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Evaluation of the adenosine deaminase (ADA) *G22A* gene polymorphism with recurrent spontaneous abortion among Egyptian patients

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Abstract

Introduction: *Adenosine and deoxyadenosine metabolism is influenced by adenosine deaminase (ADA) enzyme. ADA increases in different diseases and is considered as one of the markers for cell-mediated immunity. Pregnancy is associated with depressed cell-mediated immunity. The level of ADA expression, which seems to play a key role in maintaining pregnancy, is influenced by adenosine deaminase G22A gene polymorphism. We aimed in our study to evaluate the association of ADA G22A gene polymorphism with recurrent spontaneous abortion (RSA) in Egyptian women.*

Material and methods: *Adenosine deaminase G22A gene polymorphism was genotyped in 40 patients (age range 22-39 years) with a history of RSA, selected from those attending the Gynaecology and Obstetrics Clinic of Beni-Suef University Hospital, and 20 age-matched healthy women as a control group, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.*

Results: *In our study, no statistically significant difference was found between RSA patients and control group as regards ADA G22A genotypes (p = 0.653) and alleles (p = 0.697). A comparison of the frequencies of ADA alleles in RSA patients as regards the below-35-years-old age group revealed that ADA 2(A) allele was associated with a low risk for RSA in patients aged 35 years old or younger (p = 0.008).*

Conclusions: *In conclusion, our study revealed an age-dependent protective value of ADA 2(A) allele in recurrent spontaneous abortions among the Egyptian population.*

Key words: *Recurrent spontaneous abortion; ADA; ADA G22A gene polymorphism; PCR-RFLP.*

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Introduction

The World Health Organisation (WHO) defines abortion as pregnancy termination prior to 20 weeks' gestation or a foetus born weighing less than 500 g [1]. The number of spontaneous abortions performed worldwide has remained stable in recent years, with 41.6 million having been performed in 2003 and 43.8 million having been performed in 2008 [2]. Recurrent spontaneous abortion (RSA) refers to two or more consecutive pregnancy losses [3].

Adenosine deaminase (ADA) enzyme affects the methylation process, cell growth and differentiation, apoptosis, DNA replication, and immune functions through catalysation of the deamination of adenosine and deoxyadenosine [4]. Adenosine deaminase, a key enzyme in purine metabolism, regulates extracellular and intracellular concentrations of adenosine by irreversible deamination of adenosine into inosine [5, 6]. Adenosine deaminase is needed for the breakdown of adenosine from food and the turnover of nucleic acids in tissues. It is found in a wide variety of prokaryotes and eukaryotes in different forms [7]. Adenosine deaminase is present in virtually all mammalian cells; its primary function in humans is the development and maintenance of the immune system. The activity of ADA has been changed in diseases characterised by altered cell-mediated immunity [8]. Lymphocyte proliferation and differentiation requires ADA [9, 10]. Adenosine deaminase regulates the concentration of adenosine as the main modulator of oocyte maturation [11]. Adenosine deaminase activity is critical in maintaining a normal pregnancy

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[12, 13]. It was observed that total serum ADA activity was decreased during normal pregnancy compared with non-pregnant women [8]. One possible explanation for decreased ADA activity in normal pregnancy is due to the increase in pregnancy-related hormones such as oestradiol and cortisol, which tend to inhibit ADA activity [14]. Another possible reason is to favour increasing adenosine levels as ADA is needed for the deamination of adenosine to inosine. Adenosine in turn exhibits an inhibitory effect in platelet activation during pregnancy. Reduced serum total ADA activity may be in part associated with depressed cell-mediated immunity during normal pregnancy [15].

The human adenosine deaminase gene is widely studied because of its importance in the medical field. Gene regulation and gene therapy are being investigated to understand the clinical implications of *ADA* gene regulation [16]. Adenosine deaminase is encoded by the polymorphic *ADA* gene, which is located on chromosome 20q13.11. One of the commonest single nucleotide polymorphisms (SNP) of ADA gene is the SNP *G22A* in exon1. This SNP results in the substitution of asparagine amino acid *ADA 1(G)* allele with aspartic amino acid *ADA 2(A)* allele in position 8 of the enzyme. This amino acid substitution decreases catalytic activity of ADA. Consequently, individuals with the *ADA 2(A)* allele show reduced ADA enzymatic activity compared to homozygous *ADA 1(G)* individuals [17, 6]. Since ADA activity could be affected by gene polymorphisms, it is therefore rational to hypothesise that SNP in the *ADA* gene could influence the activity of the protein, and further affect the susceptibility to related diseases. In this study, our goal was to investigate the *ADA G22A* gene polymorphism as a genetic risk factor to recurrent spontaneous abortion in Egyptian women.

