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*Research Article*

**Adenosine Deaminase 1 rs73598374 Genetic Polymorphism and Activity in Patients with Ischemic Stroke**

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## **Abstract**

In the present work we evaluated adenosine deaminase 1 (ADA1) encoding gene (ADA1) rs73598374 single nucleotide polymorphism (SNP) and the activity of this enzyme in patients with ischemic stroke (IS) in Armenian population. A total of 98 first episode IS patients within the first 24 hours of stroke onset, and age- and sex matched 177 healthy subjects (control group) were enrolled in this study. Genomic DNA samples of study subjects were genotyped for ADA1 rs73598374 SNP using polymerase chain reaction with the sequence specific primers. ADA1 activity was determined by colorimetric assay evaluating ammonia liberated in the enzymatic reaction of adenosine deamination. Hardy-Weinberg equilibrium, Pearson's Chi-square test and Student's t-test were used for evaluation of the obtained

results. The obtained results demonstrated no significant differences in allele and phenotype frequencies of the selected genetic variant between study groups, thus indicating no association of ADA1 rs73598374 SNP with IS. Also, insignificant difference was detected when comparing ADA1 activity levels in the blood of IS patients and healthy subjects. In summary we concluded that alterations in ADA1 enzyme on both genetic and activity levels are not implicated in IS pathogenesis.

**Highlights:** ADA1 gene rs73598374 SNP is not associated with IS in Armenian population. IS patients within the first 24 hours after stroke onset and healthy subjects have practically identical blood levels of ADA1 activity.

**Keywords:** Adenosine deaminase 1, genotyping, ischemic stroke, single nucleotide polymorphism.

## **Introduction**

Kurata (1995) has shown, that adenosine deaminase (ADA, EC 3.5.4.4) is a key enzyme of the purine salvage pathway that catalyses the deamination of (deoxy) adenosine to (deoxy) inosine. Sebastião et al. (2011) have shown that in the central nervous system ADA is functioning as neuroregulator providing a link between glial cells and neurons. Hirshhorn and Ratech (1980) found two isoforms of the enzyme in mammals, ADA1 and ADA2. Variations in levels and activities of both ADA1 and ADA2 have been implicated in several diseased conditions characterized by altered immune response and inflammation, as indicated by Pérez-Aguilar et al. (2010) and Antonioli et al. (2012). The ADA1 encoding gene (ADA1) is highly polymorphic and located on human chromosome 20 (20q.11.33). Hirschhorn



et al. (1994) identified the ADA1 rs73598374 (22 G>A) functional polymorphism in exonic region resulting in substitution of asparagine (ADA1\*1) for aspartic acid (ADA1\*2). In addition Hirschhorn et al. (1994) found that ADA 1\*2 demonstrated less enzymatic activity compared with ADA1\*1.

Number of studies indicated that alterations in adenosine metabolism may be implicated in ischemia-reperfusion. Thus, Kaul et al.(2006) has shown the important role of increased ADA1 levels in reperfusion injury in patients with myocardial infarction. Moreover, administration of ADA1 inhibitors has shown to be protective in murine models of heart ischemia, as mentioned by Pearta et al. (2001). In addition, Napolioni and Predazzi (2010) and Napolioni et al. (2011) found the association between this polymorphism and the rs1800629 (-

308G>A) promoter polymorphism of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) gene. TNF- $\alpha$  is one of the most typical pro-inflammatory cytokines with both beneficial and destructive properties for the central nervous system. Watters and O'Connor (2011) demonstrated the important role of TNF- $\alpha$  in the development of ischemic stroke (IS). Moreover, Tong et al. (2010), Gu et al. (2012) and Cui et al. (2012) revealed the association of the rs1800629 polymorphism of TNF- $\alpha$  gene with IS. Taken together, these findings raise our interest in studying the possible association of ADA1 rs73598374 single nucleotide polymorphism (SNP) with IS.

In the present work we evaluated ADA1 rs73598374 SNP and ADA1 activity in patients with IS in Armenian population, using as a reference control group healthy subjects from the same

population. To our knowledge this is the first study evaluating ADA1 rs73598374 SNP in IS.

