program in Oligo software and blasted with the nucleoid blast online program (http://www.ncbi.nlm.nih.gov). The sequences of the oligonucleotides were: VEGF-A forward (5'- TGCAGATTATGCGGATCAAACC-3'), VEGF-A reverse (5'- TGCATTCACATTTGTTGTGCTGTAG-'3), VEGF-R1 forward (5'-ACTCAACTCCTGCCTTCTCTG-3') and VEGF-R1 reverse (5'-CTGCTGTCGCCCTGGTAG-3'); β2M forward (5'-CTTGTCTTTCAGCAAGGACTGG-3') and β2M reverse (5'-CCACTTAACTATCTTGGGCTGTG-3').

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After amplifying the genes using conventional PCR, the PCR product was sequenced to know the validity of our work using Applied Biosystems (SEQLAB, Germany).

Real-time PCR

Quantitative real-time PCR was performed on cDNA samples with SYBR® Premix EX TaqTM (Takara BioInc, Japan) using Rotor-Gene Q 6000 (Qiagen, Germany) and β2M as a housekeeping gene to normalize mRNA expression levels. The amplification efficiency was established by relative standard curves prepared using a ten-fold dilution series of a pooled cDNA for β2M, VEGF-A, and VEGF-R1. Relative gene expression levels of VEGF-A and VEGF-R1 were determined using standard curves.

Statistics analysis

Statistical analysis was performed using the SPSS statistics software package (SPSS 20). Results are presented as mean±SD. The normalization of the data was checked using a one-sample Kolmogorov-Smirnov test. The Mann-Whitney U test for non-parametric analyses and independent student's t-test for parametric tests was used to compare the groups' statistical differences. Pearson or Spearman coefficients were used to determine the relationship between the parameters. A P-value≤ 0.05 was considered statistically significant.

Results

Patient characteristics

Of the 38 patients, 14 had meningioma, 20 had astrocytoma, and four had metastatic tumors. Peritumoral brain edema with less than 2 cm in diameter was observed in 13 patients with meningioma tumors. Among 20 patients with astrocytoma tumor, 10 had PTBE larger than 2 cm, with one being in stage III and nine being in stage IV. Similarly, patients with metastatic tumors had PTBE larger than 2 cm. Table 1 shows the main data for the subjects.

VEGF and VEGF-R1 gene expression

The mean gene expression index of VEGF-A was respectively 78.70±3.91 and 440.22±34.12 in meningioma in stages I and II, 10.08±4.82, 68115.02±160.41, and 516831.5±224.95 in astrocytoma in stages II, III, and IV, and 3583.12±920 in metastatic tumors.

The mean gene expression index of VEGF-R1 was respectively 2.86±0.95 and 0.124±0.06 in meningioma in stages I and II, 7.86±5.24, 707.95±100.72, and 768.32±483.78 in astrocytoma in stages II, III, and IV, and 19.08±26.98 in metastatic tumors. No significant association was found between VEGF-R1 expression with age and gender in different forms of the tumors. Furthermore, no significant relationship was observed between VEGF-A and VEGF-R1 expression with meningioma grading (p= 0.1 and p= 0.4, respectively).

Table 1: Clinical features of the patients (n= 38).

Type	**Meningioma No: (%)**		**Astrocytoma No: (%)**			**Metastatic No: (%)**
Grade	**I**	**II**	**II**	**III**	**IV**	**-**
Gender						
Female	8:(61.5)	-	4:(66.7)	1:(100)	4:(30.8)	2:(50)
Male	5:(38.5)	1:(100)	2:(33.3)	-	9:(69.2)	2:(50)
Age (years)						
<40	4:(30.8)	-	3:(50)	-	2:(15.4)	1:(25)
40-60	7:(53.8)	1:(100)	3:(50)	1:(100)	7:(53.8)	3:(75)
>60	2:(15.4)	-	-	-	4:(30.8)	-
Peritumoral edema						
<2 cm	13:(100)	-	6:(100)	-	4:(30.8)	-
>2cm	-	1:(100)	-	1:(100)	9:(69.2)	4:(100)

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Variability in VEGF-A and VEGF-R1 expression

There was an increasing trend in VEGF-A expression from stage I to II in meningioma tumors (Fig. 1a). Albeit, VEGF-R1 expression showed a decreasing trend from stage I to II in these tumors (Fig. 1b). However, the increasing trend in VEGF-A and VEGF-R1 expression with astrocytoma grading did not meet a significant value in VEGF-R1 expression (p= 0.006, p= 0.07, respectively). Interestingly, there was an increasing trend in VEGF-A expression from stage II to III (p< 0.01, but not from grade III to IV (Figs. 1c and d).

