VEGF-A HAPLOTYPE IS ASSOCIATED WITH STROKE DISABILITY

HAPLÓTIPO DO VEGF-A ESTÁ ASSOCIADO COM DISABILIDADE NO AVE

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ABSTRACT

Background: Although it is recognized that vascular endothelial growth factor (VEGF-A) is involved in stroke pathophysiology, there are few studies on how VEGF polymorphisms affect susceptibility of stroke and degree of disability of disease. *Methods*: Performing TaqMan® genotyping assays, we determined genotypes and haplotypes frequencies of -2578C>A and -634G>C polymorphisms of VEGF promoter region in 100 Brazilian patients with stroke and 119 controls. *Results:* No significant differences were observed in the distributions of alleles and genotypes of two polymorphisms (P>0.05) in both groups, even when groups were classified by ethnicity (P>0.05). However, combination of polymorphisms showed that "A-2578 C-634" haplotype was significantly more frequent in white healthy subjects than stroke patients (P<0.0125), moreover the same haplotype seems to be related to stroke patients presenting higher disability scale (P=0.0071), as classified by modified Rankin Scale. *Conclusions:* These findings suggest a dual effect of "A-2578 C-634" haplotype to stroke.

Keywords: VEGF; Polymorphisms; Stroke; Haplotypes; Rankin scale; Disability; Brazilian population.

1 INTRODUCTION

When blood flow is obstructed, the brain loses its energy supply, causing damage to tissues leading to stroke. Annually, 15 million people worldwide suffer a stroke. Of these, 5 million die and another 5 million are left permanently disabled, placing a burden on family and community.[1] In Brazil, stroke is the most frequent cause of death after cancer and is more frequent than heart disease, with incidence of 100,000 deaths per year.[2] Recovery of brain functions is a difficult and incomplete process after a stroke.[3,4] It appears that revascularization (angiogenesis) and neuronal restore (neurogenesis) occur as a cellular cross-talk in ischemic area.[5] The vascular endothelial growth factor (VEGF) is an angiogenic growth factor that binds to two high-affinity receptors, fms-like tyrosine kinase (flt-1) and the

kinase domain region (KDR).[6,7] Increased synthesis of VEGF and its receptor was seen in the brain after stroke, increasing its importance to vascular response to cerebral ischemia.[8,9] The coding region of VEGF gene has 14-kb with 8 exons and 7 introns located on chromosome 6.[10] The VEGF gene includes two common SNPs polymorphisms located in promoter region, -634G>C (rs2010963) and -2578C>A (rs699947) that may influence VEGF expression.[11] These VEGF polymorphisms have been related to different cardiovascular diseases, as preeclampsia,[12,13] atherosclerosis,[14,15] myocardial infarction,[16] and also diabetes retinopathy.[17] **Aims:** The effects of -2578C>A and -634G>C polymorphisms in risk of stroke are not totally recognized, thus we investigated the association of genotypes and haplotypes of these polymorphisms with stroke. Moreover, we evaluated the association of these variations with the degree of disability as scored by modified Rankin scale (mRS).

2 MATERIAL & METHODS

2.1 Population Study

Approval for use of human subjects was obtained from the Institutional Review Board at the Santa Casa Hospital of Belo Horizonte, Minas Gerais, Brazil. A total of 219 subjects were enrolled in the study. Controls and patients with stroke were matched for gender, age and ethnicity. The study group included 100 patients presenting stroke evaluated in Center of Medical Specialties of Santa Casa Hospital of Belo Horizonte between November 2009 and August 2011. Ischemic strokes were characterized by the sudden loss of blood circulation to an area of the brain, resulting in a corresponding loss of neurologic function and CT (X-ray computed tomography) and MRI (magnetic resonance image) was done to confirm the diagnosis. The modified Rankin Scale (mRS; 0-6 scale) was used to evaluate functional outcome after stroke. The mRS indicates degree of disability of stroke patients (index ranged from 0 to 6). Score 2 designates slight disability or individuals able to look after own affairs without assistance, but unable to carry out all previous activities. Score 3 designates moderate disability and maximum score 6 indicates dead.

Individuals were recruited from the same demographic area and had no clinical evidence of any cerebrovascular disease. All clinical parameters evaluated are similar between stroke and control groups, including hypertension, diabetes mellitus and alcoholism history.

