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TUMOR NECROSIS FACTOR ALPHA AND ALPHA-1 ANTITRYPSIN GENE VARIANTS IN SERBIAN PEDIATRIC ARTERIAL ISCHEMIC STROKE PATIENTS

Valentina DJORDJEVIC[1], Aleksandra DIVAC RANKOV [1], Marija STANKOVIC[1], Vesna BRANKOVIC SRECKOVIC[2], Dragica RADOJKOVIC[1]

[1]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia
[2] Clinic for Child Neurology and Psychiatry, School of Medicine, University of Belgrade, Belgrade, Serbia

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The etiology of arterial ischemic stroke (AIS) in children is complex, and different from that in adults. Although rare, stroke in children is an important cause of mortality and morbidity. There is increasing evidence that genetic factors, including inflammation mediators, have a role in occurrence and outcome of stroke. We have chosen to assess the role of polymorphism -308G/A in the promoter of *tumor necrosis factor α* (*TNFα*) gene and S and Z mutations in *alpha 1-antitrypsin* (*AAT*) gene in the etiology of stroke in children. *TNFα* polymorphism affects plasma levels of this proinflamatory cytokine, and this could contribute to stroke pathology. It has been shown that increased AAT concentration may present a risk for AIS in children. Since S and Z mutations in *AAT* gene reduce its levels in plasma they could have a protective role in pediatric stroke.In this study twenty six children with AIS and 100 unrelated individuals from Serbian general population were investigated by PCR/RFLP for these gene variations.No statistically significant difference was observed between patients and general population in distribution of genotypes for -308G/A *TNFα* polymorphism, so its contributory role in the etiology of stroke was not evident in our group of patients.None of the tested *AAT* gene mutations were found in patients, which is in concordance with the proposed protective role of deficient AAT variants.AIS is a multifactorial disease, with many genes having a modest role in its pathophysiology, so further analyses of their combined effect are needed to elucidate genetic risk factors in the etiology and outcome of stroke in pediatric patients.

Key words: tumor necrosis factor α (TNFα), alpha 1-antitrypsin, arterial ischemic stroke, children

Corresponding author: Aleksandra Divac Rankov,,Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444, PO Box 23,11010 Belgrade, Serbia, Tel: +381 11 3976658 Fax: +381 11 3975808,E-mail: aleksandradivac@imgge.bg.ac.rs

INTRODUCTION

Arterial ischemic stroke (AIS) in children is rare, but serious condition associated with significant mortality and morbidity. Most of the adult stroke risk factors, such as atherosclerosis and high blood pressure, are extremely rare in children, but several underlying medical conditions are often associated with pediatric AIS. Some of them are cardiac pathologies, infections, protombotic, metabolic and vascular disorders (JORDAN, 2006, LYNCH *et al.* 2005, BARNES *et al.* 2004, DJORDJEVIC *et al*. 2012). However, not all children with underlying conditions develop stroke, pointing to the evident contribution of genetic risk factors.

Stroke has several steps, consisting of a cascade of events, and the allelic variants of functionally relevant genes could modulate different pathophysiological processes related to this disease. For example, it has been shown that mutations in genes involved in hipercoagubility present risk factors for stroke in children (KENET *et al.* 2010).

One of important part of stroke pathophysiology is inflammation. Acute infections, accompanied by acute phase reactions and a prothrombotic state, have been associated with an increased risk of acute vascular events, such as stroke and myocardial infarction (LALOUSCHEK *et al.* 2006). Also, activated immune cells of the central nervous system (CNS) (microglia) are considered to have a deleterious effect in stoke.

Tumor necrosis factor alpha (TNFα) is an immune mediator that regulates inflammatory response, modulates growth and cellular differentiation and activates blood coagulation. It is mainly produced by macrophages in response to injury and inflammation, and in CNS it is mostly produced by activated microglia. A single nucleotide polymorphism in the promoter of *TNFα* gene, at the position -308, shows correlation with the overexpression of TNFα (WILSON *et al.* 1997). In patients with acute ischemic stroke increased levels of TNFα have been detected (BENVENISTE *et al*. 1998; NAYAK *et al*. 2012). In addition, overexpression of TNFα has been found to be detrimental to stroke outcome (PETTIGREW *et al*. 2009, DOMAC *et al*. 2007).