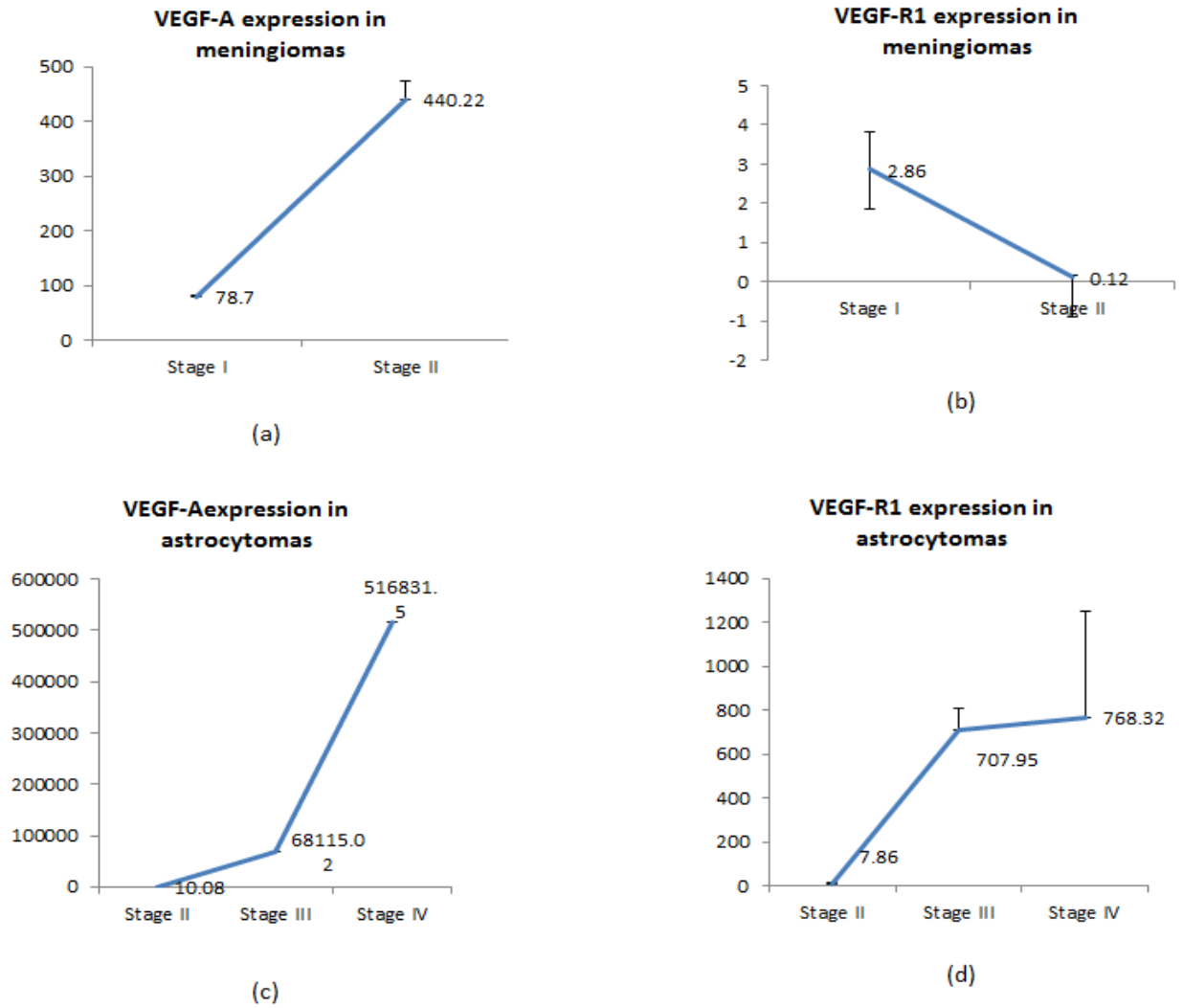

Fig. 1. Changes in VEGF-A and VEGF-RA1 expression based on World Health Organization (WHO) staging in meningiomas and astrocytomas.

VEGF-A and VEGF-R1 expression and PTBE

There was a significant correlation between VEGF-A expression and PTBE in astrocytoma (p= 0.001). However, we could not find any significant correlation between VEGF-A expression and PTBE in the meningioma and metastatic tumors. There was no correlation between the expression of VEGF-A and its receptor with the grading of the tumors. In contrast to meningioma, there was a significant relationship between PTBE and malignancy degree in astrocytoma (p= 0.01). Table 2 shows the mean gene expression index of VEGF-A and VEGF-R1 in the meningioma, astrocytoma, and metastatic tumors and their correlations based on age, gender, and PTBE.

