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Gene Expression of Adenosine Deaminase Genes 1 and 2 in Female Iraqi Patients with Autoimmune Thyroid Disease

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Abstract: The current study was carried out to investigate the correlation of gene expressions of *ADA1* and *ADA2* genes with the development of autoimmune thyroid disease (AITD) in a sample of Iraqi females. One hundred patients with AITD and 80 controls were included. Quantitative real time polymerase chain reaction (qRT–PCR) was utilized for investigation of *ADA1* and *ADA2* gene expression among patients and controls. The correlation of age and body mass index (BMI) with AITD occurrence comparing with controls was studied. Based on the results of this study, there is high expression level of *ADA1* and *ADA2* genes in patients compared with healthy controls; also, the gene expression fold ($2^{-\Delta\Delta CT}$) of *ADA1* and *ADA2* among AITD patients was recorded and according to results both genes showed up-regulation. It was concluded indicates both of age and BMI shows significant differences in patients compared with controls.

Keywords: Adenosine deaminase, qRT-PCR, ELISA, AITD, *ADA* genes.

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Introduction

Autoimmune thyroid diseases (AITD) are the most common type of autoimmune diseases (ADs) that occur in specific organ and about 2-5% of the population suffers from this disease, with wide variation between the sexes (i.e., men 1-5% and women 5-15%) (1). Examples of AITD are Hashimoto's thyroiditis (HT) and Graves' disease (GD). Both HT (cause hypothyroidism) and GD (cause hypothyroidism) are characterized by a loss of immunological tolerance, the infiltration of B cells and T cells, humoral and cell immunity against antigens from the thyroid gland, the production of autoantibodies, and the subsequent development of clinical symptoms (2).

Many studies revealed that thyroid disorders could be due to variable genetic and hormonal factors (3). Environmental risk factors that can be associated with the development of AITD include the use of certain medications, low birth weight, and excess iodine. Smoking, stress, and radiation exposure are also known to be related with the development of this condition (4). Thyroid disease risk is significantly increased by factors including family history. If you have a mother, a sister, or a daughter and any of them has had thyroid disease, your risk is slightly higher. The likelihood of developing benign goiter, especially in youth, increases with the presence of a family history of the condition (5).

Adenosine deaminase (ADA) is a polymorphic enzyme which is expressed in all human tissues. Besides controlling adenosine levels by catalyzing their deamination into inosine (6), ADA also have a pivotal

role in the mature T cell development, as a general marker of cellular immunity (7). In some autoimmune diseases, such as psoriasis (8), autoimmune hepatitis (9), inflammatory bowel disease (10), and rheumatic disease (11), high serum levels of ADA were detected. At the same time, elevated ADA activity was found in the monocytes of people with HT (12) and the peripheral blood leucocytes of GD patients (13). This suggests that serum level of ADA has the potential to be an indication for the monitoring and evaluation of GD. Moreover, oral antithyroid medications such propylthiouracil (PTU) dramatically decreased epidermal ADA activity and improved psoriatic plaques in psoriasis patients (14).

Adenosine deaminase (ADA) has demonstrated its possible medical relevance in the monitoring and diagnosis of autoimmune disorders, suggesting its role as an immune regulatory molecule. There is a close relationship between ADA and immune system development, maintenance, and function (15,16). There are two types of ADA isoenzymes: ADA1 and ADA2. ADA1 encoded by the *ADA* gene (Gene ID: 100, Chromosome 20-NC_000020.11), and ADA2 is encoded by the *ADA2* gene (Gene ID: 51816, Chromosome 22-NC_000022.11). The sum activity of ADA1 and ADA2 *in vivo* are known as total ADA activity (tADA) activity. Adenosine deaminase 1 (ADA1) is an intracellular protein that is widely expressed (17), in contrast, ADA2 is a protein found in the blood that is released by immune cells including monocytes and macrophages (18,19). Therefore, the activity of tADA in serum or plasma should rely heavily on ADA2 activity. In the existence of erythro-9-(2-hydroxy-3- nonyl) adenine (EHNA), a specific inhibitor of ADA1, ADA2 activity may be assessed. the difference between the activity of tADA and ADA2 is equivalent to ADA1 activity (20).

