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Association of IFN-γ T/A +874 Gene Polymorphism with Type-1 diabetes mellitus in Iraqi Children

Hasanain Riyadh Al- Isawi[1,2] , Faiza Safi[3] , Hayder A. Muhammed[4]
[1]-Department of Biology, Faculty of Science, University of Sfax, Tunisia
[2]-Department of Basic medical science, College of Nursing, University of Al-Qadisiyah, Iraq
[3]-Department of Pediatrics, Hedi Chaker Hospital. Faculty of Medicine, The University of Sfax. Tunisia
[4]-Department of Microbiology, College of Veterinary medicine, University of Kerbala, Iraq

Abstract:
The purpose of this case-control study was to examine the association between polymorphisms in the IFN-γ T/A +874 Gene Polymorphism and the development of Type 1 Diabetes mellitus. Samples of blood were taken from 60 people with Type 1 diabetes mellliuts and 40 healthy controls for this study serum levels were found to be significantly higher ($p>0.05$) than healthy.

The frequency of the allele and genotypes in the patient groups and control groups were determined using Allele Specific-PCR, Triiodothyronine (T3), Thyroxine (ng/ml), and Thyroid Stimulating Hormone (mU/L) levels in the blood were measured using a kit designed to do so in accordance with the manufacturer's instructions. The study show a significant ($p \leq 0.05$) increase in TSH in Patient subjects (diabetic mellitus) as compared to the control group as shown in table (4-2) and this result is in agreements with. The study also shown that there is a significant ($p \leq 0.05$) increase in both hormones (T3& T4) n the P atient subjects (diabetic mellitus) when compared to the control group. In the current study liver enzyme (AST, ALP& ALT) shown a significant ($p \leq 0.05$) increase in Patient subjects (diabetic mellitus) as compared to the control. Patients had an increased frequency of the TT homozygous and TA heterozygous genotypes compared to controls (15 vs. 22 and 21 vs. 12) respectivelly. When comparing the odds ratios (OR) of 0.38 (95% CI: 0.148 to 1.024) and 1.71 (95% CI: 0.05 to 0.51) for the two groups, with statistically significant difference ($p = 0.0539$ and $P=0.0042$) respectively. Patients also had a much higher frequency of the (A) allele than controls did (34.5 vs. 12, OR = 0.327, 95% CI = 0.14 to 0.76, $p = 0.009$).

Keywords: ***IFN-γ, Polymiophism, Liver enzymes, thyroid hormone***

Introduction:

Insulin-dependent diabetes type 1 (also known as juvenile diabetes) was the previous name for this condition, when a person has type 1 diabetes, their bodies stop producing any insulin at all, this means it starts when the immune system of the body attacks healthy cells, In type 1 diabetes, the immune system attacks and kills off the pancreatic cells (beta cells) responsible for producing insulin (Katsarou et al., 2017). The digestive process involves the dismantling of food into its constituent parts. Simple sugars, primarily glucose, are produced when carbohydrates are digested. When it comes to fueling our cells, glucose is king. Glucose, a form of sugar found in food, must enter cells from the bloodstream in order to fuel them (Qaid & Abdelrahman, 2016).

Glucose uptake by cells is triggered by insulin floating through the blood, the pancreas secretes the hormone insulin, the pancreas usually increases insulin production when blood glucose levels rise, such as after eating. when the pancreas' insulin-producing cells are destroyed, the result is type 1 diabetes (Haller et al., 2005). The result is that the patient has very little insulin left. Without insulin, blood sugar levels rise because glucose cannot enter cells. Therefore, the body is unable to convert this glucose into usable energy, high blood glucose levels are harmful to tissues and can lead to dehydration and frequent urination (Paschou et al., 2018).

One of the most important cytokines linked to the rise of autoimmune disorders is interferon gamma (IFN-γ). Immune cells like T and NK cells produce the vast majority of IFN-γ (Psarras et al., 2017). The interferon gamma (IFN-γ) plays a role in both innate and acquired immunity, controlling immune responses like antigen presentation and phagocytosis (Ye et al., 2019).

ISSN NO: 2770-2936
Date of Publication:22-04-2023

The interferon gamma gene, or IFN-γ gene, can be found at 12q15 (Hardy et al., 2004) on chromosome 12, The production level of IFN-γ was linked to an SNPs at position +874 in the first intron of the encoding gene. The +874 T/A polymorphism in the IFN-γ gene has been the subject of a number of investigations into its possible association with autoimmune disease (da Silva *et al*., 2014). However, there have been no attempts at investigating this in Iraq. individuals at risk of developing T1DM can be identified based on their genetic makeup, as genes play an important role in the development of the disease. Phenotypic polymorphism research suggests that the IFN-gene may play a role in the development of certain immune-related diseases (Shuo et al., 2023). This study provides further evidence that the IFN- + 874 A allele is a genetic risk factor for autoimmune disease like Type 1 diabetes mellitus in Iraqi children.

