Original Article

Vascular Endothelial Growth Factor-A mRNA Gene Expression in Multiple Sclerosis Egyptian Patients

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ABSTRACT

Background: Vascular endothelial growth factor-A (VEGF-A) is a pro-angiogenic glycoprotein existing in humans. There are conflicting opinions about its role in Multiple Sclerosis (MS); it can be either neuroprotective or neurotoxic. **Objective:** To investigate the VEGF-A mRNA gene expression in the peripheral blood mononuclear cells of a group of Egyptian MS patients and to correlate its levels with different clinical MS subtypes, clinical data, disability scale and brain Magnetic Resonance Imaging (MRI) findings. **Methods:** Thirty clinically definite MS patients were included in the study after being clinically evaluated. All patients underwent brain and Spinal cord MRI. VEGF-A mRNA gene expression was measured by Real Time Polymerase Chain Reaction (RT-PCR) for the patient group and 10 controls. **Results:** VEGF-A mRNA gene expression was significantly elevated in the MS group (4.62±6.7) compared to the control group (1.43±0.84) ($p<0.05$). There was no statistically significant difference in the levels of VEGF-A mRNA gene expression between the control group and each of the clinical subtypes. No significant correlation was noted between its expression and either age, disease duration, age of onset and disability scale The number of brain MRI lesions (either ≥ or <9) did not influence VEGF-A mRNA expression levels ($p>0.05$). **Conclusion:** VEGF-A mRNA is up regulated in MS patients irrespective of clinical subtype, age, disease duration, age of onset, disability scale and the number of MRI lesions. Further studies are needed to rule out the exact role of VEGF-A in MS. **[Egypt J Neurol Psychiat Neurosurg. 2014; 51(2): 137-143]**

Key Words: Multiple sclerosis – Vascular Endothelial Growth Factor – A mRNA gene

INTRODUCTION

Multiple Sclerosis (MS) is an immune mediated disease of the central nervous system (CNS) and is one of the most common neurological diseases of young adults. The etiology of MS, though still not clear, is thought to involve a complex interplay between genetic and environmental factors[1,2].

Clinically, MS is classified into four subtypes: Relapsing Remitting MS (RRMS) representing the majority of MS cases (about 85%). It is characterized by recurrent attacks of neurologic deficits resolving completely or leaving minimal deficit. Approximately 50% of RRMS patients within 10-15 years from the disease onset, convert to Secondary Progressive MS (SPMS), in which the patients had continuous progression over years, with increasing disability while relapses may not occur. Primary progressive MS (PPMS) accounts for approximately 10% of MS cases in which the function declines steadily with no relapses. Finally, Progressive Relapsing MS (PRMS) accounts for less than 5% of MS cases and is characterized by occasional relapses superimposing on progressive disease[3].

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Vascular endothelial growth factor (VEGF) is a pro-angiogenic glycoprotein existing in humans in four isoforms (VEGF A-D). VEGF-A promotes vascular neogenesis through its tyrosine kinase-receptors VEGFR1/Flt-1[4].

At the molecular level, the exact role of VEGF-A in cerebral inflammation is still far from clear. VEGF-A is proposed to be a pro-inflammatory factor with neurotoxic potential on CNS[5]. It has an important role in the feed-forward interdependence between angiogenesis and chronic inflammation[4]. Inflammatory mediators induce angiogenesis while, new blood vessels facilitate immune cell migration to the site of inflammation and increase the capacity for immune-cell adhesion, cytokine and chemokine production[6].

The capability of VEGF to downregulate the key components of tight junctions named claudin-5 and occludins, promotes blood brain barrier (BBB) breakdown[7].

Vascular permeability changes resulting from BBB disruption are considered pathological crucial events in the pathogenesis of MS since they precede the development of MS lesions[8] and lesions preferentially develop perivascularly[9,10]. The BBB disruption correlates clinically with neurologic exacerbation and radiologically with the appearance of contrast- enhancing plaques in MRI[11,12].

On the contrary, there is accumulating evidence that VEGF-A has a neuroprotective effect; it can stimulate the neuron survival and astrocyte proliferation[13,14]. Inhibition of endogenous VEGF-A can lead to increased brain lesion size[15] and administration of VEGF-A can attenuate CNS damage[16-18]. Studies on the neurodegenerative diseases namely amyotrophic lateral sclerosis and Alzheimer's disease have suggested a role for VEGF-A in the pathogenesis of these diseases[19-25].