Alpha 1-antitrypsin (AAT) is an active phase reactant and has several roles that may impact the pathophysiology of stroke. It is a physiological inhibitor of activated protein C (APC), which itself has an anticoagulatory effect. In this case, AAT could function as a regulator of thrombotic events, as the elevated AAT could theoretically decrease APC concentration subsequently leading to a prothrombotic state. Also, it has been shown that AAT has antiinflammatory and antiapoptotic functions, which could be important in stroke cascade. AAT is an endogenous inhibitor of production of proinflammatory cytokines, so its deficiency may lead to increased production of these cytokines, such as TNFα, IL-6, IL-8 and IL-1 beta (HUNT 2012). It is likely that AAT activities separate from serine protease inhibition participate in proinflammatory cytokine suppression. The most frequent deficient variants of AAT are Z (E342K, PiZ) and S (E264V, PiS). The Z mutation gives only 15% of normal AAT serum levels in homozygotes and approximately 60% in heterozygotes. Heterozygous carriers of S mutation have AAT levels 80% of the normal AAT plasma levels; whereas homozygous carriers have AAT levels that are 60% of the normal AAT plasma levels (CROWTHER *et al*. 2004).

In our previous study, we have investigated the role of mutations and polymorphisms of genes involved in hemostasis, endothelial function and vascular reactivity in a well-defined group of children with AIS (DJORDJEVIC *et al*. 2009). In this study we have focused on genes that may be involved in inflammation and protease/antiprotease balance – *tumor necrosis factor-alpha* and *alpha 1-antitrypsin* in the same group of children. Our aim was to investigate the

possible association between -308G/A polymorphism in *TNFα* and *AAT* S and Z mutations and the development of AIS in pediatric patients.

MATERIALS AND METHODS

Children with AIS were recruited from a single medical center, The Clinic for Child Neurology and Psychiatry, University of Belgrade, in the period from 1997 to 2005. The diagnosis was established based on clinical and radiological criteria using magnetic resonance imaging (MRI) and magnetic resonance and/or digital subtraction angiography. Patients with neonatal stroke, sino-venous thrombosis and hemorrhagic stroke were excluded. From 49 children diagnosed with AIS, during this period, 26 (age at diagnosis: 8.5 ± 5.1; male/female: 15/11) were available for testing and were included in this study. To define possible risk factors for AIS extensive clinical and laboratory investigations were performed including genotyping of *FV* Leiden, *FII* G20210A, *PAI-1* 4G/5G, *MTHFR* C677T, *eNOS* G894T, *ACE* I/D gene variants (DJORDJEVIC *et al*. 2009). Control group encompassed 100 unrelated subjects from Serbian general population (age: 50.9 ± 1.4; male/female: 35/65).

The study protocol was approved by local ethics committee, and informed consent was obtained from control participants and parent of all pediatric patients.

Peripheral blood was taken on 3.8% Na-citrate as anticoagulant. Genomic DNA was purified using QIAamp DNA blood mini kit (QIAGEN, Germany) according to manufacturer's protocol. Genotyping of *TNFα* -308G/A and *AAT* gene variants was performed using PCR-RFLP method as described previously (SAKAO *et al.* 2001, LUCOTTE *et al.* 1999).

The comparison of genotype distributions between children with AIS and Serbian general population were assessed by Fishers exact test. Odds ratio and 95% confidence interval were calculated. P value of less than .05 was considered significant. All statistical analyses were performed using MedCalc - version 12.4.0 software.

RESULTS

The recurrence of stroke was recorded in five (19.2%) children, while one child died of complications associated with a preexisting condition. Positive family history was present in 15.4% patients. Possible risk factors, such as infection, prothrombotic disorders, head injury, nonprogressive and progressive arteriopathy and cardiac disease, were identified in 15 (57.7%) children.

Out of 26 patients, five (19.2%) were found to be heterozygous for -308G/A *TNFα* polymorphism. None of the patients were homozygous for this polymorphism. Mutations, S and Z, in *AAT* gene were not present in any of the patients.

In the control group, *TNFα* genotypes were distributed as follows: -308G/G in 69, -308G/A in 28, -308A/A in three subjects, giving the allelic frequencies for G and A alleles of 83% and 17%, respectively. Z mutation was present in heterozygous state in three (3%) out of 100 subjects from general population, whereas S was not present in any of them.

No statistically significant differences were observed between genotypes or allelic frequencies between patient and control groups (Table 1).

The main clinical and laboratory findings, including genetic testing, for the five patients who were found to be carriers of TNFA -308G/A are given in Table 2. Most of them (80%) had no family history of stroke, but all of these patients had at least one more possible genetic risk factor that has been previously tested.