Materials and Methods

Study subjects:

Sixty children with diabetes were used for making this study in Al-Qadisiyah special clinic from January 2021 to July 2021. Their ages ranged (from 1 to 16) years old. The patients included (30 boys and 30 girls). The control group consisted of 40 healthy children (diabetes-free) (20 boys and 20 girls); aged (5-7 years).

The diagnostic & Principles criteria:

The used basic diagnostic for children with diabetes are included: (i) Fasting blood glucose. (ii) Glucose 2 H after a meal (iii) Glycosylated hemoglobin (HbA1c) and (iv) Not taken insulin injection.
The information on the patients and healthy people was obtained using a questionnaire form that was organized for each individual. Consent for participation in the study was provided verbally and in writing by every participant in Al-Qadisiyah maternity and children Teaching hospital, Diabetic clinic, Al-Qadisiyah province, Iraq.

The following types of controls were excluded as the fellowing: children with a history of autoimmune disease, and children with a history of malignancy. children undergoing a physical examination at the same hospital.

Blood sample collection:

Participants fasted overnight before having 10 mL of blood drawn. 5ml of blood was stored in test tubes containing heparin as an anticoagulant and placed in the freezer for later use in molecular investigation; the other 5ml was kept in gel tubes without anticoagulant and allowed to clot before being centrifuged for 5 minutes at (3000 RPM) to separate the serum for estimination the levels of thyroid parameters and liver enzymes.

DNA extraction and purification:

Extraction of the total DNA from blood samples using Wizard® Plus SV Minipreps DNA Purification Systems (Promega/USA) and assessment of the integrity of the DNA samples will determine by gel electrophoresis, and its Purity determined by Nanodrop spectrophotometer in a ratio of ~2.0 is generally accepted as "pure" for DNA

Polymerase chain reaction

Alelle Specific PCR method was used to analyze the genotyping of IFN-γ T/A +874 Gene Polymorphism with the primers given in table 1

Genes	Type of primers	Sequence	Product size (bp)	References
IFN-γ T/A +874 Gene Polymorphism	Allele A	5-TTCTTA CAACACAAAATCAAATCA-3	261	Mahmoud et al., 2016
	Allele T	5-TTCTTACAACACAAAATCAAATCT-3	261	
	Common primer	5-TCAACAAAGCTGATACTCCA-3	-	

ISSN NO: 2770-2936
Date of Publication:22-04-2023

Different volumes of primer (0.5 µl, 1 µl, 1.5 µl,) with different volumes of template DNA (1 µl, 2 µl, 3 µl, 4 µl, 5 µl, 6 µl) and different experiments of the reaction conditions were trailed in order to optimize the conditions of the reaction. PCR tube centrifuged for 30 seconds at 2000 xg in a micro-centrifuge in order to mix solutions well at room temperature then tubes are placed in the thermocyclerto start the reaction. Programs of the PCR protocol reaction for IFN-γ T/A +874 gene Polymorphism table (2-7).

Running the PCR Reaction :

The PCR reaction was done by mixing PCR components with DNA extraction in the table (2) and using the optimized PCR programs as shown in tables (3)

Table (2) : PCR mix reaction for genotyping Of CTLA-4 (+49A/G) gene polymorphism

Component	Volume (µl)
Outer forward primer	1.25
Inner forward primer	1.25
Reverse primer allele T	1.25
Reverse primer allele C	1.25
DNA template	5.0
Deionized water	7.5
Master mix	10

Table (2-8). Allele specific –PCR Program for Detection of IFN-γ T/A +874 gene Polymorphism.

No.	Stage	Cycle	Step	Temp.	Time
1	Initial Denaturation	1	1	92 °C	**2min.**
2	Denaturation	45	1	92 °C	**30 sec.**
3	Annealing	35	2	57°C	**30 sec.**
4	Extension	45	3	72 °C	**55 sec.**
5	Final Extension	1	1	72 °C	**5 min.**
6	**Hold Phase**			**10 °C**	

The samples were subjected to electrical migration at 75 volts for two to three hours. the acarose gel was stained for 20 minutes with ethidium bromide (EtBr) stain (the tincture of ethidium bromide was generated from the concentrated dye at a concentration of 10 mg / mL and prepared by dissolving it in distilled water to obtain a concentration of 0.5 g / ml). A camera-equipped Gel documentation equipment was used to monitor the genotypes and photograph the magnified DNA fragments.