This study was designed to investigate the expression of VEGF-A mRNA gene in the peripheral blood mononuclear cells (PBMC) of a group of Egyptian MS patients; to be correlated with different clinical MS subtypes, clinical data, disability scale and brain MRI findings of those patients.

SUBJECTS AND METHODS

Subjects

This case control study was performed in the Neurology Department of Cairo University Teaching Hospitals from January to April 2012.

Thirty consecutive clinically definite MS patients were recruited according to McDonald criteria 2010[(3)] based on the clinical, laboratory and radiological findings.

Informed consent was obtained from all patients after explaining the nature of the study.

Eight males (26.7%) and 22 females (73.3%) were recruited with a mean age 33.37±5.13 years and a mean disease duration 2.15±1.17.

The control group consisted of 10 normal healthy blood donors (5 males and 5 females) having 31.1±4.48 years old, with neither personal nor past nor family history of neurological diseases.

Methods

- **Clinical Assessment:**

MS patients underwent history taking, thorough general and neurological examination. The examining physician focused on the type of MS, disease duration, age of onset of disease, relapse status and disability scale measured by the Expanded Disability Status Scale (EDSS)[26].

Regarding the clinical subtypes: 11 had RRMS (36.7%), 11 had SPMS (36.7%), and 8 had RPMS (26.6%) with a mean EDSS score of 2.72±1.22. Out of RRMS and RPMS patients, 9/19 (47.3%) were in relapse, while 11/19 (57.8%) were in remission.

- **VEGF-A mRNA Gene Expression:**

The blood samples were received within one month from the patients in relapse [9/19 (47.3%)][(5)] and before starting the high dose corticosteroids (Solumedrol). Regarding the patients in remission [11/19 (57.8%)] and those having SPMS, the blood samples were obtained during their routine follow-up in the clinic.

Blood samples were collected in a tube containing ethylene diamine tetraacetate (EDTA), then stored at −20°C. Semiquantitative Real Time Polymerase chain reaction (RT-PCR) assays were performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Total RNA was extracted from peripheral blood (Number=40) according to the Trizol method (Invitrogen, Melbourne, Victoria, Australia). C-DNA was prepared by reverse transcription. The primer sequences for GAPDH and primer sequences for Vascular endothelial growth factor were designed using online software Primer 3.[27].

Vascular endothelial growth factor was determined using SYBR Green chemistry. Diluted (1/20) cDNA (4 µl) was added to a PCR mix containing 2.4 µl sterile water, 10 µl 2× SYBR mix (Qiagen, Clifton Hill, Victoria, Australia), and 1.5 µl each of forward and reverse primers to make up a final volume of 20 µl. Cycling conditions for amplification were 48°C for 30 minutes then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and a final step of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds in StepOne Applied Biosystem.

For each assay, analysis was performed using StepOne Analysis Software (Applied Biosystem, USA). Relative concentrations of mRNAs present were determined using the following formula: Ratio a (expression of vascular endothelial growth factor gene in patient samples) / b (expression of GAPDH in patient samples) / c (expression of vascular endothelial growth factor gene in normal samples) / d (expression of GAPDH in normal samples)

- **Magnetic Resonance Imaging:**

Brain MRI studies (1.5 T) were performed for all patients with the typical imaging parameters: axial T2 weighted spin-echo and FLAIR imaging; and sagittal and axial T1-weighted spin-echo imaging. The typical imaging parameters of the spinal cord MRI were: sagittal T1 and T2-weighted spin-echo imaging; axial T1 and T2- weighted spin-echo imaging. Brain and spinal cord MRI studies were evaluated separately by two neuro-radiologists who were unaware of the diagnoses.

Brain and spinal cord MRI lesions were evaluated according to McDonald's criteria. Thirteen patients (33.3%) displayed more than 9 brain lesions while 17(56.7%) patients had less than 9 brain lesions on the brain MRI. Meanwhile, 22 patients (73.3%) had positive spinal lesions and only 8 (26.7%) showed a normal cervical cord.

Statistical Analysis

Statistical analysis was performed using the SPSS (statistical package for social science) version 17. Data were subjected to Kolmogorov–Smirnov test to determine the distribution and method of analysis.[28] The comparisons between groups were performed using Student's test for the age, EDSS and VEGF-A mRNA gene expression level). A chi-square test was used to compare the gender. The effect of demographic and clinical variables (age, disease duration, age of onset and EDSS) on VEGF-A mRNA gene expression were determined individually using Pearson's correlation coefficient (r). A P-value less than 0.05 was considered statistically significant.