Material and methods

Patients

A total of 40 recurrent spontaneous abortion patients (age 28.4 ± 4.9 ranging from 22 to 39 years) were selected from those attending the Gynaecology and Obstetrics Clinic of Beni-Suef University Hospital during the period between August and December 2014. All patients had histories of at least two successive miscarriages. A control group of 20 women (age 27.2 ±3.0 ranging from 23 to 33 years) with at least one live birth were derived from healthy volunteers with no history of spontaneous abortion or preterm labour. The study design was approved by the Scientific Research Committee, Faculty of Medicine, Beni-Suef University. Data confidentiality was preserved according to the Revised Helsinki Declaration of Bioethics 2008 [18]. Informed consent was obtained from all participants in this study.

Patients were subjected to: 1) Full history taking including age, history of diabetes, hypertension, and polycystic ovarian syndrome (PCOS). 2) Full clinical examination including weight (Wt), height (Ht), and calculated body mass index (BMI). 3) Routine laboratory investigations including fasting blood sugar (FBS), cholesterol, triglycerides (TGs), low-density cholesterol (LDL-C), and high-density cholesterol (HDL-C) using a Flexor EL 200 automated chemistry analyser and its own kits (Holanda). 4) Follicle-stimulating hormone (FSH) using human FSH sandwich- enzyme linked immunosorbent assay (ELISA) kit (Cat No: E-EL-H1143, Thermo Fisher Scientific Inc., USA). 5) Progesterone using human progesterone competitive immunometric ELISA Kit (Cat No: E-EL-H2269, Thermo Fisher Scientific Inc., USA). 6) Cytomegalovirus (CMV) and toxoplasma using an immunochromatographic TORCH IgM Rapid Test kit (Cat No TORCH 121. Code: MST417362, Guagdong Shantou Minston Medical Instruments, China).

Methods

Gene extraction

Peripheral blood samples of the patients were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) for DNA isolation. The genomic DNA was extracted from each blood sample using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Cat No#FD0674, Lot/ 00187735, Fermentas Life Sciences, Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

Genotyping of ADA G22A Polymorphism

ADA G22A polymorphism was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR primers are forward primer 5′-GCCCGGCCCGTTAAGAAGAGC-3′ and reverse primer 5′- GGTCAAGTCAGGGGCAGAAGCAGA-3′ [19].

PCR assay was performed for each sample in a final reaction volume of 25 µl, using 5 µl DNA, 12.5 µl universal master mix, 1 µl PTPN22 1858C>T forward primer, 1 µL PTPN22 1858C>T reversed primer, and 5.5 µl distilled water (DW). The PCR conditions were as follows: initial denaturation at 94°C for 15 minutes, 36 cycles of denaturation at 94°C for 40 seconds, annealing at 66°C for 80 seconds, extension at 72°C for 80 seconds, followed by a final extension at 72°C for 8 min [19]. All reactions were done using a thermal cycler from Applied Biosystems (Perkin-Elmer 9600, USA).

After amplification, the PCR products were treated at 65°C for 20 minutes with the restriction enzymes Fast Digest TaqI enzyme supplied by Thermo Fisher Scientific Inc. (Cat No. # FD0674, Lot/ 00187735, Fermentas Life Sciences, Thermo Fisher Scientific Inc., USA) [19]. The products were then resolved on 2% agarose gel electrophoresis containing ethidium bromide, then visualised using

a UV transilluminator. DNA molecular weight marker (Cat No. # SM0373, Lot/ 244669, Fermentas Life Sciences, Thermo Fisher Scientific Inc., USA) was used to assess the size of PCR-RFLP products. *ADA G22A* Genotype was defined by the presence of two bands (245 and 152) for *ADA1/ADA1 (GG)* genotype. While *ADA1/ADA2 (GA)* genotype was defined by the presence of three bands (397, 245, and 152). The representative gel showing the typical patterns of bands is presented in Fig. 1.

Statistical methods

Data were analysed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann–Whitney test (non-parametric *t*-test). The odds ratio (OR) with its 95% confidence interval (CI) was used for risk estimation. A p-value < 0.05 was considered a significant result [20].