Estimation of Liver enzymes

Liver Aspartate transaminase AST (IU/ mL), Liver Alkaline Phosphatase ALP (IU/ L) and Liver alanine aminotransferase ALT (IU/ L) was measured using a Kit Specific to measure depending on the method of using Kit according to the instructions of the producing company (Spectrum/ Egypt).

Estimation of Thyroid hormones

Serum Triiodothyronine (T3), serum Thyroxine T4 (ng/ml) and thyroid stimulating hormone TSH (mU/ L) were measured using a Kit Specific to measure depending on the method of using Kit according to the instructions of the company (Spectrum/ Egypt)

ISSN NO: 2770-2936
Date of Publication:22-04-2023

Statistical analysis:

The results from polymerase chain reactions were analyzed with SPSS, and the significant differences between means were examined with Fisher's test at the P 0.05 level. allele frequencies, genotypes, odds ratios (OR), and confidence intervals (CI) was assessment according to www.had2know.com, the Hardy-Weinberg equilibrium was applied to the data.

Results and Discussion:

Effect of thyroid hormones on diabetic mellitus patient

The study show a significant ($p \leq 0.05$) increase in TSH in Patient subjects (diabetic mellitus) as compared to the control group as shown in table (4-2) and this result is in agreements with (Shih et al.,2012 & Jun et al., 2017). The study also shown that there is a significant ($p \leq 0.05$) increase in both hormones (T3& T4) I n the P atient subjects (diabetic mellitus) when compared to the control group. And this result is agreements with (Cooper,2001; Rugge et al.,2012; Wu, 2000).

Several studies was found that the TSH increases for people who suffer from diabetes, perhaps because high blood sugar is harmful to the body and as a defense mechanism from the body the anterior pituitary gland increases the production of TSH in order to increase the consumption of sugar by different cells (Biondi et al.,2019; Schernthaner-Reiter et al., 2021).

Thyroid hormones including T3, have been shown to play a role in regulating glucose metabolism in the body. However, the effect of T3 on glucose levels can be complex and may depend on a variety of factors such as the amount of T3 present in the body, the individual's metabolic state, and the presence of other hormones(Mullur et al., 2014 and Eom et al., 2022) .

T3 has been found to decrease blood glucose levels by increasing glucose uptake into cells and stimulating glucose metabolism, T3 has also been shown to increase insulin sensitivity, which can further contribute to a decrease in blood glucose levels (Lin & Sun., 2011). Table 2.

Table (2): Effect of diabetic mellitus on the serum Thyroid hormones

Parameters	Control subjects (Mean ± SD)	Patient subjects (Mean ± SD)	*P*-value (p (0.05 ≥
TSH (mU/ L)	2.57 ± 0.732	3.98 ± 1.23	0.042
T4 (ng/ ml)	9.8 ± 1.38	12.3 ± 2.89	0.023
T3 (pg/ ml)	2630 ± 830.93	4010 ± 660.47	0.017

Thyroxine (T4) is the main hormone produced by the thyroid gland, T4 is converted into the more active T3 in various tissues throughout the body, T3 is the more biologically active form of thyroid hormone, and it is responsible for many of the metabolic effects of thyroid hormone (Wu et al., 2005). Both T3 and T4 regulate metabolism by increasing the production of proteins, increasing the consumption of oxygen, and increasing the rate of energy consumption by cells throughout the body, also both of them increased in case of increase hormone TSH some of study shown that an increased in the amount of the TSH led to an increase in both hormones in people who suffer from diabetes (Crunkhorn & Patti, 2008) .

ISSN NO: 2770-2936
Date of Publication:22-04-2023

Effect of Liver enzymes on diabetic mellitus patient.

Table 3: Effect of serum Liver enzymes on diabetic mellitus

Parameters	Control subjects (Mean ± SD)	Patient subjects (Mean ± SD)	*P*-value (p 0.05 ≥)
AST (IU/ L)	**29.00 ±3.73**	**36.29 ± 5.00**	***.0041***
ALP (IU/ L)	216.78± 64.71	324.39 ±47.82	.0029
ALT (IU/ L)	49.80 ± 7.54	66.00 ± 2.51	.003

In the current study liver enzyme (AST, ALP& ALT) shown a significant ($p \leq 0.05$) increase in Patient subjects (diabetic mellitus) as compared to the control groups as shown in table (3) and this result is in agreements with (Maxwell et al., 1986; West et al., 2006; Mandal et al., 2018 and Islam et al., 2020).

