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IL-10 -592 C/A polymorphism association with hemorrhagic stroke/aneurysm and their risk factors

Associação do polimorfismo *IL-10* -592 C/A com acidente vascular cerebral hemorrágico/aneurisma e seus fatores de risco

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ABSTRACT

Stroke is one of the leading causes of death worldwide. Hemorrhagic strokes (HS) present higher mortality than ischemic stroke (IS) and promote disability and dependency on trivial activities in most patients. Although unclear for HS, inflammatory mediators appear to promote IS onset that anti-inflammatory cytokines, such as IL-10, could mitigate. Polymorphisms in cytokine locus can affect its expression, especially in regulation regions, or affect protein's structure and, consequently, its biological role, impacting its availability or performance and promoting pathogenesis. In this case-control study, we analyze *IL-10* -592 C/A (rs1800872) polymorphism association with HS by investigating 162 participants - 81 patients with HS, aneurysm, or both and 81 healthy controls - from Federal District, Brazil. We also evaluate this polymorphism association with known risk factors for strokes - high blood pressure, smoking, alcoholism, and diabetes. Herein, we found no association between the *IL-10* -592 C/A polymorphism and HS, nor the risk factors examined.

Keywords: Hemorrhagic stroke; Aneurysm; *IL-10* -592 C/A; Genetic polymorphism; Risk factors.

RESUMO

O acidente vascular cerebral (AVC) é uma das principais causas de morte em todo o mundo. O AVC hemorrágico apresenta maior mortalidade que o AVC isquêmico e promove incapacidade e dependência na maioria dos pacientes. Embora não esteja claro para o AVC hemorrágico, mediadores inflamatórios parecem promover o início do AVC isquêmico, que as citocinas anti-inflamatórias, como a IL-10, poderiam mitigar. Polimorfismos no *locus* da citocina podem afetar sua expressão, principalmente em regiões de regulação, ou afetar a estrutura da proteína e, consequentemente, seu papel biológico, impactando sua disponibilidade ou desempenho e promovendo sua patogênese. Neste estudo de caso-controle, analisamos a associação do polimorfismo *IL-10* -592 C/A (rs1800872) com AVC hemorrágico em 162 participantes - 81 pacientes com AVC hemorrágico, aneurisma ou ambos e 81 controles saudáveis - do Distrito Federal, Brasil. Também avaliamos a associação desse polimorfismo com fatores de risco conhecidos para AVC – hipertensão, tabagismo, alcoolismo e diabetes. Aqui não encontramos associação entre o polimorfismo *IL-10* -592 C/A e AVC hemorrágico, nem os fatores de risco examinados.

Palavras-chave: Acidente vascular cerebral hemorrágico; Aneurisma; *IL-10* -592 C/A; Polimorfismo genético; Fatores de risco.

INTRODUCTION

With an increasing burden worldwide, stroke corresponds to about 10% of deaths (Global Burden of Disease Stroke Expert Group *et al*, 2018; Hay *et al*, 2017; Naghavi *et al*, 2017) and is, together with ischemic heart disease, one of the leading causes of disability-adjusted life years (DALYs) (Hay *et al*, 2017). Combined, these diseases led to more than 85% of all deaths caused by cardiovascular diseases in 2016 (Naghavi *et al*, 2017).

Stroke is currently defined as the blockade of blood supplies in a focal area of central nervous system (CNS) organs (brain, spinal cord, or retinal) due to either occlusion (ischemic stroke) or rupture of blood vessels (hemorrhagic stroke), leading to cell death and, consequently, compromising organ function (Campbell and Khatri, 2020; Sacco *et al*, 2013). Some symptoms related to stroke are impaired speech, paralysis, and loss of vision (Moskowitz *et al*, 2010).

