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Protective Role of ADA*2 Allele of the Adenosine Deaminase Gene Against Recurrent Spontaneous Abortions in Iranian Women

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Abstract

Background: Adenosine deaminase (ADA) is involved in recurrent spontaneous abortion (RSA) while normal pregnancy is defined by suppressed cell-mediated immunity.
Objectives: This study aimed to determine the connection between single nucleotide polymorphism (SNP) G22A and protection against RSA.
Methods: In this analytical case-control study, the allele frequency of ADA G22A gene polymorphism was determined in 113 participants including 50 women with RSA and 63 healthy pregnant women using RFLP-PCR to determine if there is a statistically significant difference in the frequency of ADA genotypes between the patient group and the control group.
Results: The frequency of ADA2/ADA2 (AA) genotype was significantly different between RSA patients and controls (P = 0.004) while no significant differences were identified in the genotype frequency of ADA1/ADA1 (GG) (OR = 0.678; P = 0.228) and ADA1/ADA2 (GA) (OR = 0.976; P = 0.943). SNP G22A in the ADA gene was associated with the protection against RSA. The frequency of ADA alleles in RSA patients was compared between the age groups, and the results indicated that the ADA2 (A) allele was associated with protection against RSA in patients aged 35 years or younger (P < 0.0001).
Conclusions: The findings showed that women carrying the ADA*2 allele are protected against RSA.

Keywords: Adenosine Deaminase, Gene, Polymerase Chain Reaction, Polymorphism, Pregnancy, Recurrent, Single Nucleotide, Spontaneous Abortion

1. Background

Unexplained recurrent spontaneous abortion (RSA) is referred to as the termination of three or more pregnancies prior to the 20th week of gestation (1-4). There are several reasons for RSA including immunological, endocrine, and genetic factors (3-5%), anatomical factors, antiphospholipid syndrome (15%) (5), and infections (6). Nevertheless, about 40-50% of the cases have unknown etiologies. It is thought that certain gene polymorphisms may be implicated in the pathogenesis of unexplained RSA (3, 4, 7, 8).

Several genetic polymorphisms have been associated with RSA (9). Genetics polymorphisms in interleukin genes may lead to unexplained RSA (7, 8). It is thought that certain gene polymorphisms may be implicated in the pathogenesis of unexplained RSA (10-13).

A key purine enzyme named adenosine deaminase (ADA) is present in blood serum and most tissues, especially lymphoid tissues. ADA is essential for the maturation and function of lymphocytes, especially those of T lineage, and is required for the maturation of human blood monocytes to macrophages (14-17). ADA is one of the most important enzymes in the human reproductive system affects the metabolism of adenosine and plays a protective role in early post implantation embryonic development (18, 19). The ADA gene in the human genome is located on chromosome 20 (20q13.11) (20). The enzyme adenosine deaminase is involved in catalyzing the deamination of adenosine and deoxyadenosine into inosine and deoxyinosine, respectively. This catalytic pathway is essential for lymphocyte proliferation and differentiation (21).

A study by Martinez-Navio et al. suggested that ADA had a potential role in the generation of effector, memory, and regulatory CD4+ T cells (22). The common G22A polymorphism in the ADA gene leads to the nucleotide substitution of asparagine for aspartic acid at codon 8

(Asp8Asn) and production of ADA*2 isoenzyme with lower enzymatic activity and subsequently causes increased tissue concentrations of adenosine (11, 23-26). The increased ratio of adenosine may play a fundamental role in pregnancy maintenance (23). Much research in the European population and among Brazilian women (27) has been conducted to assess the association of G22A polymorphism with RSA.

2. Objectives

We hypothesized that the ADA*2 allele maintains the pregnancy against RSA. To examine this hypothesis, we compared the distribution of ADA genotypes in women with RSA and a control group in an Iranian population.

3. Methods

This study was approved by the Payame-Noor University Ethic Committee (Reference No. 167/D/11837) and the Department of Biomedical Sciences, Alzahra University, Tehran, Iran. All the participants signed informed consent after explaining the objectives of the study.

3.1. Study Population and Sampling

This study was carried out in 2017 - 2018 at the Department of Biomedical Sciences, Women Research Center, Alzahra University, Tehran, Iran. The sample size was calculated with an 85% power at a significance level of 95% and considering a Standard Deviation (SD) (https://clincalc.com/stats/samplesize.aspx).

In this study, 53 whole blood samples were collected from women with RSA (age range: 23 - 54 years, Body Mass Index (BMI): 29.44 $\pm$ 5.17, and mean age $\pm$ standard deviation: 37.5 $\pm$ 7.51) referring to Shahid Akbar Abadi Hospital, affiliated to the Iran University of Medical Sciences, in Tehran and 63 women as the control group (age range: 26 - 50 years, BMI: 29.1 $\pm$ 4.81, and mean age $\pm$ standard deviation: 38.52 $\pm$ 7.50).