The goal of the current work is to investigate the gene expressions of *ADA1* and *ADA2* genes and evaluate their roles in development of AITD in female Iraqi patients.

Materials and methods

Patients and controls

One hundred samples of female patients with AITD were included in this study with an age ranged between (11-74) years old, were collected from the Baghdad Medical City, Ministry of health /Baghdad/ Iraq, corresponding to eighty controls with an age ranged between (15-68) years old. A consultant letter was signed by each patient were diagnosed with thyroid disease according to patient history, symptoms and hormonal tests.

Blood collection

For RT-qPCR technique, each patient and control subject had blood drawn and placed in an Trizol-tube.

Extraction of total RNA

According to the kit manufacturer, RNA was extracted from the sample by the following methods:

Sample lysis

To homogenize the lysate in each tube, we pipetted up and down multiple times after adding of blood (0.5mL) into of TRIzol TM Reagent (0.5mL).

Three phase's separation

Before closing the tubes, the lysate within was treated with 0.2 mL of chloroform. The mixture was centrifuged for 10 minutes at 12,000 rpm to separate the lower organic phase, interphase, and colorless. The aqueous phase, that contain RNA, was separated into a fresh test tube.

Precipitation of RNA

After centrifuging at 12,000 rpm for 10 minutes, we added 500 µl of isopropanol to the aqueous phase and let

it sit for 10 minutes. A white, gel-like pellet of total RNA formed at the tube base. The supernatant is then eliminated afterward.

Washing of RNA

After adding 0.5 mL of ethanol (70%) to each tube, we gave it a quick vortex and spun it at 10000 rpm for 5 minutes. The pellet was then aspirated with ethanol before being air-dried.

RNA solubility

After the pellets were rehydrated in 100 µl of Nuclease Free Water, they were placed in an incubator at 55-60°C for 10-15 minutes.

Determine RNA yield using Fluorescence Method

The quality of the samples for further usage was evaluated by measuring the concentration of the extracted RNA using a Quantus Fluorometer. QuantiFluor Dye was diluted to a final volume of 200 µl and added to 1 µl of RNA. Values for RNA concentration were measured following a 5-minute incubation at room temperature in the dark. The units for expressing the concentration are "ng/µl " (absorption wavelength of protein and DNA). The concentration is measured in ng/µl (absorption wavelength of protein and DNA). RNA Concentration range was 15-20 ng/µl.

Primer design

The NCBI Gen Bank database was used to obtain Adenosine aminase 1 (*ADA1*) and Adenosine deaminase 2 (*ADA2*) genes as well as housekeeping gene (*β-Globin*) sequences. The Primers with probe were designed with melting temperature of 60-65°C. They also have a variety of amplicon lengths and primer lengths, Table 1. Control genes, which are often referred to as housekeeping genes, are frequently utilized to normalize mRNA levels between various samples. However, the expression level of these genes may vary among tissues or cells and may change under certain circumstances. Thus, the selection of housekeeping genes is critical for gene expression studies (21).

Table (1): Sequences of primer of the study.

Gene	Primer	Sequences 5′→3′	Annealing Temperature (^{o}C)	References
β-Globin	***F***	ACACAACTGTGTTCACTAGC	65	This study
	R	CAACTTCATCCACGTTCACC		
***ADA1*-exp**	***F***	CTGCTGAACGTCATTGGCATGG		This study
	R	GCGATCCTTTTGATAGCCTCC		
***ADA2*-exp**	***F***	CAGGAGTTCTACGAGGACAAC	60	This study
	R	CTTCGTCATGGTGCTCTCCACT		

Primer preparation

After lyophilized primers have been dissolved in nuclease-free water, a working solution for testing genes was prepared. This solution was stored at -23 degrees Celsius until use. The 10 µL of stock solution that was diluted with the 90 µL of DNase free water yielded a working solution that had a 10 pmol/µL M concentration.