Table 1. The distribution of TNFα and AAT gene variants among patient and control groups

	genotype	patients n=26	control group n=100	OR (95% CI)	p
TNFα	-308G/G	21	69	1.89 (0.65-5.46)	0.24
	-308G/A	5	28	0.61 (0.21-17.80)	0.37
	-308A/A	0	3	0.53 (0.03-10.50)	0.67
	-308G/A + -308A/A	5	31	0.68 (0.24-1.97)	0.48
	G	47	166	1.93 (0.71-5.20)	0.20
	A	5	34	0.52 (0.19-1.40)	0.20
AAT	M/M	26	97	1.90 (0.10-38.00)	0.67
	M/Z	0	3	0.52 (0.03-10.50)	0.67
	Z/Z	0	0	-	-
	M/S	0	0	-	-
	S/S	0	0	-	-

Table 2. Clinical and genetic data of TNFα -308G/A polymorphism carriers

patient number	sex	age at diagnosis	No of recurrences	underlying disease and/or additional risk factors	family history	genetic data							
						TNFα-308G/A	*AAT* (S and Z)	*FV* Leiden*	*FII* G20210A*	*MTHFR* C677T*	*eNOS* G894T*	*PAI-1* 4G/5G*	*ACE* I/D*
4	F	13	3	Hypoplastic ICA artery	Neg	M/N	N/N	M/N	N/N	M/N	M/M	M/M	M/M
9	M	1	1	Infection, talasemia	Neg	M/N	N/N	N/N	N/N	M/M	N/N	M/N	N/N
12	F	16	1	-	Neg	M/N	N/N	N/N	N/N	M/N	N/N	M/N	M/M
14	M	16	1	Postvaricella arteriophaty	Neg	M/N	N/N	N/N	N/N	N/N	M/N	M/M	M/N
33	F	11.5	1	-	Pos	M/N	N/N	N/N	N/N	M/N	N/N	M/N	M/N

*M mutant allele; N wild type allele; * DJORDJEVIC et al. (2009)*

DISCUSSION

Since pediatric stroke is an important cause of childhood mortality and morbidity, and the nature of risk factors in children differ from those in adults further elucidation of its risk factors is needed. Several genetic risk factors for spontaneous ischemic stroke in children have been identified in recent years, such as lipoprotein A levels, prothrombotic mutations (*FV* Leiden, *FII* G20210A, *MTHFR* C677T) and protein C deficiency (GOLDENBERG *et al*. 2013; KENET *et al*. 2010; MARTINEZ *et al*. 1993).

There is an increasing body of evidence about the role of inflammation in etiology and/or progression of stroke. In the present study we tested the possible role of genes involved in vascular inflammation – *TNFα* and *AAT*. We found no difference between distribution of tested genotypes between patient and control groups.

Most of the studies on involvement of TNFα polymorphism have been done on adult patients, but even there the results are still conflicting (UM *et al*. 2003, RUBATTU *et al*. 2005, GU *et al*. 2013). TNFα, as a proinflammatory cytokine, has been shown to have an important role in proinflammatory and procoagulant states of endothelium. Several studies suggest a direct role of TNFα in the course of acute stroke. Stimulation of TNFα has a critical role in increasing the sensitivity to induction of brain ischemia and hemorrhage. Additionally, there is evidence that blocking of TNFα has an impact on improvement of brain ischemia in experimental models (SIRAN *et al*. 2001). There have been studies showing a direct contributory role of *TNFα* polymorphisms on ischemic stroke in several adult populations (UM *et al* 2003, RUBATTU *et al*. 2005), but the results are still conflicting and varying between different populations.

In pediatric patients, several studies have assessed the role of *TNFα* polymorphisms in stroke pathology, yielding controversial findings (KARAHAN *et al.* 2005; SHI *et al*. 2009; HOPPE *et al*. 2007). In Turkish and Chinese pediatric stroke populations, no association with the polymorphism -308G/A was found. Our results showed no statistically significant difference between distributions of *TNFα* -308G/A polymorphism between patient and control group. However, G allele was more frequent in patients (OR 1.93; 95%CI 0.7-5.2) indicating its possible role in pediatric stroke pathogenesis. These results are in concordance with study of stroke in children with sickle cell anemia that showed association of *TNFα* G/G genotype with increased risk (HOPPE *et al*. 2007).

The combined role of *TNFα* -308G/A genotype with fever for increasing the risk of stroke was reported in one study (LALOUSCHEK *et al*. 2006) in adult patients. In our study, out of five patients who were carriers of this polymorphism, two had infection before the onset of stroke and three had a fever, so this polymorphism could have had a contributory effect in this instance.

There are several roles of AAT which could have an effect on pathophysiology of stroke: physiological inhibition of APC, inhibition of proteolitic enzymes in vascular tissue, antiinflammatory and antiapoptotic functions. Several studies have found increased levels of AAT in adult patients with cerebral infarction and cerebral ischemia, as well as in patients with acute ischemic heart disease. But so far, the studies on the role of AAT in adult stroke have had inconsistent results.