RESULTS

The demographic and clinical characteristics of the subjects are summarized in Table (1).

Correlations among VEGF-A mRNA gene expression and the clinical data of the patients:

VEGF-A mRNA gene expression was significantly elevated in the MS group (4.62±6.75) compared to the control group (1.43±0.84) ($p<0.05$).

There was no statistically significant difference ($p>0.05$) between the control group and each of: RRMS (4.07±0.84), SPMS (4.07±7.55) and RPMS (3.79±5.03) ($p>0.05$). In addition, VEGF-An mRNA expression levels were not significantly different between RPMS and SPMS ($p>0.05$) (Table 2).

In addition, there was no significant correlation between VEGF-An mRNA expression with either the age of the patients ($r=0.005$, $p>0.05$), disease duration ($r=0.391$, $p>0.05$), age of onset ($r=0.054$, $p>0.05$) and EDSS ($r=0.132$, $p>0.05$) ($p>0.05$) (Table 3).

Correlation among VEGF-An mRNA gene expression and the number of brain T2 MRI lesions:

The number of brain MRI lesions did not influence VEGF-A mRNA expression levels; there was no statistical significant difference in the levels of VEGF-A mRNA expression between the patients having 9 or more brain T2 MRI lesions (4.90±5.61) compared to those having less than 9 MRI lesions (4.68±7.85) ($p>0.05$) (Table 4).

Table 1. Clinical, laboratory and radiological characteristics of MS patients compared to control subjects.

			MS Subjects (30)	Control subjects (10)	P-value
Demographic Characteristics	Age (years) mean ±SD		33.37±5.13	31.1±4.48	*p*>0.05
	Sex	Male (N) (%)	8/30 (26.7%)	5/10 (50%)	Odds ratio 2.750
		Female (N) (%)	22/30 (73.3%)	5/10 (50%)	*p*>0.05
Clinical Characteristics	EDSS (years)	Mean ±SD, Median (minimum–maximum)	2.72±1.22, 2.5 (1-6)		
	Disease Duration (years)	Mean ±SD	2.15±1.17		
		Median (minimum–maximum)	2 (0.7-5)		
	Age of onset of disease (years), mean ±SD		31.20±4.49		
	Relapsing Status	Patients in relapse (N) (%)	9/19 (47.3%)		
		Patients in remission (N) (%)	11/19 (57.8%)		
	Brain MRI	More than 9 lesions (N) (%)	13/30 (33.3%)		
		Less than 9 lesion (N) (%)	17/30 (56.7%)		
	Cervical	Positive (N) (%)	22/30 (73.3%)		
		Negative (N) (%)	8/30 (26.7%)		
	VEGF-A mRNA gene expression	Mean ±SD, Median (minimum–maximum)	4.62±6.75 1.52 (0.01-25.99)	1.43±0.84 1.62 (0.44-2.64)	<0.05

P-value: MS cases compared to the control group, ***EDSS*** Expanded Disability Status Scale, ***N*** number, ***MS*** Multiple Sclerosis, ***VEGF-A*** Vascular Endothelial Growth Factor-A, ***MRI*** Magnetic Resonance Imaging

Table 2. VEGF-A mRNA Gene Expression Level Among MS Disease Subgroups Compared to Healthy Control Subjects

		Controls	RRMS	SPMS	RPMS
VEGF-A mRNA gene expression	Number	10	11/30 (36.7%)	11/30 (36.7%)	8/30 (26.6%)
	Mean±SD	1.43±0.84	4.07±7.55	4.07±7.55	3.79±5.03
	Median (minimum– maximum)	1.62 (0.44-2.64)	1.52 (0.01-27)	1.51 (0.07-25.09)	1.22 (0.06-12.13)
	p-value[a]		> 0.05	> 0.05	> 0.05
	p-value[b]			> 0.05	

[a] MS subgroups compared to the control group [b] RRMS compared to SPMS
RRMS Relapsing Remitting Multiple Sclerosis, ***RPMS*** Relapsing Progressive Multiple Sclerosis, ***SPMS*** Secondary Progressive Multiple Sclerosis, ***VEGF-A*** Vascular Endothelial Growth Factor-A

Table 3. Correlation between VEGF-A mRNA Gene Expression Level among MS patients; age, disease duration, age of onset and EDSS.