Real Time Quantitative PCR (RTq–PCR)

The quantitative PCR technique was performed using the AUSTRALIA system. The goal of the quantitative PCR technique was to determine the cycling threshold (Ct) using the 2xqPCR Master Mix Kits. Each reaction was performed twice. The needed volume of components was estimated as in Table 2. As can be seen in Table 3,

the cycling protocol was also optimized for the subsequent cycles.

Table (2): Components of qPCR utilized in the study.

Master mix components	Volume / µl
qPCR Master Mix	5
RT mix	0.25
MgCl2	0.25
Forward primer	1
Reverse primer	1
Nuclease Free Water	0.5
RNA	2
Total volume	10

Table (3): Thermal profile of genes expression for *ADA1* and *ADA2*.

Step	Temperature (ºC)	Duration	Cycle
Enzyme activation	95	5 min	1
Denature	95ºC	20 sec	40
Annealing	60*, 65**	20 sec	
Extension	72ºC	20 sec	1

*** For *ADA2*, ** For *ADA1*.**

The threshold cycle was calculated using the software used by real-time cycler. Each sample was executed twice and the values of mean were estimated. The expression analysis of required genes was normalized against the housekeeping gene. Results were reported as a folding change in gene expression based on the ΔΔCt method's recommendations for data processing (22):

For each sample, the difference between the CT values (ΔCt) for each gene of target and the housekeeping gene was calculated

$\Delta Ct_{control} = CT_{gene} - CT_{Housekeeping\ gene}$

$\Delta Ct_{patient} = CT_{gene} - CT_{Housekeeping\ gene}$

The difference in ΔCt values represented as (ΔΔCt) for the genes of Interest was calculated as follow:

$\Delta\Delta Ct = \Delta Ct_{patient} - \Delta Ct_{control}$

The fold-change in gene expression was calculated as follow: Fold change=$2^{-\Delta\Delta Ct}$

Estimation of body mass index (BMI)

The difference ΔCT values (Ct) between target and housekeeping genes were determined for each sample, as following:

$\Delta Ct_{patient} = CT_{gene} - CT_{Housekeeping\ gene}$

$\Delta Ct_{control} = CT_{gene} - CT_{Housekeeping\ gene}$

For the genes of interest, we determined the difference in ΔCt values, which then expressed as (ΔΔCt) as follow:

$\Delta\Delta Ct = \Delta Ct_{patient} - \Delta Ct_{control}$

This formula was utilized to estimate the fold-change in gene expression: **Fold change=$2^{-\Delta\Delta Ct}$**

Estimation of body mass index (BMI)

The BMI was estimated from the weight and height of each patient and control and according to the following formula (21):

$$BMI = \frac{Weight\ (kg)}{Height\ (m^2)}$$

Table (4): The classification based on BMI (21).

BMI (kg/m^2)	Classification
< 18.5	Under-weight people
18.5 to 24.9	Normal-weight
25.0 to 29.9	O ver-weight
> 30	Obese people

Statistical analysis

The proportions and Frequencies tests were then used to compare the mean and standard deviation of the various categorical variables. The Pearson correlation analysis was then used to examine the levels of interaction between the variables. A value of less than 0.05 was regarded as significant.

Results and discussion

RNA extraction

All samples had their whole RNA extracted. Total RNA concentrations varied between 15 and 20 ng/µl.

cDNA reverse transcription

RNA extraction was followed by a round of complementary DNA reverse transcription on day 2. cDNA for the ADA1 and ADA2 genes, as well as the housekeeping -Globin gene, were all required, so a shared primer reaction was used to generate all three. The subsequent qPCR efficiency analysis revealed how well cDNA was concentrated. All of the procedures resulted in perfect yield, which is indicative of highly effective reverse transcription. The optimum annealing temperature (Ta) for each primer was determined using the (Melting temperature) Tm value provided by this formula:

Ta = Tm - (2-5) ºC.