2.2 Allelic Discrimination

Genomic DNA was isolated from the buffy coat of centrifuged whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Alameda, CA) according to the manufacturer's instructions. Two clinically relevant polymorphisms in the VEGF gene were studied: C-2578A (rs699947) and G-634C (rs2010963). Genotypes were determined using the Taqman® Allele Discrimination assay (Applied Biosystems, Foster City, CA, USA). These assays use probes and primers designed by Applied Biosystems assay-on-demand services (assay ID: C_8311602_10/-2578; C_8311614_10/-634). TaqMan® polymerase chain reaction (PCR) amplification was performed in 10 µl volumes (10 ng dried DNA, 1x TaqMan master mix, 900 nM of each primer and 200 nM of each probe) in 48-well polypropylene reaction Eco plates and fluorescence from PCR amplification was detected using a Eco RT-PCR system (Illumina Inc., CA, USA) and analyzed with its software. The PCR assay was carried out following the manufacturer's instructions (Applied Biosystems) that include: one step of 10 min at 95^{o} C (Ultra Pure AmpliTaq Goldw DNA Polymerase Enzyme Activation) followed by 40 cycles of DNA denaturation at 92 ^{o} C for 15 s and annealing/extension at 60^{o} C for 1 min. Genotyping success rate was 100% and no discordant genotypes were observed among samples and duplicate of genotype controls.

2.3 Statistical analysis

Contingency table-based analyses or the unpaired Student *t*-test were used for comparisons of nominal variables. Chi-square (X^2) test for trend was used to evaluate the distribution of haplotypes, genotypes and allele frequencies between groups. Data were reported as the mean ± S.D or percentage. A probability value $P<0.05$ was considered the minimum level of statistical significance. The estimating haplotype (EH) software program (ftp://linkage.rockfeller.edu/ott/eh.htm) was used to estimate the haplotypes frequencies in each group. In addition, to identify which specific haplotypes are associated with increased susceptibility to stroke or associated with Rankin Scale, differences in haplotype frequency were further tested using a contingency table, and value of $Pc < 0.0125$ (0.05/number of haplotypes=4) was considered significant to correct for the number of comparisons made.

3 RESULTS

The clinical characteristics of controls and stroke patients are shown in Table 1. The groups were matched for gender characteristic and stroke risk factors, as hypertension, diabetes mellitus and alcoholism (P>0.05); however age, smoking and ethnicity parameters presented significant differences between groups (Table 1). The clinical characteristics of stroke individuals according to degree of disability were also represented in Table 1. All clinical parameters were similar between less and more affected stroke patients (*P*>0.05), excepting the percentage of hypertensives, smokers and males (*P* <0.05).

Table 1 - Demographic characteristics of the study volunteers.

Parameter	**Control**	**Stroke**	***P***	**Stroke**		***P***
				mRS<2	**mRS≥2**	
	n=119	n=100		n=65	n=35	
Gender (Male %)	49.6	56.0	NS	26.1	48.6	<0.05
Ethnicity (White %)	64.7	45.0	<0.05	44.6	48.6	NS
Age (years; mean±SD)	54.7±9	63.9±13	<0.05	62.6±13	66.4±12	NS
Hypertension (%)	47	44	NS	86	40	<0.05
Diabetes mellitus (%)	22	22	NS	25	14	NS
Smoking (%)	23	38	<0.05	59	100	<0.0001
Alcoholism (%)	12	13	NS	9	20	NS

mRS - modified Ranking Scale. P was evaluated using contingency table-based analysis to gender, ethnicity, and risk factors, as hypertension, diabetes mellitus, smoking and alcoholism and using unpaired Student t-test to age. NS: Non-significant

The frequencies of C-2578A and G-634C genotypes and alleles in patients with stroke and controls are displayed in Table 2. The distribution of genotypes for the two polymorphisms studied here showed no deviation from Hardy–Weinberg Equilibrium (HWE), even when the groups were classified by ethnicity (P>0.05). No significant differences were observed in genotypes and allele frequencies for the both polymorphisms between stroke and controls. The same observation was found when controls and stroke patients were sub-grouped by ethnicity (P>0.05). We also observed a lack of difference between genotypes and alleles frequencies when stroke patients were classified by degree of disability (mRS<2 and mRS≥2; all P>0.05). The estimated haplotype frequencies for the control group (all subjects) and the patients (all subjects) with stroke shown in Table 3 were similar (P>0.05). Furthermore, when controls and stroke groups were classified by ethnicity, it was observed that haplotype overall distribution was not significantly different between white controls and

white stroke groups (P=0.05) and non-white control versus stroke (P>0.05). However, the haplotype "A-2758 C-634" presented a significant higher frequency in white controls when compared to white stroke individuals (P=0.0194). In addition, we observed that stroke patients classified by higher degree of disability (mRS≥2) presented a higher frequency of "A-2758 C-634" haplotype when compared to less affected individuals (P=0.002), besides this two groups show a significant difference regarding overall haplotype distribution (P=0.0152).