Hemorrhagic stroke (HS) has higher fatality and Years of life lost (YLLs) compared to ischemic stroke (IS) (Hay *et al*, 2017), despite the latter being the most common type of stroke (Moskowitz *et al*, 2010). Moreover, 74% of HS survivors become dependent on others for basic activities (Van Asch *et al*, 2010). One known risk factor correlated with HS is elevated systolic blood pressure (Gakidou *et al*, 2017), which could trigger an aneurysm (Albuquerque, 2013; Tajiri *et al*, 2012). In contrast, IS is correlated with hypertension, diabetes, hypercholesterolemia, cigarette smoking, atrial fibrillation, and valvular heart disease, among other risk factors (Amarenco *et al*, 2006; Bonita *et al*, 1999; Hart and Pearce, 2000; Lawes *et al*, 2004; Lawes *et al*, 2004).

Inflammation of the central nervous system (CNS) plays a critical role in stroke pathology, characterized by damage and activation of resident immune cells, such as glial cells. When these cells sense the damage, they, in turn, release inflammatory mediators, such as cytokines and chemokines, that recruit more immune cells to the harmed site. Although this help is necessary to “clean the mess” and promote repair, massive or prolonged recruitment could trigger off the breakdown of the blood-brain barrier, cerebral edema, cerebral hypertension, and ischemia (Cederberg and Siesjö, 2010; Iadecola and Anrather, 2011; Patterson and Holahan, 2012; Wang and Doré, 2007).

Artery inflammation is also associated with IS, as experimental models present peaking levels of inflammatory mediators such as IL-6, TNF, INF-γ, CXCL1, and

CXCL2, and as IS onset in humans also correlates with increased amounts of TNF and IL-6 (Chapman *et al*, 2009; Ferrarese *et al*, 1999; Kes *et al*, 2008; Offner *et al*, 2006). As opposed, HS association is unclear. For instance, although some studies have shown that experimental HS exhibit increased inflammatory mediators (Agnihotri *et al*, 2011; Di Napoli *et al*, 2011; Mracsko and Veltkamp, 2014), the causes of the inflammatory process are not considered (Fu *et al*, 2015).

Conversely, the anti-inflammatory cytokines, such as IL-10, soften inflammation and prevent tissue damage, counteracting the effects of pro-inflammatory mediators (Ouyang and O'Garra, 2019). As some pro-inflammatory cytokines may influence IS onset, anti-inflammatory mediators may prevent IS /HS pathogenesis.

Hence, factors like polymorphisms that affect the expression or function of these mediators could influence the disease's onset, severity, or both. For instance, *IL-6* rs1800795 polymorphism correlated significantly with susceptibility to IS in Asians but not in Caucasians (Chen and Yang, 2019). TGF- β1 -509 C/T and codon *10* Leu/Pro polymorphisms were associated with an increased risk of stroke (Sie *et al*, 2006), and elevated TGF- β1 levels were detected in the brain of IS deceased patients (Krupinski *et al*, 1996). Our group previously showed that *TNF-a* -308 A/G genotype is related to a reduced risk of HS/aneurysm in a Brazilian population from Brazil's capital (Borges *et al*, 2018).

IL-10 is produced by almost all lymphocyte subsets and has an essential role in attenuating inflammation, minimizing damage, re-establishing homeostasis, and avoiding inappropriate inflammation (Ouyang and O'Garra, 2019). With this in mind, the lack or diminished IL-10 could also have a role in developing some inflammatory diseases such as stroke and, consequently, HS. Some studies with IS models have shown the IL-10's neuroprotective role (Garcia *et al*, 2017). Indeed, Rui and colleagues (2020) suggest that lower IL-10 serum levels are associated with a higher risk of IS in Chinese individuals, though no correlation could be confirmed for HS.

Regarding *IL-10* polymorphisms, *IL-10* -819 T/C polymorphism is unrelated to IS risk (Zuo *et al*, 2020), whereas *IL-10* rs1800896 polymorphism correlated significantly with the susceptibility to IS (Chen and Yang, 2019) and *IL-10* -1082 A/G polymorphism contributes to a decreased IS risk (Zuo *et al*, 2020). *IL-10* rs1800871 and 1800872 were associated with intracranial aneurysm incidence, although they did not correlate with rupture (Sathyan *et al*, 2015). As no research was found that seems to

have analyzed *IL-10* polymorphisms with HS, this case-control study investigates for the first time the correlation between *IL-10* -592 C/A (rs1800872) polymorphism (Turner *et al*, 1997) and HS/aneurysm.