Tm =4 (G+C) + 2 (A+T).

Melting points for both the backward and forward primer were also determined using the aforementioned formula. Based on a comparison of the forward and reverse primer annealing temperatures, The lowest temperature (ºC) was determined (22). The RT-qPCR was utilized for relative quantitative expression of interested genes (*ADA1* and *ADA2*) and housekeeping gene (*β-Globin*). As shown in Tables (4 and 5), respectively, the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method was utilized for this purpose.

The following figures (1 and 3) were show the plots of amplification of *ADA1* and *ADA2* genes using RT-qPCR, respectively, in addition to their melt curves, that shown in Figures (2 and 4), in which the amplicons were represented as a single peak.

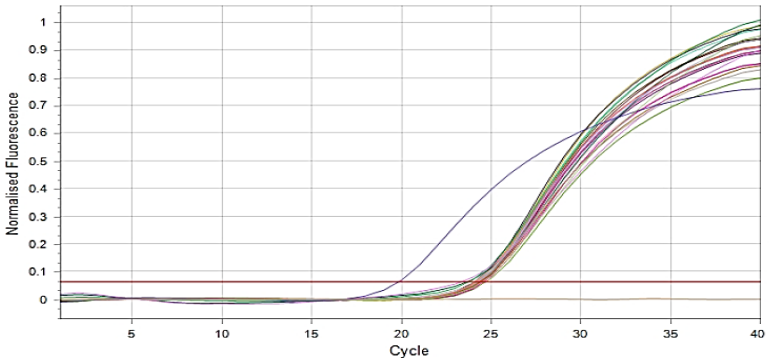

Figure (1): Plots of *ADA1* Amplification by RT-qPCR.

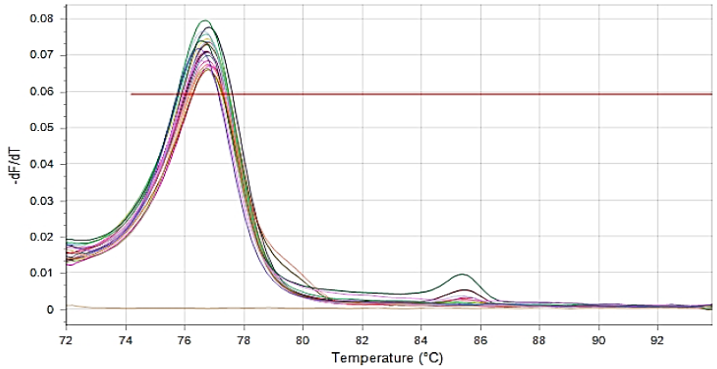

Figure (2): Melt curve of *ADA1* gene amplicons after analysis of RT-qPCR display one. Threshold 0.059 starting at 74.18°C.

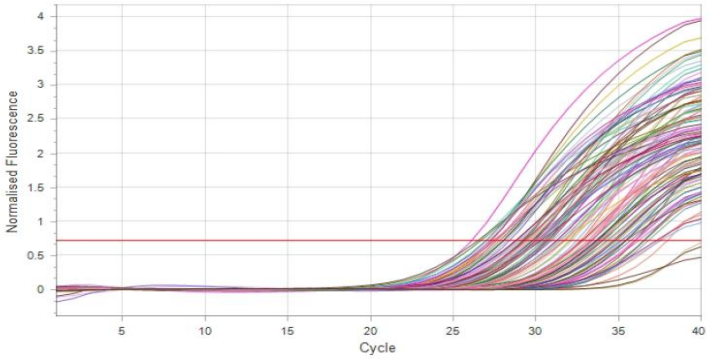

Figure (3): Plots of *ADA2* Amplification by RT-qPCR.