	R	P-value
Age (years)	0.005	> 0.05
Disease Duration	0.391	> 0.05
Age of onset (years)	0.054	> 0.05
EDSS	0.132	> 0.05

EDSS Expanded Disability Status Scale, ***MS*** Multiple Sclerosis, ***r*** correlation coefficient, ***VEGF-A*** Vascular Endothelial Growth Factor-A

Table 4. VEGF-A mRNA Gene Expression Level Among MS Patients with ≥ 9 lesions compared to those with < 9 lesions in Brain T2 MRI.

		MS patients with ≥ 9 Brain MRI lesions	MS patients with < 9 Brain MRI lesions
VEGF-A mRNA gene expression	Number	13/30(33.3%)	17/30 (56.7%)
	Mean ±SD,	4.90±5.61	4.68±7.85
	Median (minimum– maximum)	2.30 (0.01-16)	1.002 (0.06-25.99)
	p-value	>0.05	

MRI Magnetic Resonance Imaging, ***MS*** Multiple Sclerosis, ***VEGF-A*** Vascular Endothelial Growth Factor-A,

DISCUSSION

VEGF-A is a potent inducer of blood vessel growth and it was isolated as a vascular permeability factor responsible for angiogenesis. Its functions are mediated by VEGF-receptor (VEGFR) 2/KDR/Flk-1.[5] Alterations in the VEGF/VEGFR system were observed in various inflammatory diseases including MS[30]. Anti-VEGF therapy has been approved for the treatment of various solid tumors and angiogenic ocular diseases[31]. The pro-inflammatory properties of VEGF-A render it a potential therapeutic target in autoimmune disease[4].

Recently, Argaw et al.[12] administrated systemically the selective endothelial nitric oxide synthase inhibitor "Cavtratin" during the acute clinical phase of experimental autoimmune encephalitis (EAE) in mice abrogated VEGF-A-induced BBB disruption. They showed that blockade of endothelial VEGF-A signaling could be a mean by which clinical severity and inflammatory tissue damage could be restricted.

Nevertheless, MacMillan et al.[31], demonstrated that the anti-VEGF-A monoclonal antibody "Bevacizumab" can inhibit angiogenesis and it is able to ameliorate the vascular and T-cell responses during EAE.

Based on the findings of Iacobaeus et al.[32], who cited that PBMC constitute the major source expressing VEGF-A, we decided to investigate the VEGF mRNA gene expression levels in PBMC of a group of MS patients.

Our results showed elevated VEGF mRNA gene expression in PBMC of MS patients, compared to the controls which is in agreement with Su et al.[(10)], Graumann et al.[33] and Kirk et al.[34], who showed an elevated expression of VEGF-A in CNS tissue and serum of animal models as well as MS patients. On

the contrary, Tham et al.[5] showed that the levels of VEGF-A mRNA were significantly lower in the CSF of MS group compared to controls while, no VEGF-A expression difference in PBMC between MS patients and controls was noticed. Seabrook et al.[2] showed decreased levels of VEGF-A protein in rat spinal cord tissue during EAE relapse and a reduced expression in neurons upon immunohistochemistry. Iacobaeus et al.[32] did a study on a larger group of patients and had found evident reduction of VEGF-A mRNA in CSF in MS patients either RRMS or SPMS compared to controls, while, VEGF-A mRNA levels in PBMC were similar in RRMS and controls, but significantly reduced in SPMS.

It is known that the net effect of VEGF-A can vary depending on the target tissue, the timing and the concentration. The neuroprotective role of VEGF-A critically depends on the proper dosage and may be compromised by angiogenesis[35].

Concerning the clinical subtypes, we could not show a significant difference between the control group and each of: RRMS, SPMS and RPMS. In addition, no difference of VEGF-A mRNA expression levels between RPMS and SPMS. Our results were in agreement with Tham et al.[5] and Su et al.[10]. However, Iacobaeus et al.[32], showed that SPMS patients are characterized by a prominent decrease of VEGF-A mRNA expression in PBMC compared to both RRMS and controls. They attributed their result to an underlying disease mechanisms involving VEGF-A and operating specifically in progressive MS. The authors mentioned that they measured VEGF-A protein levels with ELISA in a small subset of patients; revealing a non significant trend towards decreased levels in SPMS patients compared to RRMS and controls. The fact that we included RPMS and it did not show a difference in VEGF-A gene expression levels compared to other subgroups doubts the results of[(32)] and warrants the need for another large study involving all MS subtypes.