All RSA cases were diagnosed based on at least two recurrent spontaneous abortions in less than 20 weeks of gestational age. The control group women were enrolled based on the following criteria: regular menstrual cycles, no history of pregnancy loss, and a history of at least two natural pregnancies. Controls and patients with RSA had a single partner during their reproductive age. Patients with RSA were confirmed to be negative for the chromosomal abnormalities, infectious diseases, history of diabetes, and polycystic ovarian syndrome (PCOS). Controls and patients with RSA were originally from different parts of Iran. From all participants, 5-ml venous blood samples were collected in ethylene-diamine-tetra-acetic acid (EDTA) tubes for DNA extraction.

3.2. Genetic Analysis

Genomic DNA extraction from the blood was carried out by the salting-out method as described by Miller et al. (28). Then, genomic DNA of blood samples was determined with a spectrophotometer at 260 nm. The samples were diluted to standard concentrations and stored at -20°C until gene analysis.

3.2.1. Verification of ADA*1 and ADA*2 of the ADA Gene

The verification of the ADA*1 and ADA*2 alleles was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. A gene amplification reaction (25 μl of total volume) was performed for each sample of genomic DNA under the following conditions: 2 μl of genomic DNA, 1.25 μl of sense primer (GCCCGGCCCGTTAAGAAGAGC), 1.25 μl of anti-sense primer (GGTCAAGTCAGGGGCAGAAGCAGA), 2 μl of DMSO, 1.5 μl of $MgCl_2$ (25 mM), 5 μl of 10x PCR Buffer, 4.0 μl of dNTPs (1.25 mM), 0.5 μl of *Taq* DNA polymerase, and 7.5 μl of deionized water (CinnaGen Co. Tehran, Iran). The amplification conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 8 min. PCR was carried out in a PTC-1148 programmable thermal controller (BioRAD, USA). The amplified 397-*bp* fragment was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The PCR product (15 μl) was incubated at 65°C with 0.3 mL of *Taq I* (3000 U, Fermentas), 2.5 μl of enzyme buffer (10x, Fermentas), and 2.5 μl of deionized water overnight. The DNA fragments were subjected to electrophoresis on a 3.5% agarose gel stained with ethidium bromide. The ADA*2 allele (22A) was identified by the absence of the *Taq I* restriction site and the presence of the 397 *bp* fragment. The PCR product of the ADA*1 allele (G22) was split into two fragments (245 *bp* and 152 *bp*) (Figure 1).

3.3. Statistical Analysis

Statistical analysis was performed by StatPages.info online program. Data were analyzed using the chi-square test to detect differences in the distribution of ADA genotypes and alleles concering RSA. Moreover, the odds ratios (OR) and 95% confidence intervals (CIs) were calculated to estimate the effect of ADA genotypes and alleles on the RSA risk. Significant differences in the ADA genotypes and alleles were indicated at a P value of < 0.005.

4. Results

The demographic characteristics of the RSA patients and controls are presented in Table 1. The groups were matched for age and BMI (P > 0.05) while there was a significant difference in the number of pregnancies and the number of children (P < 0.05). Concerning the ADA2/ADA2 (AA) genotype frequency, there was a statistically significant difference between the RSA patients and controls (P = 0.004) while no significant differences were identified in the genotype frequencies of ADA1/ADA1 (GG) (OR = 0.678; P = 0.228) and ADA1/ADA2 (GA) (OR = 0.976; P = 0.943) (Table 2; Figure 2). Concerning the ADA1 (G) (OR = 1.842; P = 0.128) and ADA2 (A) (OR = 0.543; P = 0.128) allele frequency, there was no statistically significant difference between the RSA patients and controls (Table 2; Figure 3). The comparison of ADA allele frequency among RSA patients concerning the age groups of younger and older than 35 years showed a highly significant difference between the groups (P > 0.0001). The ADA1 (G) allele was associated with a low risk of RSA in patients aged 35 years or younger while the ADA2 (A) allele was not associated with a low risk of RSA in this age group (Table 3; Figure 4).

Table 1. Demographic Characteristics of RSA Patients and Controls[a]

Characteristics	RSA Patients	Controls	P Value
Age (years)	37.5 ± 7.51	38.52 ± 7.50	0.56
BMI (Kg/m^2)	29.44 ± 5.17	29.1 ± 4.81	0.68
Live births	None	1.73 ± 0.61	0.000
Previous pregnancy losses	3.10 ± 1.60	None	0.000

Abbreviations: RSA, recurrent spontaneous abortion; BMI, body mass index.
[a]Values are expressed as means ± SD.