Diabetes mellitus can increase oxidative stress in the liver. When blood glucose levels are elevated for prolonged periods of time, it can lead to the production of reactive oxygen species (ROS) in the liver, which can cause oxidative stress. The ROS can damage cellular components such as proteins, lipids, and DNA, leading to inflammation and tissue damage(Mohamed et al.,2016 & Mendes-Braz et al., 2018) .

Diabetes can also cause an increase in advanced glycation end-products (AGEs), which are compounds formed when glucose reacts with proteins in the body. These end products AGEs can increase oxidative stress by producing ROS and impairing the body's antioxidant defenses, also AGEs can lead to an increase in osmotic pressure, which can contribute to tissue damage and inflammation. The accumulation of AGEs in the liver can also impair liver function and contribute to the development of liver disease. Chronic oxidative stress can lead to liver damage and contribute to the development of liver disease. (Leung et al., 2016 & Rungratanawanich et al.,2021).

Also there is some evidence to suggest that diabetes mellitus can increase apoptosis in the liver (Inoguchi et al., 2022). Some Studies have shown that high blood glucose levels in diabetes can activate a number of cellular pathways that contribute to apoptosis in liver cells, a chronic exposure to high glucose levels can lead to the activation of a protein called caspase-3, which is involved in the apoptotic pathway (Meisse et al.,2002). Diabetes mellitus itself does not directly cause organized cell death in the liver, the high blood glucose levels associated with diabetes can activate a number of cellular pathways that contribute to apoptosis in liver cells, especially when diabetes is poorly managed. For this reason, we notice an increase in the level of liver enzymes in the blood serum in people who have diabetes and this agreement with (Biondi et al., 2019).

Genotyping and allele frequencies of IFN-γ T/A +874 Gene Polymorphism:

The IFN-γ T/A +874 Gene Polymorphism have been linked to diabetic Mellitus. Polymorphism of Allele Specific-PCR of IFN-γ T/A +874 was represented in Figure 1. The results of IFN-γ T/A +874 gene polymorphism were a clear band with a molecular size 349 bps..The size of amplicon 261 bp was determined and compare with DNA ladder 100 - 1500 bp.

Genetic polymorphism of IFN-γ T/A +874 which were classified into three genotypes:

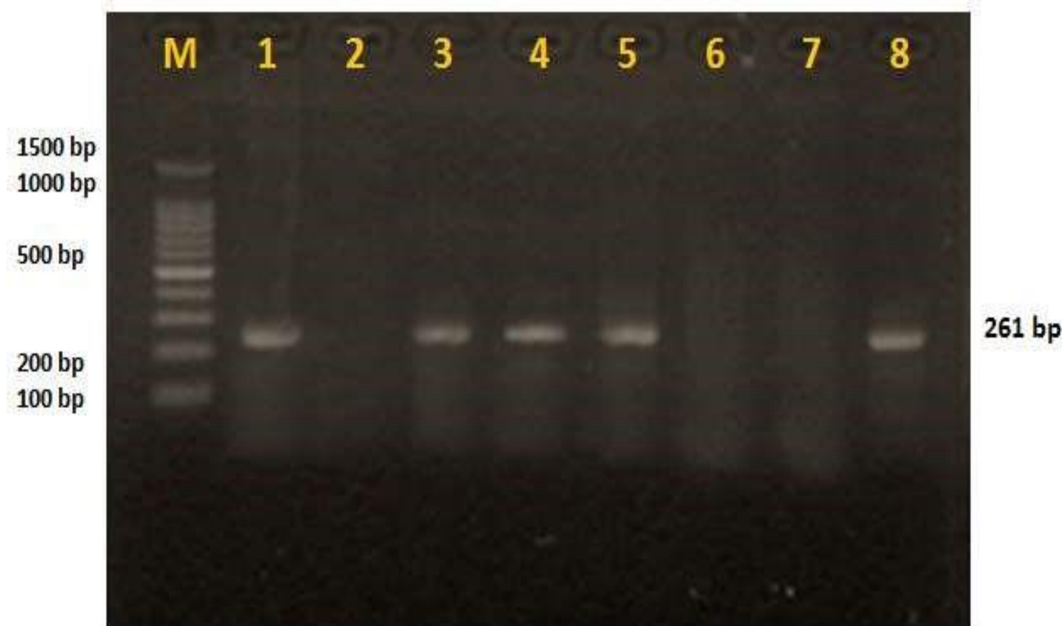
1. The major genotype group (TT) homozygous for the allele T.
2. The minor genotype group (AA) homozygous for the allele A.
3. Heterozygous (TA).