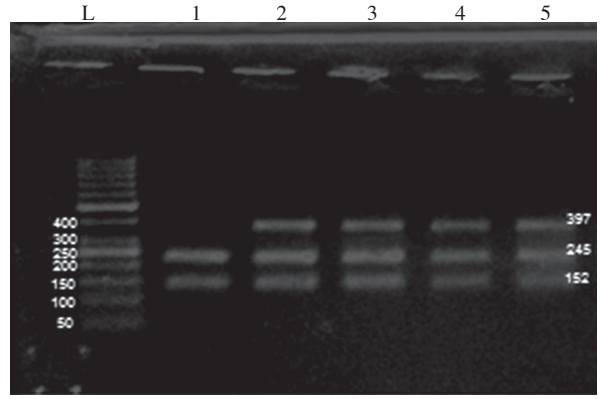
Results

By evaluating the demographic and biochemical characteristics among participants, the patient group showed statistically significant increases versus the control group as regards BMI, FBS, cholesterol, TGs, LDL-C, FSH, and progesterone (p = 0.001, 0.007, 0.001, 0.005, 0.001, 0.001, and 0.001, respectively). There was a statistically significant decrease in HDL-C in the patient group versus the control group (p = 0.001). Both the patient group and control group were age matched (p = 0.637) (Table 1).

Among the patient group, the frequency distribution of comorbidities including PCO, diabetes, hypertension, CMV, and toxoplasma was (50.0%, 30.0%, 15.0%, 15.0%, and 30.0%, respectively) (Table 2).

As regards *ADA G22A* genotypic frequency, there was no statistically significant difference between the patient group and the control group (p = 0.653). The *ADA1/ ADA2 (GA)* genotype was the most common genotype among the control group (95.0%) and the patient group (87.5%) (Table 3, Fig. 2). As regards *ADA* allelic frequency, there was no statistically significant difference between the patient group and the control group (p = 0.697). The *ADA1 (G)* allele was the most common allele in the patient group (56.3%) and the control group (52.5%) (Table 3, Fig. 3).

There was no statistically significant difference in allelic frequency of *ADA G22A* gene polymorphism among RSA patients as regards the co-morbidities, including PCO, diabetes, hypertension, CMV and toxoplasma (p = 0.822, 0.806, 0.636, 0.636, and 0.806, respectively) (Table 4).



Lane L: Gene ruler 50 pb DNA ladder (50-500 bp).
Lane 1: *ADA1/ADA1(GG)* represented by two bands (152, 245).
Lanes 2, 3, 4, and 5: *ADA1/ADA2 (GA)* represented by three bands (152, 245, and 397).

Fig. 1. PCR-RFLP analysis of ADA G22A gene polymorphism using TaqI restriction enzyme by agarose gel electrophoresis

Table 1. Demographic and biochemical characteristics between RSA patients and controls

Parameters	Control Mean ± SD	Patient Mean ± SD	*P*-value
Age (years)	27.2 ±3.0	28.4 ±4.9	0.637
BMI (kg/m^2)	27.6 ±2.7	31.2 ±3.8	0.001
FBS (mg/dl)	89.6 ±10.4	110.3 ±30.5	0.007
Cholesterol (mg/dl)	150.8 ±28.8	193.7 ±47.3	0.001
TGs (mg/dl)	125.8 ±15.0	160.8 ±54.0	0.005
HDL-C (mg/dl)	55.9 ±4.4	45.1 ±9.0	0.001
LDL-C (mg/dl)	117.8 ±18.0	143.6 ±27.2	0.001
FSH (mIU/ml)	15.1 ±3.4	6.9 ±4.2	0.001
Progesterone (ng/ml)	16.8 ±8.3	8.8 ±5.5	0.001

Table 2. Frequency distribution of comorbidities among RSA patients

Comorbidity	Patient group Frequency *n* (%)
PCO	20 (50)
Diabetes	12 (30)
Hypertension	6 (15)
CMV	6 (15)
Toxoplasma	12 (30)

Table 3. Genotypic and allelic frequencies of *ADA G22A* among RSA patients and controls

ADA genotypes and alleles		Patient frequency *n* (%)	Control frequency *n* (%)	OR (95% CI)	*P*-value
ADA Genotypes	ADA1/ADA1 (GG)	5 (12.5%)	1 (5.0%)	2.714 (0.295-24.954)	0.653
	ADA1/ADA2 (GA) ADA*1/*1	35(87.5%)	19(95.0%)		
ADA alleles	ADA1(G) allele	45(56.3%)	21(52.5%)	1.163 (0.543-2.492)	0.697
	ADA2 (A) allele	35(43.8%)	19(47.5%)		

