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TNFA gene in Brazilian patients with hemorrhagic stroke or cerebral aneurysm

Gene TNFA *em pacientes brasileiros com acidente vascular encefálico hemorrágico ou aneurisma cerebral*

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ABSTRACT

Introduction: Many cerebrovascular diseases display a relation with inflammatory processes. Furthermore, the influence of several polymorphisms has been studied to improve the knowledge of physiological mechanisms of the nervous system. **Objectives:** The aim of this study was to identify if there was an association between a polymorphism in -308 position of the *TNFA* gene and the development of hemorrhagic stroke or aneurysm in Distrito Federal, Brazil. **Methods:** We collected the clinical information and the medical records from hemorrhagic stroke or aneurysm patients. The occurrence of stroke or aneurysm was confirmed by computed tomography (CT) or magnetic resonance image (MRI). The *TNFA* genotypes were determined by polymerase chain reaction restriction fragment length polymorphism. **Results:** The AG genotype appears to decrease the occurrence of hemorrhagic stroke or aneurysm in people between 45-63 years. Our study was the first to investigate this association in a Brazilian sample, although a previous report showed a similar effect with ischemic stroke in a Chinese population. **Conclusion:** The *TNFA* -308 AG genotype is associated with a decreased risk of aneurysm or hemorrhagic stroke in a population from the capital of Brazil, Distrito Federal.

Key words: tumor necrosis factor-alpha; single nucleotide polymorphism; stroke; intracranial hemorrhages; intracranial aneurysm.

INTRODUCTION

Stroke is a major global health problem that imposes a huge socioeconomic burden[1]. According to estimates of the World Health Organization (WHO), 6.7 million people died worldwide due to stroke[2] in 2015. Stroke is a leading cause of mortality and disability in Brazil and Latin America overall, probably in consequence of changes in the population lifestyle and increasing life expectancy[3].

Stroke is primarily classified into two types: ischemic (80%-85% of cases) and hemorrhagic (15%-20% of cases). Given its multifactorial nature, stroke is related with hypertension, diabetes, atherosclerosis, genetic influences, among other factors[3-6].

It is well known that inflammatory factors are closely related with ischemic stroke and cerebrovascular diseases[7]. Patients with stroke have shown an increase in pro-inflammatory cytokines concentrations in their serum and cerebrospinal fluid[8-10]. Punctual factors, such as polymorphisms in promoter region of cytokine or cytokine receptor genes, might modulate the levels of its final product. Therefore, the *TNFA* polymorphisms could potentially influence the etiology of stroke. Even though some studies were conducted with this polymorphism in relation to other diseases, none were about hemorrhagic stroke (HS) or aneurysm[11, 12].

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine common in the central nervous system. The human *TNFA* gene is mapped to chromosome 6p21.33[13], and it has two polymorphisms in its promoter regions, described at positions -308 (G/A) and -238(G/A). The one present at position -308 (G/A) has its A allele associated with higher levels of tumor necrosis

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factor (TNF) expression[14]. A meta-analysis of 18 studies with 8,075 patients found no significant association between ischemic stroke and *TNFA*-308G/A and *TNFA*-238G/A. However, the *TNFA* -308G/A demonstrates a protective factor against ischemic stroke, according to subgroup analysis of East Asians[15]. Therefore, we oriented the study to *TNFA*-308G/A polymorphism. Among its functions, it activates proteolytic enzymes that damage endothelial cells, resulting in rupture of the aneurysm or HS[16]. Through these effects, TNF-α may be involved in the pathogenesis of HS. Hence, this case-control study was conducted to investigate the effect of *TNFA*-308G/A (rs1800629) genetic variants on the development of HS/aneurysm in a Brazilian population.

METHODS

Subjects

This study composed of 162 individuals was conducted at Faculdade de Ceilândia of Universidade de Brasília (UnB), Brazil. The samples were obtained from a hospital-based case-control study completed in two years (January, 2011 to December, 2012). A total of 81 patients diagnosed with aneurysm/HS (48 females and 33 males; mean age 54 ± 9 years) were recruited for this study. All patients had clinical signs consistent with the WHO definition of stroke and confirmed by imaging [computed tomography (CT) or magnetic resonance image (MRI)][17].