## **Materials and Methods**

### ***Study Population***

In total, 98 first episode IS patients (males/females: 50/48, mean age $\pm$ SD: 64 $\pm$ 12 years), and age- and sex matched 177 healthy subjects (males/females: 90/87, mean age $\pm$ SD: 62 $\pm$ 11 years) were enrolled in this study. All subjects were of Armenian nationality living in Armenia. The patients were recruited from the “Armenia” and “St. Gregory the Illuminator” Medical Centers of the Ministry of Health of the Republic of Armenia (MH RA). According to TOAST classification developed by Adams et al

(1993), all IS patients had large vessel atherothromboembolic subtype of IS. Diagnosis of IS was based on clinical history and neurological examination and was confirmed by brain computer tomography (CT) imaging and basal laboratory tests. Stroke severity was scored using the National Institutes of Health Stroke Scale. All IS patients presented anatomically relevant CT hypodense areas in subcortical parts of cerebral hemispheres. Patients had moderately severe IS with a median baseline score of 14. Among them 54 had hyperlipidemia, 62 had arterial hypertension, and 29 had arterial fibrillation. Healthy subjects were recruited among the blood donors of the Erebouni Medical Center MH RA and had no family or own history of ischemic cerebrovascular event or myocardial infarction. No special studies have been performed to assess the progress of atherosclerotic process in this group. Exclusion criteria for all

subjects include diabetes mellitus, neuropsychiatric disorders, coronary artery disease, myocardial infarction, vasculitis, oncological, inflammatory, infectious, immune system disorders hematological diseases, severe renal or liver failure, gynecologic or urologic diseases, any other serious medical disorder or treatment, use of immunomodulators and any surgical intervention in the previous 12 months.

### ***Collection of Blood Samples, Separation of Blood Plasma and Isolation of Genomic DNA***

Ten ml of venous blood was collected from each patient and healthy subject using EDTA as anticoagulant. Blood from the patients was collected within the first 24 hours of stroke onset, before any medication was applied. Collected blood was divided

in two aliquots; one was used for separation of plasma and another - for isolation of DNA.

Plasma was obtained by centrifugation of blood (1500g x10min, 4°C) and kept frozen at -30°C until further use.

Genomic DNA was isolated from fresh blood according to the standard phenol-chloroform method, developed by Sambrook and Russell (2001), and stored at -300C until further use.

### ***Genetic Analysis***

All DNA samples were genotyped for ADA1 rs73598374 SNP using polymerase chain reaction with the sequence specific primers (PCR-SSP) as follows:

Specific primer for G allele (forward):  
5'CGCGCTCACTTTGGGCTTGTC;

Specific primer for A allele (forward):  
5'CGCGCTCACTTTGGGCTTGTT;

Constant primer (reverse): 5'TAGTTTTTCGGGTCGGCGGG.

All primers for the PCR-SSP were designed using the genomic sequences in the GenBank (<http://www.ncbi.nlm.nih.gov>, GenBank ID: 6347). Amplification reaction was carried out under the conditions described by Bunce et al (1995). Further, the allele-specific amplicons were visualized by electrophoresis in 2% agarose gel stained with ethidium bromide. Randomly selected samples (n=25; 10% of the total number) were analyzed

twice to check for confidence of genotyping and in each case the complete concordance was obtained.

### ***Determination of the ADA1 Activity in the Blood Plasma***

ADA1 activity was determined by evaluating ammonia liberated in the enzymatic reaction of adenosine deamination according to Andreasyan et al (2005). One of two identical enzyme assay mixtures contained a selective inhibitor of ADA1, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). Ammonia amount was measured using the phenol-hypochloride colorimetric method registering the absorbance of each assay mixture against blank at 625 nm. The absorbance for the EHNA containing mixture was taken as ADA2 activity. Difference between this value and a value registered in the mixture without EHNA was considered as ADA1



activity. Solution of ammonium sulfate was used as a standard to calculate ADA1 specific activity (U/L).

### ***Statistical Analysis***

The distribution of genotypes for ADA1 rs73598374 SNP was checked for correspondence to the Hardy-Weinberg (H-W) equilibrium. In order to investigate potential association of the selected SNP with IS, the allele (gene) and phenotype frequencies (carriage rates) in patients and healthy subjects groups were compared using Pearson's Chi-square test. The odds ratio (OR), 95% confidence interval (CI), and Pearson's p-value were calculated. Statistical power of the association study was calculated according earlier described approach by Lalouel and Rohrwasser (2002). Unpaired two-tailed Student's t-test was

used for evaluation of intergroup differences in the blood activities of ADA. Group statistics was presented as mean $\pm$ SD. P values  $\leq$  0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., USA) software.