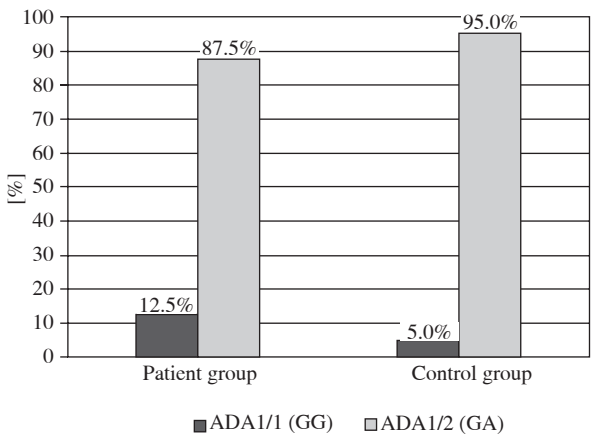


Fig. 2. Genotypic frequency of *ADA G22A* gene in RSA patients and controls

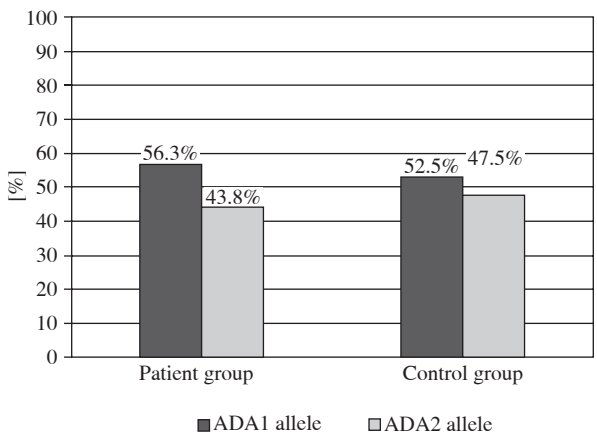


Fig. 3. Allelic frequency of *ADA G22A* gene in RSA patients and controls

Table 4. Allelic frequency of ADA G22A gene polymorphism as regards comorbidities among RSA patients

Variable	ADA1(G) (*n* = 45) Frequency *n* (%)	ADA2(A) (*n* = 35) Frequency *n* (%)	*P*-value
PCO			
Present	23 (57.5)	17 (42.5)	0.822
Absent	22 (55)	18 (45)	
Diabetes Present	13 (54.2)	11 (45.8)	0.806
Absent	32 (57.1)	24 (42.9)	
Hypertension			
Present	6 (50.0)	6 (50.0)	0.636
Absent	39 (57.4)	29 (42.6)	
CMV			
Positive	6 (50.0)	6 (50.0)	0.636
Negative	39 (57.4)	29 (42.6)	
Toxoplasma			
Positive	14 (58.3)	10 (41.7)	0.806
Negative	31 (55.4)	25 (44.6)	

Table 5. Allelic frequency *ADA G22A* gene polymorphism as regards the age group 35 years old and below among RSA patients

Alleles	35 – age group		*P*-value
	≤ 35 years	> 35 years	
ADA1 (G) Frequency *n* (%)	37 (51.4)	8 (100.0)	0.008
ADA2 (A) Frequency *n* (%)	35 (48.6)	0 (0.0)	

A comparison of *ADA* allelic frequency among patients as regards the age group 35 years old and below showed a high statistically significant difference ($p = 0.008$). *ADA 2(A)* allele was associated with a low risk for RSA in patients aged 35 years old or younger (Table 5, Fig. 4).

Discussion

There are different types of abortion, including early, late, spontaneous, induced, therapeutic, and threatened

abortion [2]. Our study evaluated the association between *ADA G22A* gene polymorphism and RSA in an Egyptian population. In our results, no statistically significant difference was found as regards genotypic and allelic frequencies between RSA the patient group and the control group. Yet, the *ADA 2(A)* allele was associated with a low risk of RSA in patients aged 35 years or younger, suggesting an age-dependent protective value of *ADA 2(A)* allele in RSA among the Egyptian population.