Among the 60 patients, 15 samples (25%) were the TT genotype, 21 samples (35%) were the TA genotype, and 24 samples (40%) were the AA genotype. T allele and A allele have 42.5% and 57.5%, respectively. The distribution of genotypes was out Hardy-Weinberg equilibrium ($\chi^2 = 0.024$; $P < 0.05$).

The genotype frequency of IFN-γ T/A +874 in cases revealed that 25%, 35%, and 40% of cases were TT wild-type, TA heterozygous, and AA homozygous mutation, respectively. Moreover, an allelic frequency

was 42.5% and 57.5% respectively, in controls. The genotype frequency of the described SNP was tested for in consistent with Hardy-Weinberg equilibrium (HWE). IFN-γ T/A +874 SNP was clear to be harmonious with HWE in the control group, indicating that the distribution of genotypes in our population is constant from generation to generation. Table (4).

The codominance model has shown a significant variation ($P < 0.05$) between Patient and control groups ($P=0.0539$ and $P=0.0042$). In the dominant model, there was significant difference ($p= 0.0022$). The recessive model has shown significant difference ($p= 0.02$). The additive model confirmed with significant association ($p= 0.0013$). Analysis of alleles has shown significant association ($p= 0.009$) table (8).



Electrophoresis of Allele Specific-PCR results of IFN-γ T/A +874 Polymorphism. Lane M is marker; lane (1,5 and 5,6) is DNA fragment (TT genotype), with single band at 261 bp; lanes 3,4 are TA genotype, with one band of 261 bp; lanes 7,8 are AA genotype with one band of 261 bp.

Table (7): Hardy Weinberg equilibrium equation of CTLA-4 (+49A/G) polymorphism

Genotype	Actual Alleles values	Expected values	Alleles	X2	P value
CTLA-4 (+49A/G)					
TT	22	19.6			
TA	12	16.8		3.265	0.07
AA	6	3.6			

Table (): Displays the genotype and allele frequencies.

IFN-γ T/A +874 Gene Polymorphism	Patient n=60	Control n=40	Unadjusted OR (95% CI)	P value	Adjusted OR (95%CI)	P value
Codominant						
TT(Reference)	15	22				

ISSN NO: 2770-2936
Date of Publication:22-04-2023

TA	21	12	0.389 (0.148 to 1.024)	0.055	0.58 (0.53-3.65)	0.0539
AA	24	6	0.171 (0.05 to 0.51)	0.0018	0.242 (0.041-0.94)	0.0042
Dominant						
AA+TA	45	18	0.2 (0.073 to 0.54)	0.0019	0.43 (0.28-4.58)	0.0022
Recessive						
TT+TA(Reference)	36	34				
AA	24	6	0.02 (0.46-3.06)	0.0015	1.12 (0.33-2.16)	0.61
Additive						
2(AA)+TA	69	24	(0.11 to 0.237 0.53)	0.0013	0.45 (0.092-3.41)	0.0017
Allele frequency						
T	25.5	28	0.327 (0.14 to 0.76)	0.009	0.587 (0.27 to 1.57)	0.0026
A	34.5	12				

Patients had an increased frequency of the TT homozygous and TA heterozygous genotypes compared to controls (15 vs. 22 and 21 vs. 12) respectivelly. When comparing the odds ratios (OR) of 0.38 (95% CI: 0.148 to 1.024) and 1.71 (95% CI: 0.05 to 0.51) for the two groups, with statistically significant difference (p = 0.0539 and P=0.0042) respectively. Patients also had a much higher frequency of the (A) allele than controls did (34.5 vs. 12, OR = 0.327, 95% CI = 0.14 to 0.76, p = 0.009).

Despite the fact that earlier studies has demonstrated IFN-γ dysregulation in pulmonary tuberculosis patients (Areeshi et al., 2021 and Álvarez et al., 2023), to the best of our knowledge, this is the first study to investigate whether or not there is a connection between the IFN-γ +874 T/A polymorphism and the vulnerability to Type 1 diabetes mellitus in an Iraqi Population.

The IFN-γ gene, which encodes human interferon, is around 6 kb in size and is situated on chromosome 12 (12q14). Natural killer (NK) cells and T lymphocytes produce interferon (IFN-), which is encoded by IFN- (also called IFNG)(Mah & Cooper, 2016).

The expression and secretion of this cytokine, which has a major effect on the course of an infection, may be affected by a single nucleotide polymorphism (SNP) at the +874 A>T (rs2430561) position in the first intron of the IFN-γ gene. Patients with diabetes who are homozygous for allele A have been shown to produce considerably less IFN-γ gene than those who contain either one or two copies of allele T.

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