There are sparse results about the involvement of AAT in pediatric AIS. In a study by Burghaus et al. it has been shown that increased levels of AAT (above 90th age-dependent percentiles) present an additional risk for development of AIS in children (BURGHAUS *et al.* 2006). Unfortunately, since it is not a part of the routinely done panel of laboratory workup in

pediatric stroke patients, we did not have the data on AAT concentration in our patients. Since elevated level of AAT in AIS has been detected, it could be speculated that presence of deficient mutations in *AAT* could have a protective role. Our results could be interpreted to be in concordance with these findings (wild type *AAT* genotype: OR 1.90; 95%CI 0.10-38.0), since we found none of the patients to be the carriers of two most common AAT deficient variants. This result could also be due to fairly low overall frequency of these variants in our population (DIVAC *et al.* 2004). Additionally, the limitation of our study, like others concerning childhood stroke, is a relatively small sample size. Further studies are needed to better evaluate and understand the role of AAT in AIS.

In conclusion, our findings suggest that *TNFα* and *AAT* S and Z variants do not have direct effect on the etiology of AIS in Serbian pediatric patients, but still could be additional factors important in the complex and intricate interplay of factors that contribute to the development of stroke in children. All patients who were carriers of *TNFα* -308G/A polymorphism had at least one other possible genetic risk factor for development of stroke, so this goes in support of AIS being a polygenic disease. The pathophysiology of stroke is under the influence of many factors, both acquired and genetic, and further analyses of their combined effect will give a better picture of their influences on onset and outcome, as well as possible treatment of stroke.

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GENSKE VARIJANTE FAKTORA NEKROZE TUMORA-ALFA I *ALFA1-ANTITRIPSINA* KOD PEDIJATRIJSKIH PACIJENATA SA ARTERIJSKIM ISHEMIJSKIM MOŽDANIM UDAROM U SRBIJI

Valentina DJORDJEVIC [1], Aleksandra DIVAC RANKOV [1], Marija STANKOVIC [1], Vesna BRANKOVIC SRECKOVIC [2], Dragica RADOJKOVIC[1]

[1]Institut za molekularnu genetiku i genetičko inženjerstvo, Univerzitet u Beogradu, Beograd, Srbija
[2] Klinika za dečiju neurologiju i psihijatriju, Mediciski fakultet, Univerzitet u Beogradu, Beograd, Srbija

Izvod

Etiologija arterijskog ishemijskog moždanog udara (AIS) kod dece je veoma kompleksna i razlikuje se od one kod odraslih. Iako je redak, moždani udar kod dece predstavlja značajan uzrok mortaliteta i morbiditeta. Sve je više podataka o ulozi genetičkih faktora, uključujući tu i medijatore inflamacije, u nastanku i ishodu moždanog udara. U ovoj studiji, odabrali smo da ispitamo ulogu polimorfizma -308G/A u genu za *faktor nekroze tumora-alfa* (eng. *Tumor Necrosis Factor α - TNFα*), kao i mutacija S i Z u genu za *alfa1-antitripsin* (*AAT*) u etiologiji moždanog udara kod dece. Polimorfizam -308G/A u genu za *TNFα* dovodi do povećanja koncentracije ovog proteina u plazmi, što bi moglo da doprinese patologiji moždanog udara. Pokazano je i da povišena koncentracija AAT može da predstavlja rizik za nastanak moždanog udara kod dece. S obzirom da mutacije S i Z u genu za *AAT* dovode do smanjenja koncentracije ovog proteina u plazmi, one bi mogle da imaju protektivnu ulogu kada je u pitanju moždani udar.Genske varijante *TNFα* (-308G/A) i *AAT* (S i Z) su ispitivane u grupi od 26 dece sa AIS i 100 odraslih osoba PCR/RFLP metodom.Nije nađena statistički značajna razlika u učestalosti -308G/A *TNFα* polimorfizma između pacijenata i kontrola, tako da u našoj grupi pacijenata *TNFα* najverovatnije nije imao značajnu ulogu u razvoju bolesti. Ni kod jednog pacijenta nije pronađena nijedna od ispitivanih mutacija u genu za *AAT*, što je bilo u skladu sa potencijalnom protektivnom ulogom ovih varijanti.AIS je multifaktorijalno oboljenje, u čijoj patologiji veliki broj gena ima -ulogu, tako da je potrebna dalja analiza zajedničkog delovanja većeg broja genskih varijanti da bi se rasvetlila njihova uloga kao genetičkih faktora rizika i njihovog uticaja na razvoj i ishod moždanog udara.

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