Table 2 - Genotypes and alleles frequencies of the two polymorphisms analyzed in stroke patients, as classified by modified Rankin Scale and controls.

SNP	**Control**			**Stroke**					
	All	**White**	**Non-white**	**All**	**White**	**Non-white**	**mRS≤2**	**mRS≥2**	**P**
2578C>A	% (n=119)	% (n=77)	% (n=42)	% (n=100)	% (n=45)	% (n=55)	% (n=65)	% (n=35)	
CC	29 (34)	30 (23)	26 (11)	36 (36)	35 (16)	36 (20)	34 (22)	41 (14)	NS
CA	51 (61)	48 (37)	57 (24)	53 (54)	62 (28)	47 (26)	55 (36)	50 (18)	NS
AA	20 (24)	22(17)	17 (7)	10 (10)	3 (1)	17 (9)	11 (7)	9 (3)	NS
Allele	n=238	n=154	n=84	n=200	n=90	n=110	n=130	n=70	NS
C	54 (129)	54 (83)	55 (46)	62(125)	64 (58)	61 (67)	62 (80)	66 (46)	NS
A	46 (109)	46 (71)	45 (38)	38 (75)	46 (32)	39 (43)	38 (50)	34 (24)	NS
-634G>C	% (n=119)	% (n=77)	% (n=42)	% (n=100)	% (n=45)	% (n=55)	%(n=65)	% (n=35)	
GG	49 (58)	47 (36)	51 (22)	51 (51)	49 (22)	53 (29)	52 (34)	48 (17)	NS
GC	41 (49)	41 (31)	43 (18)	43 (43)	44 (20)	41 (23)	43 (28)	43 (15)	NS
CC	10 (12)	12 (9)	6 (3)	6 (6)	7 (3)	6 (3)	5 (3)	9 (3)	NS
Allele	n=238	n=154	n=84	n=200	n=90	n=110	n=130	n=70	NS
G	70 (166)	68 (105)	73 (61)	72 (144)	71 (64)	73 (80)	74 (96)	68 (48)	NS
C	30 (72)	32 (49)	27 (23)	28 (56)	29 (26)	27 (30)	26 (34)	32 (22)	NS

mRS- Modified Ranking Scale. P was evaluated using χ^2-tests. NS: non-significant.

Table 3 - Estimated haplotype of VEGF -2578C>A and -634G>C polymorphisms in stroke patients, as classified by modified Rankin Scale and controls.

Haplotypes	Control				Stroke							
	All	White	Non-white	OR (95%CI)[§]	All	OR (95%CI)[#]	White	Non-white	OR (95%CI)[§]	mRS <2	mRS ≥2	OR (95%CI)[§§]
-2578 - 634	% (n=238)	% (n=154)	% (n=84)		% (n=200)		% (n=90)	% (n=110)		% (n=130)	% (n=70)	
C G	32 (76)	28 (43)	37 (32)	0.63 (0.36-1.1)	37 (74)	0.8 (0.53-1.2)	37 (34)	38 (42)	0.98 (0.55-1.7)	36 (47)	40 (28)	2.1 (1.24-3.7)
C C	39 (93)	40 (62)	37 (32)	1.1 (0.6-1.9)	36 (72)	1.14 (0.77-1.7)	35 (30)	36 (40)	0.87 (0.49-1.5)	38 (49)	30 (21)	1.4 (0.75-2.6)
A G	23 (55)	26 (40)	16 (	2.1 (1.0-4.3)	25 (50)	1.14 (0.73-1.7)	28 (26)	22 (24)	1.45 (0.76-2.7)	26 (34)	23 (16)	1.2 (0.6- 2.4)
A C	6 (14)	6 (9)	10 (8)	0.6 (0.22-1.6)	2 (8)	1.1 (0.45-2.7)	0 (0)*	4 (4)	0.13 (0.007-2.5)	0 (0)	7 (5)**	0.05 (0.002-0.84)

*P<0.0125 (0.05/4) was considered as significantly different compared to white control and **P<0.0125 compared to mRS<2 group. §Odds ratio between white and non-white classified groups, # between controls and stroke groups, and §§ between mRS<2 and mRS≥2 classified groups.