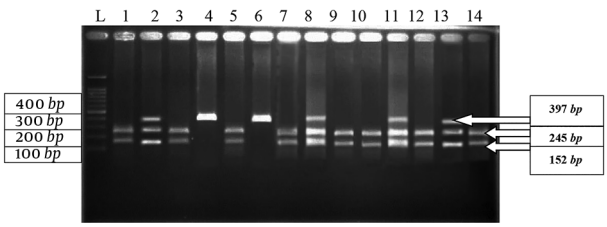

Figure 1. PCR-RFLP analysis of ADA gene polymorphism using TaqI restriction enzyme by agarose gel electrophoresis. Lane L: Gene ruler 100-*bp* DNA ladder (100 *bp* -1500 *bp*). Lanes 1, 3, 5, 7, 9, 10, 12, and 14: ADA1/ADA1 (GG) represented by two bands (152 *bp* and 245 *bp*). Lanes 2, 8, 11, and 13: ADA1/ADA2 (GA) represented by three bands (152 *bp*, 245 *bp*, and 397 *bp*). Lanes 4 and 6: ADA2/ADA2 (AA) represented by one band (397 *bp*).

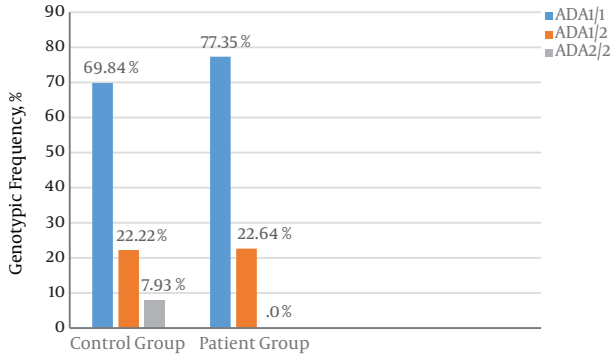

Figure 2. Genotype frequency of ADA gene in RSA patients and controls

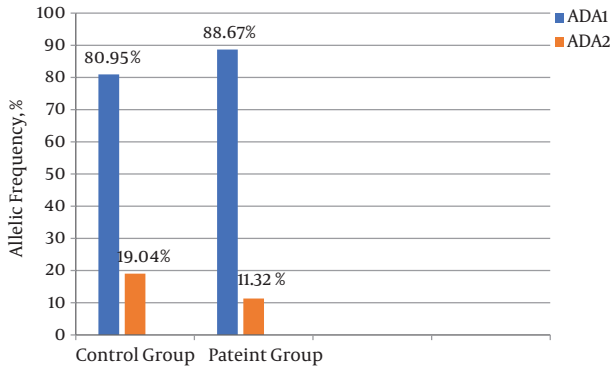

Figure 3. Allele frequency of ADA gene in RSA patients and controls

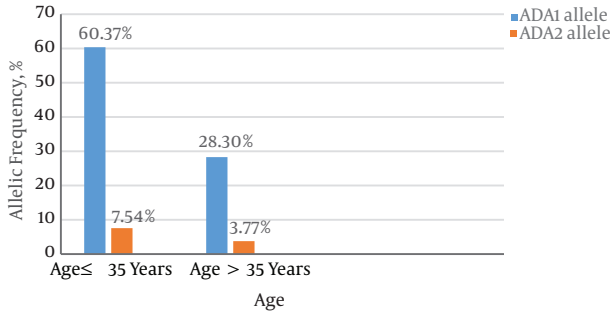

Figure 4. Allele frequency of ADA gene polymorphism in the age groups of younger and older than 35 years among RSA patients

5. Discussion

The present study was carried out to determine if there is a relationship between the SNP G22A of the ADA gene and its protective role in Iranian women with a history of RSA.

The result of this study showed no statistically signif-

Table 2. Frequencies of ADA Gene Alleles and Genotypes Among RSA Patients and Controls

ADA Genotypes and Alleles	Control Frequency, No. (%)	Patient Frequency, No. (%)	OR (95% CI)	P Value
ADA genotypes				
ADA1/ADA1 (GG)	44 (69.84)	41 (77.35)	0.678 (0.343 - 1.339)	0.228
ADA1/ADA2 (GA)	14 (22.22)	12 (22.64)	0.976 (0.477 - 1.998)	0.943
ADA2/ADA2 (AA)	5 (7.93)	-	lnf (1.545 - lnf)	0.004
ADA alleles				
ADA1 (G) allele	102 (80.95)	94 (88.67)	1.842 (0.781 - 4.397)	0.128
ADA2 (A) allele	24 (19.04)	12 (11.32)	0.543 (0.228 - 1.281)	0.128

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 3. Allele Frequency of ADA Gene Polymorphism Based on Age Among RSA Patients

Alleles	Age Group		P Value
	≤ 35 Years, No. (%)	> 35 Years, No. (%)	
ADA1 (G)	64 (60.37)	30 (28.30)	0.000
ADA2 (A)	8 (7.54)	4 (3.77)	0.248

icant differences in allele frequency between the control group and the RSA patients group. In addition, no significant difference was found in the genotype frequencies of ADA1/ADA1 and ADA1/ADA2. However, significant differences were identified in the genotype frequencies of ADA2/ADA2. The results of our study showed a relationship between ADA1 allele and the lower RSA risk in 35-year-old or younger women patients. Our results also suggest a cooperative effect of ADA2/ADA2 (AA) genetic polymorphism on protection against RSA in Iranian women.