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Table 2. Comparison of VEGF-A and VEGF-R1 expression in meningiomas, astrocytomas and metastatic tumors based on age, gender and peritumoral edema.

Type	Meningiomas		Astrocytomas		Metastatic	
Gene expression (mean±SD)	**VEGF**	**VEGFR**	**VEGF**	**VEGFR**	**VEGF**	**VEGFR**
Gender						
Female	311.98±282.62	89.96±88.53	3550134±39641.41	962.36±782.17	5265.271	
Male	542.16±94928	124.98±76.68	53657±16277	360.56±228.39	3504.52	
Age (years)						
<40	246.26±116.37	178.37±25.05	115.08±15.38	27.1±9.5		
40-60	645.34±109.56	73.53±13.58	6146364±15406	1195.79±216	438489±1245	19.08±6.9
>60	205.28	5.06	24416±3139	858.68±530		
Peritumoral edema						
<2 cm	150.95±92.86	246.26±116	949.26±542	236.99±64.3		
>2cm	2.124	645.34±109	155.38±115	2415±115	31399±2441	19.08±2.6

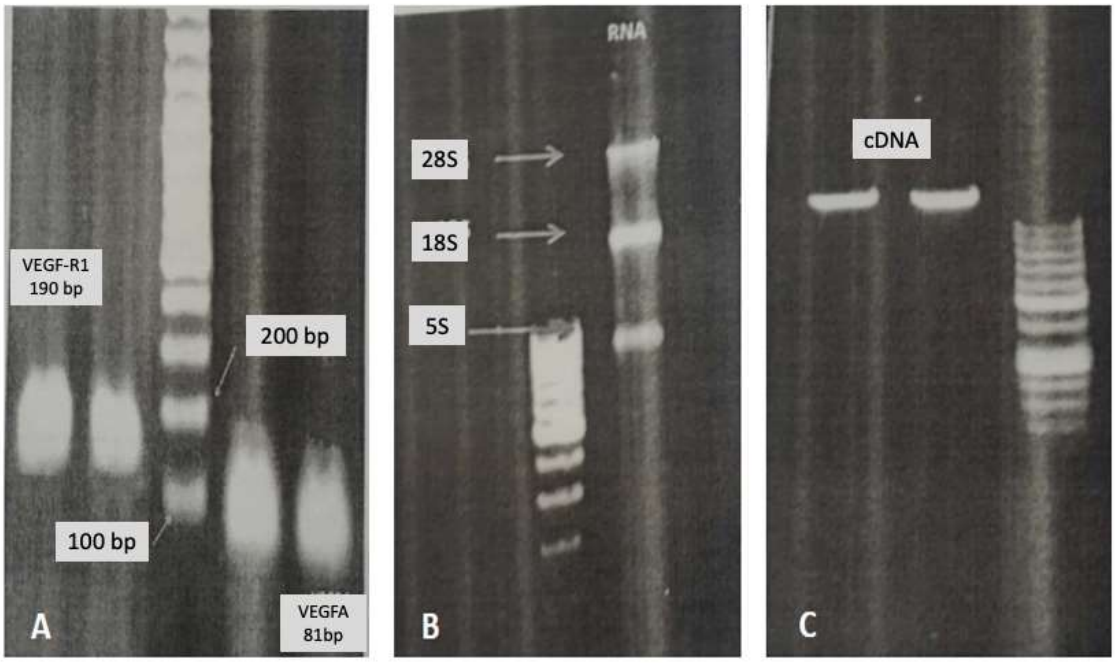

Fig. 2 A. The illustration of PCR products of VEGF & VEGF-RA1 with designed primers which the reliability confirmed by oligonucleotide sequencing. **B.** The quality of RNA extraction for VEGF & VEGF-RA1 **C.** The quality of cDNA made for the genes of interest.

Discussion

Many studies have focused on VEGF in cancer studies as a prognostic factor (12). Malignant brain tumors are one of the primary threatening diseases for human beings. VEGF has been considered a primary regulator of angiogenesis and a critical factor in tumor growth (13). However, main factors involved in VEGF expression are yet to be identified.

In the current study, VEGF-A expression was observed in glioblastoma and astrocytoma with low malignancy measures. This result may suggest that other factors might also be involved in VEGF-A expression. Several studies suggested hypoxia as one of the well-known regulators for VEGF production (14, 15). Findings in the present study showed the maximum expression of VEGF-A in malignant

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glioma (astrocytoma). Huang et al. (2005) investigated VEGF and VEGFR expression in 12 different forms of tumors, and reprted that VEGF (121) and VEGFR (165) were expressed in all types of tumors. However, the highest VEGF level was reported in glioblastoma and metastatic kidney tumors (6). VEGFR (189) expression was not an important marker in few tumors, and the expression of VEGF receptors was not correlated with VEGF. Moreover, VEGF and VEGFR were highly expressed in different patterns of glioblastoma. In meningioma, VEGF expression was low whereas VEGFR expression was high, although it was inverse in metastatic tumors (6).