The control group comprised 81 (43 females and 38 males; mean age 52 ± 6 years) age- and sex-matched healthy individuals recruited from volunteers and healthy individuals accompanying the patients in the general outpatient department (OPD). The sample was calculated by estimating a prevalence of 20% of HS in the adult population, a sampling error of 5% and a 95% confidence interval (CI), in 112 patients evaluated according to the criteria previously described. A total of 78 participants were included. We considered a sample of 81 patients with HS in order to compensate for any loss.

Informed consent was obtained from all subjects before information collection. This study was approved by the institutional ethical committee. Participants under the age of 18 without diagnosis of aneurism or stroke and parentage correlation with the control group were excluded.

Clinical characteristics

Detailed history was taken and clinical evaluation was done. Patients were questioned about having arterial hypertension (blood pressure was measured) or diabetes, current smoking, alcohol consumption. Scales were used to measure severity, stages of disease and incapacity of patients: Rankin scale (RS) for measurement of motor capacity[18], Barthel index (BI) score for mobility and personal cares[19], and the Glasgow scale for assessing the level of consciousness of the patient[20].

Laboratory methods

Each patient and control subject provided a 5 ml peripheral venous blood sample after enrollment. Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood using the Invisorb Spin Blood Mini Kit (250) by Invitek (catalog #CA10-0005, batch #1031100300). The genotyping of *TNFA* -308G/A (rs1800629) polymorphism was performed using polymerase chain reaction (PCR) combined with restriction fragment length polymorphism analysis. The forward and reverse primers used to amplify *TNFA* -308G/A (rs1800629) were 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and 5'-TCC TCC CTG CTC CGA TTC CG-3', respectively. Amplification was performed using the following cycling program: an initial denaturation step at 95°C for 10 min, then 38 cycles of denaturation at 94°C for 1 min, annealing at 57°C for one minute, and extension at 72°C for one minute, followed by a final extension at 72°C for eight minutes. PCR products of 107 bp were subsequently incubated for 90 minutes at 37°C with NcoI. The G allele was cut into two fragments of 87 bp and 20 bp, while the A allele remained undigested. These fragments were visualized on a 3.5% agarose gel stained with ethidium bromide and exposed to ultraviolet light.

Statistical analysis

The genotype and allele frequencies in HS patients were compared to those in control subjects using the chi-squared test in recessive and dominant models. The Armitage trend test was performed to see if there is a dosage effect in a risk allele for HS. An association of clinical features with each genotype was analyzed by the chi-squared. A *p*-value of less than 0.05 was considered significant.

RESULTS

No significant difference was found between the control and case groups in terms of gender ($p = 0.429$) and age ($p = 0.185$). We analyzed the presence of *TNFA* -308G/A polymorphism in

the aneurysm case group when compared with the HS case group and the control group. No specific association was found (**Table 1**). Nevertheless, the AG genotype is associated with lower risk of aneurysm or HS [odds ratio (OR) = 0.35; 95% CI 0.13-0.96 ($p = 0.035$)] (**Table 2**). Secondly, the genotype distribution is not at Hardy-Weinberg equilibrium in the case group ($p < 0.01$).

TABLE 1 – Association of *TNFA* -308G/A (rs1800629) polymorphism with HS or aneurysm

	Aneurysm		HS		Control		
	n	%	*n*	%	*n*	%	*p*-value
GG	21	63.6	37	77.1	47	58	0.09
AG	3	9.1	3	6.3	15	18.5	0.102
AA	9	27.3	8	16.7	19	23.5	0.493
Allele							
G	45	68.2	77	80.2	109	67.3	0.07
A	21	31.8	19	19.8	53	32.7	

HS: hemorrhagic stroke.

TABLE 2 – Association of *TNFA* -308G/A (rs1800629) polymorphism with HS or aneurysm

	Aneurysm/HS		Control			
	n	%	*n*	%	OR (CI)	*p*-value
GG	58	71.6	47	58	1.82 (0.95-3.51)	0.07
AG	6	7.4	15	18.5	0.35 (0.13-0.96)	0.035*
AA	17	21	19	23.5	0.87 (0.41-1.82)	0.708
Allele						
G	122	75.3	109	67.3	1.48 (0.91-2.41)	0.11
A	40	24.7	53	32.7	0.67 (0.41-1.09)	0.11