Hitoglou et al. (2004) reported that total ADA and isoenzyme ADA1 and ADA2 in serum and peripheral blood lymphocytes were significantly higher in women with RSA than in non-pregnant women. Their results showed the cell-mediated immunity role of ADA in RSA (29). In a similar study by Bonyadi et al., the protective roles of the G22A polymorphism (ADA*2 allele) and AG genotype were confirmed against RSA (30). Yoneyama et al. (2003) reported that ADA is an early indicator of trophoblast cell differentiation, and it is enriched in trophoblast cells of the placenta (17).

The protective role of ADA*2 allele against RSA was also reported by Nicotra et al. Early observations in their study showed the defensive effect of ADA-2 allele against the development of autoantibodies in RSA. The results obtained by Nicotra et al. showed that women with a history of several births had more ADA*2 allele than women who had a history of one birth or no live birth (31).

The comparison of the frequency of ADA alleles in RSA patients at various ages indicated that the ADA2 (A) allele was associated with a reduced risk of RSA among patients aged 35 years or younger. This study showed the protective role of ADA2 (A) allele in spontaneous abortion among an Egyptian population (32). Lee et al. reported the protective effect of *ADA 2 (A)* allele against RSA (33).

The results by Camargo et al. indicated that G22A polymorphism was not associated with ankylosing spondylitis (34). Safranow et al. studied the possibility of ADA*2 allele protection against coronary artery disease. The results showed that the ADA*2 allele frequency was lower in the patient group than in the control group. It has been suggested that the ADA*2 allele reduces the genetic susceptibility to coronary artery disease, and it has a major role in preventing the progression of heart failure (10, 35, 36).

Nicotra et al. conducted a study on the joint effect of genetic modulations of ACP1 and ADA on spontaneous abortion and found a common effect for ACP and ADA polymorphisms on protection against or susceptibility to spontaneous abortion. The results showed that women with low activity of ADA and high activity of ACP1 had the least sensitivity to RSA while women with high ADA activity and low ACP1 showed the highest sensitivity to RSA (37). The results of Salehabadi et al. showed that PCOS was not associated with G22A polymorphism. It was also found that total ADA, ADA1, and ADA2 activity were significantly lower in the PCOS group than in the control group. It was shown in this study that the low level of ADA activity might be effective in PCOS pathogenicity by maintaining adenosine at higher concentrations that could affect the follicular growth (38).

In a similar study by Nunes et al. on the ADA2 allele of the ADA gene and RSA in Brazilian women, they found no statistically significant differences in ADA alleles and genotypes between women with RSA and controls while the maternal ages were considered significant. Statistical differences were determined between ADA alleles and genotypes while they were not associated with RSA. Nunes et al. suggested that the ADA2 allele inhibited RSA in women over 35 years of age (1), possibly due to ADA function in blood flow regulation, platelet aggregation, and neurotransmission (39). The ADA presence in human uterus and the pla-

centa has a major role in the reduction rate of early pregnancy failure; thus, the ADA*2 allele has a protective role in genetic susceptibility to RSA. On the other hand, increasing the ADA*2 allele levels participate in vascular integrity by increasing uterine and placental blood flow (37).

It is suggested that ADA polymorphism be investigated in different geographic areas with larger sample sizes in order to determine the association of ADA polymorphism in larger populations. We also suggest this test be conducted as a regular test for patients with unknown etiologic RSA.

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Footnotes

Authors' Contribution: Atefeh Mohammadi and Sepideh Khodaverdi did blood sample collection and patients with RSA data regarding the disease. Naeimeh Najafi performed the DNA extraction, restriction enzyme and PCR performance. Naeimeh Najafi, Ali-Reza Ahmadi, and Reza Hajihosseini analyzed the data of genotypic, allelic frequencies of ADA gene among RSA patients, and allelic frequency ADA gene polymorphism and also major contributor in writing the manuscript. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare no conflict of interest in this study.

Ethical Approval: The present study and its procedures were approved by the Department of Biochemistry, Payame-Noor University of East Tehran (Reference No. 167/D/11837), and the Department of Biomedical Sciences, Alzahra University, Tehran, Iran.

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