Several studies have been performed to find the relationship between VEGF expression and malignancy measures (7, 12, 13, 16). In 2009, Holobilkova et al. demonstrated that VEGF expression increased in high stages of tumors; however, there was no correlation between VEGF and VEGFR expression and malignancy measure in astrocytoma (16). Higher VEGF expression in the high stage of astrocytoma may subsequently lead to activation of survival, angiogenesis, and cell migration (16). Similarly, in the present study, no relationship was found between VEGF-R1 expression and tumor and malignancy degree. In contrast, Xiang et al. demonstrated that the expression of VEGF and its receptors (Flt1 and Flk1) was correlated with malignancy in astrocytoma. VEGF, Flt1, and Flk1 can be considered important indicators of the malignancy potential for diffusely infiltrating astrocytoma (13). In a study by Lamszus et al. on gliomas, VEGFR-1 expression was correlated with the malignancy stages.

Furthermore, VEGFR-1 and VEGF-A expression levels were 12-fold and 30-fold higher in glioblastomas than in diffuse astrocytoma, respectively (7). In contrast to the relationship between VEGFR and the malignancy stages, which is not completely clear, the correlation between VEGF and the malignancy stages and angiogenesis development in brain tumors have been revealed by several studies (7, 13, 16, 17). Variation in the results of such projects might be due to different methodologies and populations.

In the current study, an increasing trend in VEGF-A expression and a decreasing trend in VEGF-R1 expression were found in meningioma from stage I to II. On the other hand, an increased level was observed in VEGF-A and VEGF R-1 expression in astrocytoma ($p= 0.006$, $p= 0.07$, respectively). Moreover, there was a significant increasing trend ($p< 0.01$) in VEGF-A expression from stage II to III, but not from stage III to IV. Dickinson et al. in a retrospective study on a variety of spontaneous canine CNS tumors found an increased VEGF expression in high stages of astrocytic (stage IV) and oligodendroglial (stage III) tumors and a lower VEGF expression in low stages of astrocytoma and meningioma (stage I) (18). Therefore, VEGF and VEGF-R are helpful to induce capillary growth into the tumor.

In this study, no significant correlation was found between the expression of VEGF-A and its receptor with gender and age, which is in agreement with the results of previous studies (19, 20).

It is well known that PTBE might be a serious barrier in treating brain tumors (21). In our study, the effect of VEGF-A as a predominant factor was considered on the growth and permeability of vessels in brain tumors and PTBE. A significant increase in VEGF-A expression was found in astrocytoma with PTBE development. In meningioma, PTBE pathogenesis is still unknown. Several studies showed a significant correlation between VEGF expression and the presence of PTBE in stage II and III meningiomas (22-24). However, in our study, most of the patients were in stage I meningioma, and we could not find a significant correlation between PTBE and VEGF-A expression. In 2008, YaSuo Ding et al. evaluated VEGF protein and gene expression in human meningioma and peritumoral brain sites. They found a higher VEGF expression in the tumor compared to its surrounding tissues. However, VEGF was not observed in peritumoral brain sites. VEGF protein levels in this site were correlated with the extent of PTBE, suggesting that VEGF macromolecules were secreted by the tumor tissue and entered into the peritumoral normal brain tissue to induce edema (21).

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Moreover, in a study conducted by Berkman et al., VEGF expression significantly increased in the highly vascular and edema-associated CNS neoplasms, suggesting a significant role for VEGF in developing CNS tumor neovascularity and PTBE (25). Most of the studies on glioma have shown a direct correlation between VEGF expression and the malignancy stage. Gliomas in the low malignancy stage have lower VEGF expression whereas glioblastoma development is accompanied by a 50- to 100-fold increase in mRNA VEGF expression. It appears that other conditions, such as lack of functional suppressor gene P53, regulate VEGF levels (3).

The findings of this study demonstrate that VEGF-A and its receptor as permeability and growth factors are expressed in meningioma and astrocytoma and metastatic brain tumors. The high expression of these factors, particularly VEGF-A, is associated with the high proliferation of tumor cells and progression toward the upstage of malignancy. Furthermore, VEGF-A expression in astrocytoma is directly correlated with the formation of PTBE and the malignant stage. A better understanding of VEGF regulatory mechanisms involved in tumor angiogenesis and edema formation is helpful to develop more prognostic methods for detecting malignancy and introducing therapeutic approaches in different types of malignant brain tumors.

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