*HS: hemorrhagic stroke; *significant* p < *0.05.*

Traditional cerebrovascular risk factors were analyzed, such as history of hypertension, smoking status and alcohol consumption. According to the collected data, a history of systemic arterial hypertension (SAH) was found more often in cases than in control individuals (both $p < 0.001$), both in the absence of allele A (cases 70.7% vs. 14.9%) and the presence of the allele A (82.6% vs. 2.9%). Besides increasing the risk of aneurysm or HS, independently of the presence or absence of the A allele (GG: OR = 13.78, CI = 5.16-36.79; GA + AA: OR = 156.75, CI = 16.31-1506.47). However, smoking status and alcohol consumption (GG: $p = 0.584$; AG + AA: $p = 0.920$) (GG: $p = 0.442$; AG + AA: $p = 0.354$) did not show statistical significance between case and control groups, independent of the presence or absence of the A allele (**Table 3**).

For the *TNFA* -308G/A polymorphism analysis in relation to different scales that measure severity, stage and incapacity of patients, the AG and AA genotypes were combined in a single group for comparison with the GG genotype. No specific association was found between Glasgow scale, Barthel index and Rankin scale and the presence of different genotypes (**Table 4**).

According to the logistic regression analysis, the binary factors were considered: SAH, smoking status, alcohol consumption and Rankin scale (RS) did not remain significant factors, only the constant was statistically significant (**Table 5**).

In **Table 6**, there was no association between the *TNFA* -308G/A polymorphism genotypes and the presence of changes in the neuroimaging ($p = 0.371$). The most common change in neuroimaging was subarachnoid hemorrhage, both in the GG genotype and in the AG + AA genotypes, 48.7% and 12.8%, respectively.

TABLE 3 – Clinical characteristics of case and control subjects

		TNFA -308G/A											
		GG						AG + AA					
		Aneurysm/HS		Control				Aneurysm/HS		Control			
		n	%	*n*	%	OR (CI)	*p*-value	*n*	%	*n*	%	OR (CI)	*p*-value
SAH	Yes	41	70.7	7	14.9	13.78 (5.16-36.79)	< 0.001*	19	82.6	1	2.9	156.75 (16.31-1506.47)	< 0.001*
	No	17	29.3	40	85.1			4	17.4	33	97.1		
Smoking status	Yes	24	41.4	16	34	1.36 (0.61-3.04)	0.442	8	34.8	8	23.5	1.73 (0.54-5.57)	0.354
	No	34	58.6	31	66			15	65.2	26	76.5		
Alcohol consumption	Yes	15	25.9	10	21.3	1.29 (0.52-3.21)	0.584	7	30.4	10	29.4	1.05 (0.33-3.33)	0.92
	No	43	74.1	37	78.7			16	69.6	24	70.6		

*HS: hemorrhagic stroke; SAH: systemic arterial hypertension; OD: odds ratio; CI: confidence interval; *significant* p < *0.05.*

TABLE 4 – Association between *TNFA* -308G/A (rs1800629) polymorphism genotypes and outcome in case group by Glasgow scale, Rankin scale and Barthel index

		GG		AG + AA		
		n	%	*n*	%	*p*
Glasgow	Intermediate coma	5	8.6	3	13	0.644
	Superficial coma	1	1.7	1	4.3	
	Normality	52	89.7	19	82.6	
Rankin scale	No symptoms at all	17	29.3	3	13	0.091
	No significant symptoms	32	55.2	14	60.9	
	Slight disability	2	3.4	0	0	
	Moderate disability	1	1.7	2	8.7	
	Moderately severe disability	5	8.6	1	4.3	
	Severe disability	1	1.7	3	13	
Barthel index	Severe disability	6	10.3	4	17.4	0.778
	Moderate disability	5	8.6	1	4.3	
	Slight disability	28	48.3	11	47.8	
	Functional independence	19	32.8	7	30.4	

DISCUSSION

This is the first report to evaluate the influence of *TNFA* -308G/A polymorphism upon the risk of HS or aneurysm in a Brazilian population. Part of the results shows a protective effect in the AG genotype patients; this same effect was verified in a Chinese population with the AG genotype[21]. Author implied that it might indirectly explain the high serum level of the cytokine TNF-α previously reported in ischemic stroke cases[21]. However, it differs from a more recent study in which *TNFA* -308G/A gene polymorphisms seem to not contribute to the risk of ischemic stroke[22].