MATERIAL AND METHODS

This investigation is an observational, descriptive, case-control study in patients diagnosed with hemorrhagic stroke (HS), intracerebral aneurysm, or both and in healthy individuals without a recorded HS/aneurysm residing in the Federal District (Brazil).

Research participants

In total, we analyzed one hundred and sixty-two (162) individuals through a case-control-based hospital study conducted from January 2011 to December 2012.

The case group consisted of 81 HS/aneurysm patients over 18 years. All patients had clinical signs consistent with the World Health Organization definition of stroke and confirmed by imaging (computed tomography or magnetic resonance image) (Hatano, 1976). This group consisted of 48 patients (59.3%) with HS and 33 (40.7%) with an aneurysm. In contrast, the control group comprised 81 age and sex-matched healthy individuals over 18 years with no prior HS/aneurysm history recruited from volunteers and healthy individuals accompanying the patients in the general outpatient department (OPD) not related to the case group. To be included, the participants in the control group should have performed some biochemical tests, such as serum glucose, at least 15 days prior. Patients under 18 years without an HS/aneurysm diagnosis and related to the control group were excluded.

We obtained the informed consent form (ICF), and within it, the consent to publish from all research participants prior to collecting information and 10 mL of their venous blood. The study strictly followed the ethical research procedures approved by FEPECS Research and Ethics Committee, Federal District, Brazil (Opinion No. 0095/2010), as well as the 1964 Helsinki Declaration and its later amendments. All participants, residents of the Federal District, Brazil, had a detailed clinical history and clinical developments followed.

Clinical features

We conducted a detailed history and clinical evaluation. Patients were questioned about having arterial hypertension (and their blood pressure measured), diabetes, age, sex, smoking habits, and alcohol consumption.

The patients' functional assessment employed scales to measure the severity, stages of the disease, and incapacity of patients: the Modified Rankin Scale (mRS) (Banks and Marotta, 2007) for measurement of motor capacity.

Technical and Laboratory Procedures

Participants had approximately 10 mL of their venous blood collected by peripheral venipuncture. From this blood, their genomic DNA (Deoxyribonucleic acid) was extracted using Invitek's Invisorb Spin Blood Mini Kit - 250 (catalog # CA10-0005, lot # 1031100300, Germany). The average DNA concentration obtained was 20 ng/µL, as estimated by the spectrophotometer (NanoDrop 2000/2000c - Thermo Fischer Scientific).

Then, their DNA was subjected to the PCR (Polymerase Chain Reaction) - RFLP (restriction fragment length polymorphism) strategy for SNPs distribution analysis. The oligonucleotide sequences used to evaluate the *IL10* -592 A/C (rs1800872) polymorphisms frequency were, respectively: sense 5'-GGTGAGCACTACCTGACTAGC-3' and antisense 5'-CCTAGGTCACAGTGACGTGG-3'. These primers flank the IL10 promoter region at position -592. Each PCR reaction mix contained: 4.0µL of 2.5ng/µL genomic DNA, 2.5µL of 10x buffer (10mM Tris and 50mM KCl), 0.5µL of 50mM MgCl2 (Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil); 0.5µL of 2.5mM dNTPs (deoxyribonucleotide triphosphate; Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil); 0.5µL of 5U/µL Taq-Polymerase (Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil), 1.5µL of each 10µM forward and 10µM reverse primer (IDT technologies), completing the mix to a final volume of 25µL with Milli-Q water.

The thermocycling conditions were initial denaturation at 94˚C for 5 minutes, followed by 30 cycles of denaturation at 94˚C for 60 seconds and primers annealing at 56˚C for 60 seconds, and fragments extension at 72˚C for 45 seconds. The final extension was performed at 72˚C for 7 minutes and cooling for 4 minutes. The equipment used was the TC-96/G/H(b) Life Express ThermalCycler.

The PCR product (fragment: 412bp) was digested with the RsaI enzyme (Invitrogen®). The A allele has a RsaI restriction site that permits the 412bp fragment to be cleaved into two of 236bp and 176bp, while the C allele does not have the restriction site for this enzyme. The digestion products were submitted to an electrophoretic run on a 3% agarose gel, stained with ethidium bromide 0.1%, at a 100W power for 20 minutes.