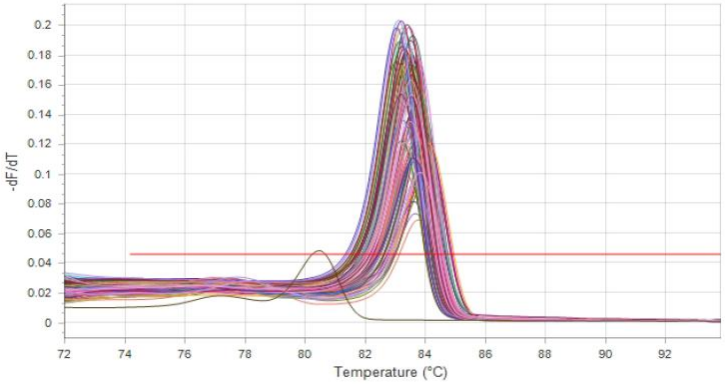

Figure (4): Melt curve of *ADA2* gene amplicons after analysis of RT-qPCR display one peak. Threshold 0.046 starting at 74.18°C.

Expression level of *ADA1* and *ADA2* gene in the studied group

The gene expression of both *ADA1* and *ADA2* was studied, the control and patient group as summarised in the following table and both genes were statistically significant at level of 0.001. The mean±SD of expression level (ΔCT, ΔΔCt and fold change) of *ADA1* and *ADA2* among AITD patients and controls were (3.359 ± 1.859 *vs.* 0.4155± 3.347, $p<0.0001$) and (0.6909± 1.673 *vs.* 0.1408± 1.722, $p<0.0014$), (3.359 ± 1.859 *vs.* 0.4155± 3.347, $p<0.0001$) and (0.6909± 1.673 *vs.* 0.1408± 1.722, $p<0.0014$) as well as (12.10 ± 9.516 *vs.* 5.949± 8.501, $p<0.0001$) and (2.106±1.774 *vs.* 1.065± 1.137, $p<0.0001$), respectively, as shown in Table5 , 6 and 7.

Table (5): Expression level (ΔCT) of *ADA1* and *ADA2* in groups of AITD patient and control.

	ADA1		*ADA2*	
	Mean±SD ΔCt Control	**Mean± SD ΔCt Patients**	**Mean±SD ΔCt Control**	**Mean±SD ΔCt Patients**
	0.4155± 3.347	3.359 ± 1.859	0.1408± 1.722	0.6909± 1.673
***p* Value**	**<0.0001**		**<0.0014**	

Table (6): Expression level (ΔΔCt) of *ADA1* and *ADA2* in groups of AITD patient and control.

	ADA1		*ADA2*	
	Mean ΔΔCt Control ± SD	**Mean ΔΔCt Patients ± SD**	**Mean ΔΔCt Control ± SD**	**Mean ΔΔCt Patients ± SD**
	-0.4155± 3.347	-3.359 ± 1.859	0.1408± 1.722	-0.6909± 1.673
Sig.	**		**	
***p* Value**	**<0.0001**		**0.0014**	

Table (7): Fold Change of *ADA1* and *ADA2* in groups of AITD patient and control.

	ADA1		ADA2	
	Mean Folding Control ± SD	**Mean Folding Patients ± SD**	**Mean Folding Control ± SD**	**Mean Folding Patients ± SD**
	5.949± 8.501	12.10 ± 9.516	1.065± 1.137	2.106±1.774
Sig.	**		**	
***p* Value**	**<0.0001**		**<0.0001**	

Thyroid disorder has a multifactorial etiology, and the right combination endogenous, environmental and genetic factors are needed for the initiation of disease process (25). In terms of immune system development and upkeep, ADA is a crucial regulating molecule (26). Humans have been revealed to contain two distinct enzymes, ADA1 and ADA2, both of which have the same activity. The finding that ADA1 loss-of-function mutations account for roughly 15% of hereditary immunodeficiencies sparked interest in the role of this gene (27). Parents of patients with a mutation in the ADA2 gene, CECR1, and low plasma ADA2 levels exhibited a normal distribution of cell subsets, with the exception of CD16+ monocytes. Major subsets of immune cells are impacted by ADA2 deficiency, albeit the specific cells affected vary amongst ADA2-deficient patients (28). The function of the enzyme is to convert deoxyadenosine, which is produced when DNA is broken down, to another chemical known as deoxyinosine. This chemical is not harmful, and it can be toxic to lymphocytes. When the *ADA* gene is defective, the levels of deoxyadenosine that are produced are reduced or eliminated. In the thymus, immature lymphocytes are known to be vulnerable to toxic levels of deoxyadenosine. This buildup can cause these cells to die, which can lead to the development of severe combined immunodeficiency. The number of these cells in the lymphoid tissues can also decrease (29). Graves' disease results from the production of IgG antibodies that bind to and activate the thyroid-stimulating hormone (TSH) receptor on the surface of thyroid follicular cells leading to diffuse thyroid enlargement and increased roduction of thyroid hormones (30).