4 DISCUSSION

VEGF is important for initiating angiogenesis and is strongly involved in risk and progression of stroke by multiple processes. For example, experimental models pointed to a close association between VEGF and ischemic stroke. It has been described that in the ischemic rat brain, VEGF exerts an acute neuroprotective effect, enhancing newborn neurons survival, and promoting angiogenesis, which could improve functional recovery from stroke [8], and that, intranasal VEGF administration promotes angiogenesis and behavioral recovery of cerebral ischemic rats.[18] It has also been described that VEGF may participate differently depending on the phase of stroke, thus early post-ischemic administration of VEGF increases blood-brain barrier leakage, hemorrhage and lesions, while VEGF markedly augments angiogenesis and reduces neurological deficits when lately administrated.[19]

While these data support the functional role of VEGF to stroke, the effect of VEGF gene polymorphisms to vascular accident and the degree of severity of lesions has not been totally elucidated. The present study is one of the few that investigated a possible association between stroke and VEGF genotypes and haplotypes. Our results pointed to an absence of

association between stroke and individual VEGF polymorphisms. Some authors demonstrated recently that there was no significant difference in allele and genotype distributions of C-2578A with the risk of acute cerebral infarction when compared with controls.[20] In other hand, it has been recently described that C-2578A and G-634C genotypes contribute to risk of stroke.[21] However these investigations were made in Asian populations. Genotype and allele frequencies of the VEGF polymorphisms may vary among different populations. Indeed, -2578A allele frequency in white population is higher than in Asians.[22] The ethnic denomination of the Brazilian population enrolled in this study was not genetically determined, however some studies has established that the auto-denotation as white gender is predominantly associated to European markers in our region.[23] In addition, it was described that the distribution of VEGF polymorphisms presents interethnic differences in Brazilian population.[24] In the present study, it was found that despite of control group has a significant higher number of self-reported white subjects than stroke group, these interethnic differences are not related to differences in genotype frequencies of both polymorphisms. An earlier study enrolled in the same region, evaluating the importance of VEGF polymorphisms to hypertensive status corroborates our present data.[25] In this study, the authors suggest that individual VEGF C-2578A and G-634C polymorphisms may not be related to hypertension. Nevertheless, the reduced number of individuals evaluated in our study may also explain the absence of VEGF genotype correlation with stroke. This limitation may also have resulted in age and ethnicity differences observed between controls and stroke groups.

Our study also used haplotype analysis to investigate the importance of these genetic markers to stroke. It was found no correlation among overall haplotype distribution between stroke and controls ($P>0.05$). However, the “A-2578 C-634” haplotype may present a dual effect in stroke, since it seems to be protective against stroke in white individuals ($P<0.0125$), and may be also related to a deleterious effect after stroke occurrence. In fact, the distribution of this haplotype according to severity of stroke was significant ($P=0.0071$). As earlier mentioned, the VEGF might present different biological effects depending on the stage of stroke.

Other authors have demonstrated in our region that the combination of C-2578A, G-1154A and G-634C polymorphisms in haplotypes determined an association with hypertensive status.[25] In this work, the ‘C-A-G’ haplotype was protective against hypertension in white subjects, and the ‘C-A-C’ haplotype was related to hypertension.[25] Furthermore, it has been described that VEGF haplotypes also contribute to stroke. The “C-A-

C" haplotype of C-2578A, G-1154A and C936T polymorphisms, respectively, reduces the risk of acute cerebral infarction.[20] Other study described that among other haplotypes of the same individual polymorphisms, the "C-A-C" haplotype was also protector to risk of stroke.[21] The differences among studies may be explained by the lower frequency of -2578A allele in Asian population, which turns difficult the detection of some haplotypes. Thus, it is clear that haplotypes are involved in susceptibility to cardiovascular disease, particularly to stroke. Some studies concerning the functional role of VEGF polymorphisms to *in vivo* VEGF production were performed. The G-634C polymorphism was shown to affect VEGF expression at the post-transcriptional level.[26,27] Therefore, the -634G allele reduces the translation of a bioactive and secreted VEGF isoforms *in vivo* Lambrechts et al.[28] The haplotypes "–2578A –1154A –634G" and "–2578A –1154G –634G" have been described as risk factors to a neurodegenerative disorder (amyotrophic lateral sclerosis, ALS), indeed the combination of at-risk alleles –2578A and –634G in genotypes may be related to a reduced VEGF expression *in vitro* and to a lower VEGF plasma levels in ALS patients.[28] Our data showed that -2578A -634C haplotype is related to the high severity of stroke. However, it was not evaluated the contribution of genotypes and haplotypes of these polymorphisms to the VEGF plasma production in stroke individuals, thus it remains uncertain how functional A-2578C and C-634G polymorphisms regulate levels of plasma VEGF in our population.

We concluded that genotypes of individual VEGF polymorphisms may not be determinant to stroke episodes. In other hand, "A-2578 C-634" haplotype has a dual effect in stroke. This haplotype is more frequent in healthy individuals than stroke patients, suggesting a protective effect to stroke. However, it seems to be related to a severe status of disease after stroke occurrence, suggesting a deleterious role to stroke outcome.

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