Statistical Analysis

Estimation of the genotypic frequencies

Genotypic and allele frequencies were determined by direct counting using the SPSS version 28.0 program. The chi-square test compared these frequencies distribution to detect genotype associations between the evaluated groups – the HS/aneurysm (case) and the control groups.

Data analysis of the study's subjects

The research participants' specific clinical characteristics frequencies were estimated: smoking, alcoholism, systemic arterial hypertension (SAH), and diabetes. Subsequently, the clinical prognosis measured by the Modified Rankin Scale (mRS) for HS/aneurysm was statistically associated (chi-square) according to each genotype. A p-value of less than 0.05 ($p < 0.05$) was considered significant.

RESULTS

We first investigated whether this study's control and case (HS/aneurysm) groups are divergent and could affect the results by evaluating the participants' global characteristics. No difference was found between the groups' sex ($p = 0.531$) and age ($p = 0.192$), indicating a homogeneous sample population. Notably, most research participants were women in this study, 59.3% in the case group and 53.1% in the control group, and the sample population's average age was 53.49 years (± 5.94) in the case group and 52.27 years (± 5.75) in the control group. The *IL-10* -592 C/A (rs1800872)'s CC, CA, and AA genotype frequencies were also similar between the groups (Table 1). The genotypes in the control group follow the Hardy–Weinberg equilibrium (HWE) ($p = 0.310$).

Table 1. *IL-10* -592 C/A (rs1800872) polymorphism genotypes' distribution in stroke and control groups.

IL-10 -592 C/A (rs1800872)	Groups				P[1]
	Stroke		Control		
	N	%	N	%	
CC	39	48.1%	40	49.4%	0.331
CA	37	45.7%	31	38.3%	
AA	5	6.2%	10	12.3%	

[1] Chi-square test

Chronic diseases and certain habits are generally risk factors for stroke onset. Hence, we examined the relation between Systemic Arterial Hypertension (SAH), diabetes, smoking status, and alcohol consumption with the genotypes (Table 2). Patients with neither CC, CA nor AA genotype were associated with any chronic condition or habit analyzed, with p-values in all cases above 0.05.

Table 2. Association between *IL-10* -592 C/A (rs1800872) polymorphism genotypes and clinical aspects in the stroke and control groups.

		Groups												
		Stroke							Control					
		IL-10 -592 C/A (rs1800872)						P[1]	*IL-10* -592 C/A (rs1800872)					P[1]
		CC		CA		AA			CC		CA		AA	
		N	%	N	%	N	%		N	%	N	%	N	%	
Sex	Female	22	56.4%	22	59.5%	4	80.0%	0.601	20	50.0%	14	45.2%	9	90.0%	0.041*
	Male	17	43.6%	15	40.5%	1	20.0%		20	50.0%	17	54.8%	1	10.0%	
SAH	Yes	28	71.8%	29	78.4%	3	60.0%	0.613	6	15.0%	2	6.5%	0	0.0%	0.261
	No	11	28.2%	8	21.6%	2	40.0%		34	85.0%	29	93.5%	10	100.0%	
Diabetes	Yes	2	5.1%	1	2.7%	0	0.0%	0.772	0	0.0%	0	0.0%	0	0.0%	NA
	No	37	94.9%	36	97.3%	5	100.0%		40	100.0%	31	100.0%	10	100.0%	
Smoking status	Yes	16	41.0%	15	40.5%	1	20.0%	0.642	11	27.5%	10	32.3%	3	30.0%	0.909
	No	23	59.0%	22	59.5%	4	80.0%		29	72.5%	21	67.7%	7	70.0%	
Alcohol consumption	Yes	10	25.6%	10	27.0%	2	40.0%	0.794	7	17.5%	9	29.0%	4	40.0%	0.261
	No	29	74.4%	27	73.0%	3	60.0%		33	82.5%	22	71.0%	6	60.0%	

*P<0.05 in the control group; [1] Chi-square test; NA: Not applicable; SAH: systemic arterial hypertension

Further analysis of the possible association between *IL-10* -592 C/A and HS/aneurysm prognostics, per the modified Rankin scale (mRS), demonstrated that most patients had a good prognostic regardless of their genotype (87.7%) (Table 3).