Furthermore, it was reported that three distinct primer combinations were used to successfully amplify the ADA gene (ADA3, ADA4, and ADA5). Patients with Graves' or Hashimoto's disease had considerably higher

expression levels of all the investigated genes in their leukocytes compared to controls; a more than twofold increase in expression of the TK1, ADA4, and ADA5 genes was detected. Gene expression analysis for purine and pyrimidyne salvage enzymes has the potential to shed light on the molecular basis of autoimmune illnesses and aid in the diagnosis and tracking of AITD(31).

Correlation of BMI with AITD patients and control groups

As shown int table 8, body mass index (BMI) was estimated. A high significant difference in BMI of patients was observed in compared with heathy control, with $p<0.0001$.

Table (8): Means of BMI parameter in AITD patients and control groups.

			BMI				*p*-value
			Low	Normal	Overweight	Obesity	
Study Groups	Patient	No.	22	32	27	8	0.0001
		%	24.7%	36.0%	30.3%	9.0%	
	Control	No.	0	7	22	2	
		%	0.0%	22.6%	71.0%	6.5%	

Many people with hypothyroidism experience first-stage weight gain. Pharmacological treatment aiming at replenishing the lack of thyroid hormones and normalizing TSH levels is the mainstay of care for hypothyroidism (including autoimmune diseases). Even after achieving euthyroidism (normalization of thyroid hormones and TSH levels within laboratory limits), research shows that 82% of treated women still have excess body weight, and 35% of them suffer from obesity (32). Inflammatory illnesses such as obesity and Hashimoto's are linked. We're curious about elimination diets and the potential anti-inflammatory effects and clinical improvement associated with their implementation because both diseases are characterized by persistent low-grade inflammation and an overproduction of pro-inflammatory cytokines like TNF-alpha and IL-6. Experiment and clinical evidence show that chronic inflammation can increase extracellular water levels and cause Water retention (33). Subcutaneous edema is caused by water accumulation in the glycosaminoglycans of connective tissue, which is also seen in people with Hashimoto's illness. In patients with autoimmune diseases, bodily water retention is considerably higher than in healthy controls (p 0.05) (34). The two clinical diseases of obesity and hypothyroidism are strongly connected together. The link has grown more pertinent in the backdrop of an exceptional rise in the prevalence of obesity worldwide. Patients often attribute their weight gain on a thyroid disorder. A new perspective suggests that shifts in thyroid-stimulating hormone (TSH) may be subsequent to weight gain. There is new evidence that links obesity to thyroid autoimmunity, with the adipocyte hormone leptin serving as the connecting link (35). The result shows (as in Table 9) that there is a significant difference in age of patients compared with control group with $p<0.05$.

Table (9): Means of age parameter in AITD patients and control groups.

Parameter	Patients (mean± SD) (n=100)	Controls (mean± SD) (n=80)	*p*-value
Age (years)	42.63±12.97	38.94±12.82	0.0460

Females have a higher risk of developing autoimmune thyroiditis, and the incidence of the disease rises with age in younger children and teenagers. Overt and subclinical hypothyroidism are diagnosed at rates comparable to those of euthyroid goiter (36). Hypothyroidism is the most common form of thyroid disease, and it is 10 times more common in women than in men, regardless of age (37).

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