### ***Ethical Clearance***

All subjects or their legal representatives gave their informed consent to participate in the study approved by the Ethical Committee of the Institute of Molecular Biology NAS RA (IRB #00004079).

## Results

### *ADA1 rs73598374 SNP in Patients with IS and Healthy Subjects*

Distribution of ADA1 rs73598374 genotypes in the groups of IS patients and healthy subjects were in compliance with H-W equilibrium.

The allele and phenotype frequencies of the studied genetic variant in the groups of IS patients and healthy subjects are shown in Table 1. The results obtained indicated that the ADA1 rs73598374\*A minor allele was insignificantly more frequent in patients with IS than in healthy subjects (11% vs 8%,  $p=0.35$ , OR=1.36, 95% CI: 0.75-2.45). Accordingly, the carriers of the

rs73598374\*A minor allele were insignificantly overrepresented in patients compared to healthy subjects (21% vs 16%,  $p=0.34$ ,  $OR=1.36$ , 95% CI: 0.73-2.51). Statistical power of the present study, indicating the difference in the carriage of the ADA1 rs73598374\*A allele between the patients and healthy subjects reached 20.33%.

### ***ADA1 Activity in the Blood Plasma of Patients with IS and Healthy Subjects***

Comparative determination of the ADA1 activity levels in IS patients and healthy subjects demonstrated insignificantly higher mean value of the ADA1 activity in patients as compared to healthy subjects (patients vs healthy subjects:  $2.82 \pm 2.50$  vs  $2.43 \pm 1.18$ ;  $p=0.36$ ). No significant difference in the mean values

of ADA1 activity between males and females in both groups was detected (patients, male vs female:  $2.96 \pm 2.32$  vs  $2.22 \pm 2.07$ ,  $p=0.27$ ; healthy subjects, male vs female: ( $2.24 \pm 1.35$  vs  $2.56 \pm 1.05$ ,  $p=0.40$ ). The activity levels of ADA1 in mutant allele carriers were lower than normal homozygotes both in IS patients (GG:  $3.26 \pm 3.83$  vs GA+AA:  $2.41 \pm 0.83$ ,  $p = 0.334$ ) and healthy subjects (GG:  $2.25 \pm 1.25$  vs GA+AA:  $1.71 \pm 0.94$ ,  $p = 0.233$ ), although the difference did not pass the significance threshold. Furthermore, we found no relation between ADA1 activity levels and the patients' comorbidities (hyperlipidemia, arterial hypertension and arterial fibrillation,  $p > 0.05$ ).

## **Discussion**

In this study we compared the distribution of ADA1 rs73598374 SNP and ADA1 activity levels in patients with IS and healthy subjects in Armenian population. The obtained results revealed no significant differences in allele and phenotype frequencies of the selected genetic variant between study groups, thus indicating no association of ADA1 rs73598374 SNP with IS. In addition we did not observe significant decrease in ADA1 levels between mutant allele carriers and normal homozygotes both in IS patients and healthy subjects. Thus our finding suggest that ADA1 rs73598374 polymorphism is not associated with IS in Armenian population.

Also, no significant difference was detected when comparing ADA1 activity levels in the blood of IS patients within the first 24 hours of stroke onset and healthy subjects. While earlier Tavilani et al (2008) reported higher levels of ADA1 activity in female patients with IS, compared to male patients, in Iranian population, we did not observed this in case of both IS-affected and healthy subjects in Armenian population. The mentioned inconsistency may reflect population differences between study subjects.

Since our study refers to one given population (Armenian), the results should be replicated in other populations/ethnic groups. Another limitation of the present study is relatively small sample size (98 patients and 177 healthy subjects). However, even with actual samples used we can conclude that there is no association

between ADA1 polymorphism and IS. First of all, ADA1 rs73598374 allelic frequency is similar to frequencies reported for European population previously by Hirschhorn (1994). Moreover, the statistical power of 20.3% indicates 79.3% chance that the distribution of ADA rs73598374 genotypes among IS patients is similar to healthy subjects.

## **Conclusions**

In summary we concluded that alterations in ADA1 enzyme on both genetic and activity levels are not implicated in IS pathogenesis.



## **Acknowledgement**

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Conflict of interest statement: The authors declare that they have no conflict of interest.

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