Our results are in accordance with Nunes *et al.* 2011 [19], who reported that the frequencies of *ADA* genotypes and alleles were similar between an RSA patient group and a control group, and they were not associated with the occurrence of RSA. Nicotra *et al*. 2007 [21] observed that the frequency of the *ADA 2(A)* allele was lower in European women who suffered from RSA than in those who did not, suggesting a protective effect of *ADA 2(A)* allele against RSA and higher fertility rates among women with *ADA 2(A)* allele regardless of age, in Italian women. The protective effect of *ADA 2(A)* allele against RSA was also reported by Lee *et al*. 2007 [13]. Nunes *et al*. 2011 [19] reported that *ADA* genotypes and alleles were compared between the RSA patient group and control group when controlling for maternal age. Their results did not reveal statistically significant differences in the frequency of the *ADA 2(A)* allele among those aged 35 years or younger, whereas an association between this allele and low risk for RSA was observed for women older than 35 years. Hirschhorn *et al*. 1994 [22] reported that the presence of an *ADA 2(A)* allele reduces ADA enzyme expression to between 15 and 20% compared to that found in the homozygous *ADA1/ADA1 (GG)* genotype, which enables an increase in adenosine levels. Carriers of at least one *ADA 2(A)* allele have higher levels of circulating and intracellular adenosine [23]. Chen *et al*. 2013 [24] found that *ADA G22A* gene polymorphism leads to a 30% reduction in ADA enzyme activity and may cause an increase in tissue concentrations of adenosine as a physiologic substrate for ADA. Nicotra *et al*. 2007 [21] reported that the presence of adenosine in the uterus and the placenta could contribute to a reduced rate of early loss of zygotes or foetuses, and they attributed that to adenosine action as a hormone that regulates blood flow, neurotransmission, and platelet aggregation as well as being a potent vasodilator. Bahadir *et al*. 2011 [25] reported that altered ADA activity and consequently adenosine level may be useful for clinical diagnosis and observation of high-risk pregnancies in which cell-mediated immunity has been altered. Accordingly, carriers of at least one *ADA 2(A)* allele, with reduced ADA enzyme expression and an increase in adenosine levels, have reduced risk of RSA [23]. Interestingly, based on both enzymatic and non-enzymatic roles of adenosine deaminase (ADA) in the male reproductive system, such as regulating adenylate cyclase activity by decreasing adenosine concentration, helping the interaction between prostasomes and spermatozoa, the role of *ADA G22A* gene polymorphism and the activities of ADA isoenzymes were investigated in fertile and infertile men by Fattahi *et al*. 2015 [26]. The authors reported that the frequency of *GG* genotype was significantly higher and *GA* genotype was lower in infertile males compared with fertile men. Also, the ADA activity with *GG* genotype was higher than *GA* carriers in the general population, revealing a protective role of *GA* genotype, which had lower ADA activity against infertility among men.

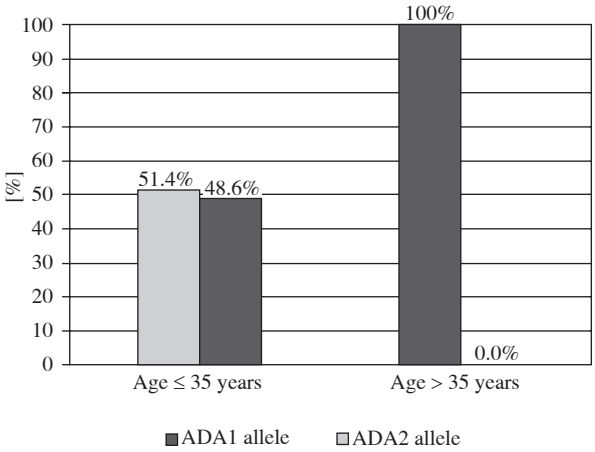


Fig. 4. Allelic frequency of *ADA G22A* gene polymorphism as regards the age group 35 years old and below among RSA patients

In conclusion, the results of our study revealed the effective role of the *ADA 2(A)* allele in reducing the risk of RSA, especially in the childbearing period of age (35 years or younger), suggesting an age-dependent protective value of *ADA 2(A)* allele in RSA. It is very important to examine a larger number of samples from different populations to draw more reliable conclusions. We recommend that evaluations of ADA levels at different times during the gestational period among women carrying distinct *ADA* genotypes could help to clarify the potential effects of the *ADA 2(A)* allele during successful pregnancies, and could shed additional light on the biological and clinical impact of this enzyme on RSA and in assisted reproduction. The clinical significance and the regulatory mechanism of the increased ADA activity in complicated pregnancies with altered cellular immunity remain to be investigated.

The authors declare no conflicts of interest to declare.

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