Furthermore, we confirmed that history of hypertension increases the risk of HS or aneurysm, independently of A allele absence or presence. This association is already well known and documented[23-26].

This is the first study to demonstrate associations between stroke or aneurysm functional outcome and *TNFA* -308G/A polymorphism. Our results find no association using the incapacity status scales.

TABLE 5 – Multivariate logistic regression for genotyping (presence of GG genotype) according to the factors included in the model

		β	E.P.	Wald	gl	*p*	Exp(β)	95% CI (EXP[β])	
								Lower	Upper
Step 1[a]	Systemic arterial hypertension [(1)]	0.659	0.667	0.974	1	0.324	1.933	0.522	7.15
	Smoking status[(1)]	-0.012	0.56	0	1	0.984	0.988	0.33	2.965
	Alcohol consumption[(1)]	0.316	0.558	0.321	1	0.571	1.372	0.459	4.098
	Rankin scale[(1)]	0.631	0.722	0.762	1	0.383	1.879	0.456	7.74
	Constant	-1.603	0.723	4.91	1	0.027*	0.201		

*[a] insert variables on step 1: SAH, smoking status, alcohol consumption, Rankin scale; *significant* p < 0.05.*
SAH: systemic arterial hypertension; CI: confidence interval.

TABLE 6 – Association between *TNFA* -308G/A (rs1800629) polymorphism genotypes and neuroimaging of case group

	GG		AG + AA		
	n	%	*n*	%	*p*-value
Middle cerebral artery	8	20.5	4	10.3	0.371
Right frontal parietal cavernous hemangioma	0	0	1	2.6	
Aneurysm clip with post-surgery infection	1	2.6	0	0	
Left temporal intraparenchymal hematoma	3	7.7	3	7.7	
Subarachnoid hemorrhage	19	48.7	5	12.8	
Diffuse subarachnoid hemorrhage	1	2.6	0	0	
Thalamic hemorrhage with ventricular flood	1	2.6	0	0	
Expansive point angle lesion	3	7.7	0	0	
Basal ganglia	3	7.7	0	0	

CONCLUSION

In summary, the findings of this work show that the *TNFA* -308 AG genotype is associated with a decreased risk of aneurysm or HS. The present study has some limitations. All patients and control subjects were selected from one hospital, thus there may be some selection bias. Besides that, other polymorphisms and genetic alterations may influence the development of aneurysm or HS. Therefore, conducting additional studies incorporating a large number of subjects might clarify the association between *TNFA* -308G/A polymorphism and the risk of aneurysm or HS.

RESUMO

Introdução: *Muitas doenças cerebrovasculares relacionam-se com processos inflamatórios, portanto, a influência de vários polimorfismos em doenças tem sido estudada para melhorar o conhecimento sobre os mecanismos fisiológicos do sistema nervoso.* ***Objetivo:*** *Identificar a associação entre um polimorfismo na posição -308 do gene* TNFA *e o desenvolvimento de acidente vascular encefálico hemorrágico (AVEH) ou aneurisma em pacientes de uma base hospitalar do Distrito Federal, Brasil.* ***Métodos:*** *Foram coletados os prontuários e as informações clínicas de pacientes com AVEH ou aneurisma. A caracterização dos grupos caso foi confirmada por tomografia computadorizada (TC) ou ressonância nuclear magnética (RNM). Os genótipos do gene* TNFA *foram determinados por técnica do polimorfismo de comprimento dos fragmentos de restrição do produto obtido pela reação em cadeia da polimerase (PCR).* ***Resultados:*** *O genótipo AG parece diminuir a ocorrência de AVEH ou aneurisma em indivíduos entre 45 e 63 anos. Nosso estudo foi o primeiro a investigar essa associação em uma amostra brasileira, embora um relatório anterior tenha mostrado efeito semelhante com o acidente vascular encefálico isquêmico em uma população chinesa.* ***Conclusão:*** *O genótipo* TNFA *-308 AG está associado à diminuição do risco de aneurisma ou AVEH em uma população da capital do Brasil, Distrito Federal.*

Unitermos: *fator de necrose tumoral alfa; polimorfismo de nucleotídeo único; acidente vascular cerebral; hemorragias intracranianas; aneurisma intracraniano.*

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