Likewise, patients with a bad prognostic presented a similar disregard towards the genotype (p = 0.544).

Table 3. Association between *IL-10* -592 C/A (rs1800872) polymorphism genotypes and Modified Rankin scale (mRS) in the stroke group.

		IL-10 -592 C/A (rs1800872)								P[1]
		CC		CA		AA		Total		
		N	%	N	%	N	%	N	%	
mRS	Poor Prognosis	6	15.4%	3	8.1%	1	20.0%	10	12,3	0.544
	Good Prognosis	33	84.6%	34	91.9%	4	80.0%	71	87,7	

[1] Chi-square test.

DISCUSSION

Immune responses and inflammation have emerged as crucial factors contributing to ischemic stroke (IS) onset. This statement has also been extrapolated to HS, although lacking experimental evidence (Fu *et al*, 2015).

The immune response can promote IS pathogenesis prior to the stroke event if something induces an unwanted immune response previously, which would lead to lymphocytes recruitment to brain vessels, activation and triggering of pro-inflammatory mediators that might generate thrombosis, which could contribute to the occurrence of occlusion or ruptures (Stark and Massberg, 2021). These events that promote the absence of blood supply lead to cellular death and tissue damage in the affected area. Dead cell components, in turn, are recognized by resident immune cells, such as microglia, and promote their activation and augment their secretion of inflammatory mediators to resolve the damage. Factors released by ischemic neurons can attract lymphocytes as NK cells, for example, stimulating them to produce IFN-γ and TNF-α, which promote excitotoxicity. NK cells also secrete IFN-y and GM-CSF that activate microglial cells, astrocytes, and macrophages, which in turn produce inflammatory mediators that promote and exacerbate inflammation in affected the site in the first hours of stroke (Chamorro *et al*, 2007; Gan *et al*, 2014; Prass *et al*, 2003). This exacerbated inflammation can also provoke further tissue damage and worsen the clinical condition.

IL-10 -592 C/A (rs1800872) polymorphism is located in the promotor region and, like *IL-10* -1082 G/A and -819 C/T, affects IL-10 expression and production. For example, the −1082 G allele was associated with increased IL-10 production upon Concanavalin A stimulation (Turner *et al*, 1997). Different haplotypes also can be involved in the diseases' pathogenesis, severity, or both, as in the case of systemic lupus erythematosus association with specific *IL-10* alleles (Eskdale *et al*, 1997; Lazarus *et al*, 1997; Llorente *et al*, 1995; Llorente *et al*, 1997).

Nevertheless, we did not verify any association between *IL-10* -592 C/A polymorphism with HS/aneurysm, its prognosis, nor specific chronic diseases or habits considered risk factors for stroke in the sample from Brazil's capital population. Aihara and colleagues (2001) postulated that the IL-10 effect could be overwhelmed by the pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β, and TNF-α, which have shown an impressive expression in HS (Aihara *et al*, 2001; Kooijman *et al*, 2014; Song *et al*, 2019). Therefore IL-10 could be unable to prevent these mediators' harmful effects.

Further studies may be necessary to confirm these findings with larger sample size and different populations. Other anti-inflammatory mediator polymorphisms, e.g., *TGF- β*, could impact HS pathogenesis and should also be considered for future studies to evaluate their participation in HS's pathogenesis, progression, or both. Even though we are unable to determine *IL-10* -592 C/A polymorphism influence on HS/aneurysm, it is the first time this possible association has been evaluated.

CONCLUSION

Our findings suggest no association with *IL-10* -592 C/A (rs1800872) polymorphism with HS/aneurysm, prognosis, or risk factors (high blood pressure, diabetes, smoking, and alcoholism). More studies with larger sample sizes and divergent populations are required to certify these data. It might also be critical to evaluate other *IL-10* polymorphisms with hemorrhagic stroke, aneurysm, or both and associate them with their product's levels to fill the gaps in understanding stroke pathogenesis.

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