We could not demonstrate any significant correlation between VEGF-A levels expression and any of disease severity measures (age, disease duration, age of onset and EDSS) also, there was no VEGF-A expression difference between the 2 groups with more or less than 9 brain T2 MRI lesions. Our results are in agreement with Su et al.[10] and Iacobaeus et al.[32].

Finally, Iacobaeus et al.[32] were unable to demonstrate any haplotype dependent association between VEGF-A gene expression levels and the overall risk of MS. This result warrants future studies on larger and better characterized clinical materials to rule out a genetic association between VEGF-A and MS.

In conclusion, we demonstrated that VEGF-A mRNA is up regulated in PBMC cells of MS patients irrespective of clinical subtype, age, disease duration, age of onset, EDSS and the number of MRI lesions.

Further larger studies are needed on a more stratified MS subgroups to find out the exact role of VEGF-A in MS and the possibility to apply an anti-VEGF therapy in this disease.

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الملخص العربي

التعبير الجيني لجين عامل النمو المبطن للأوعية الدموية في الخلايا الدموية البيضاء وحيدة النواة في مجموعه من مرضى التصلب المتناثر المصريين

الخلفية: التصلب المتناثر هو مرض مناعي يصيب الجهاز العصبي المركزي ويعد واحدا من أكثر الأمراض العصبية شيوعا بين الشباب .مسببات مرض التصلب المتناثر لا تزال غير واضحة، ولكن يعتقد أن المرض ينتج عن تفاعل معقد بين العوامل الوراثية والبيئية. عامل النمو البطاني الوعائيأ هو بروتين يساعد على تكوين الأوعية الدموية، على المستوى الجزيئي، دور عامل النمو البطاني الوعائي أ في الالتهاب الدماغي لا يزال غير واضح حيث أن بعض الأبحاث تقترح أن يكون عاملا مواليا للالتهاب ومؤذيا للخلايا العصبية بينما هناك أدلة أخرى تشير إلى أنه يحمى الخلايا العصبية ويحفز على بقاءها.

أهداف البحث: التحقيق من التعبير الجيني لجين عامل النمو المبطن للأوعية الدموية في الخلايا الدموية البيضاء وحيدة النواة في مجموعة من مرضى التصلب المتناثر المصريين وربط ذلك بمختلف الأنواع الفرعية للمرض،و البيانات السريرية، ومقياس الإعاقة إلى جانب نتائج الرنين المغناطيسي للمرضى.

المنهج: ثلاثون من مرضى التصلب المتناثر تم إدراجهم في البحث بعد تقييمهم سريريا، وقد خضع جميع المرضى للرنين المغناطيسي على الدماغ والحبل الشوكي. كما تم قياس التعبير الجيني لجين عامل النمو المبطن للأوعية الدموية بواسطة الوقت الحقيقي لتفاعل البلمرة المتسلسل لمجموعة المرضى و10 من الضوابط.

النتائج: التعبير الجيني لجين عامل النمو المبطن للأوعية الدموية كان مرتفعا في مجموعة المرضى بفارق ذا دلالة إحصائية مقارنة بالمجموعة الضابطة بينما لم يكن هناك فروق ذات دلالة إحصائية في مستويات التعبير الجيني للجين بين المجموعة الضابطة وكل من الأنواع الفرعية لمرض التصلب المتناثر كما لوحظ عدم وجود ارتباط ذو دلالة إحصائية بين التعبير الجيني وبين عمر المرضى، ومدة المرض، والعمر عند بداية المرض ومقياس الإعاقة وكذلك عدد الإصابات الظاهرة (سواء أكثر أو أقل من تسعه) بالرنين المغناطيسي على الدماغ.

الخلاصة والتوصيات: مستوي التعبير الجيني لجين عامل النمو المبطن للأوعية الدموية مرتفع في مرضي التصلب المتناثر مقارنة بالمجموعة الضابطة بغض النظر عن النوع الفرعي للمرض، والعمر، ومدة المرض، والعمر عند بداية المرض، ومقياس الإعاقة وكذلك عدد الإصابات الظاهرة بالرنين المغناطيسي على الدماغ. هناك حاجة إلى مزيد من الدراسات على مجموعه اكبر وأكثر تنوعا لمرضى التصلب المتناثر لمعرفة الدور الحقيقي لعامل النمو المبطن للأوعية الدموية وإمكانية تطبيق العلاج المضاد له في هذا المرض.