Association Between TLR4-rs1927911-C/T Gene Polymorphism and Autoimmune Thyroid Disease in Sample Iraq Children

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Abstract
Autoimmune thyroid disease is an immune problem whose incidence has increased in the past decades among individuals of all ages. Five ml of blood was collected from 60 patients from children with thyroiditis disease and 60 uninfected people for comparisation with average age of the two groups (8.17±1.5 and 9.6±1.2 years)respectively, 2 ml of each sample was used for polymerase chain reaction to detect TLR4-rs1927911-C/T gene polymorphism by amplification-refractory mutation system (ARMS-PCR) and remaining (3ml) for TLR4 Enzyme Linked Immunosorbent Assay and thyroid hormones tests. Our results indicated that C allele of TLR4 (rs1927911) polymorphism was more observed frequently in patients compared to healthy people, at a statistically significant level ($P < 0.05$). Enzyme Linked Immunosorbent Assay tests also showed that the level of TLR4 in the serum of patients was higher than it was in the serum of healthy people (P=0.001). We conclude from the results of this study that the TLR4-rs1927911-C/T gene polymorphism is associated with autoimmune thyroid disease in among Iraqi children, and may be a cause of the disease and its incidence in both sexes.

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Keywords: Autoimmune Thyroid Disease, Genes, Polymorphism, Allele, Graves' Disease

1. Introduction

Autoimmune thyroid disease (AITD) It is an autoimmune problem that affects various organs of the body, and its incidence ranges between 2-5%, and this percentage varies according to gender, as it is approximately 5-15% in females and 1-5% in males [1]. The causes of immune diseases are considered somewhat ambiguous, but identifying genetic and environmental influences can lead us to modern therapeutic and diagnostic developments and determine the role of autoimmunity that underlies these diseases [2]. Infection, iodine excess or deficiency, and extreme fatigue are contributing factors that increase the risk of developing autoimmune thyroid disease in people with a genetic influence [3]. Cancer of the thyroid gland and colon is considered in the future to be a complication of AITD of the Hashimoto's HD and Graves' disease GD types, which has been confirmed by Taiwanese studies in this regard [4]. The relationship of TLR4 with various diseases has been previously studied, and it has been shown that it has an effect on persistent infections and various cancers, as well as between it and the autoimmune thyroid gland [5]. The methods of autoimmunity, genes, genetic aspect, and triggers indicated by recent studies provide more information for understanding autoimmune diseases, as the genetic aspect of AITD may pose a worrying problem in its early stages compared to its late stages. An example in this regard is HLA. A strong risk factor shared by autoimmune diseases. These genes are considered an important predictor of the severity of diseases, their response to biological drugs, and the development of ways to manage them [6]. In the chromosome 9 (9q33.1), on its long arm, is the TLR4 site. On this gene there is an encoded protein that senses the causes and effects of immune diseases and overweight and activates the innate immune side. TLR4 is located on the surface of innate immune cells or their cytoplasmic vesicles, where it accepts patterns that are related to pathogens, known as infectious agents [7].

There are approximately 3575 morphological variations of the TLR4 gene, as the TLR4 site (rs1927911) is in a non-coding segment and the allele has a low frequency for TLR4, and this was confirmed by studies conducted previously [8]. In samples of children infected with AITD, there are alleles for HLA when compared with control samples from non-infected individuals. The frequencies of these alleles are somewhat high. This has been confirmed by studies conducted previously [9]. Statistical results in previous studies have shown is that AITD in its early stages is more closely linked and affected by genetic factors than it is in its later cases. Our study examined the possible associations of the polymorphisms of the TLR4-rs1927911-C/T gene with three genotypes in a sample of Iraqi children.

2. Materials and Methods

Our study was conducted on 120 child, including 60 children suffering from autoimmune thyroid diseases, aged between 5-15 years, who were admitted to Al-Diwaniyah Teaching Hospital between January and August 2024.The pediatric patients were diagnosed clinically by physician as having AITD.The other group consisted of 60 age-matched, healthy children with no history of other systemic diseases, and they were studied as a control group for comparison. Other cases of autoimmune, hematological or endocrine diseases were excluded and the focus was on clinically diagnosed AITD patients. Our study was in accordance with the ethical principles stipulated in the Declaration of Helsinki 1975 and the ethics of scientific research of the College of Dentistry, Al-Qadisiyah University. Informed verbal consent was obtained from all participants before starting the study. Five ml of blood was drawn from each participant's vein using medical syringes, and 2ml of this blood was stored in an EDTA tube. TLR4-rs1927911-C/T gene polymorphism detected by ARMS-PCR technique (BioRad-USA). Remaining 3 ml was left to clot in gel tubes for a quarter of an hour at room temperature. Then it was separated into serum using a centrifugal separator at a speed of 3000 rounds per minute over a period of 10 minutes, and the result was divided into three parts using a fine laboratory pipette. This division was in order to avoid re-freezing the samples upon use because it would distort the results. It was stored at -20 degrees Celsius for use in future analysis. Using an enzyme-linked immunosorbent assay (ELISA) (Mabtech USA) Kit test for TLR4, the concentration of serum TLR4 was determined. (Fig.1) show the study design of this research.

Statistical Analysis

In this research, SPSS Statistical Package for the Social Sciences, version 26, was the mathematical analysis tool used to present, analyze, and describe the study data and results. The arithmetic mean and standard deviation were used to describe the quantitative variables. While percentages and frequencies played a role in describing their qualitative counterparts, the independent t-test was used to compare the results of the two groups. Pearson's correlation coefficient was calculated between the two quantitative variables, and a $p \leq 0.05$ was statistically significant.

3. Results

The age and gender variables for the two study groups are shown in [Table 1]. The average age of the two groups, patients and healthy people, was (8.17±1.5 and 9.6±1.2 years), respectively, and no statistically significant change was found with regard to this variable ($P = 0.301$). While the two groups included a number of males and a number of females as follows: the number of males to females among patients was 12:48 and the number of males to females among healthy people was 20:40. The statistical discrepancy was not large for the gender variable compared to the healthy group ($P = 0.098$), but the discrepancy is clear between females and males, as shown by the statistical ratios in [Table 1].The results of the study regarding the Thyroid-stimulating hormone (TSH) in two group of patients and healthy people were as follows (4.81±0.61 and 1.71±0.32), respectively, and the difference was clear and highly effective ($P = 0.001$). While the results of tetraiodothyronine (T4) for the two groups of patients and healthy people were (86.82±17.1 and 34.45±7.23), respectively, the contrast was important and clear ($P = 0.001$). However, the difference is neither noticeable nor significant between the two groups with regard to triiodothyronine
(T3) examination. The polymorphic distributions of the TLR4 gene (rs1927911) were identified through T-ARMS-PCR technology, where three genotypes were identified, which are TT, CT, and CC, as shown in [Fig.2]. These distributions are consistent with Hardy-Weinberg equilibrium in the two groups. It was found that the frequency of TLR4 (rs1927911) C/T and CC is higher in AITD when compared to healthy controls, where the variance was large and influential ($P < 0.05$). At the same time, a higher frequency of TLR4 polymorphism (rs1927911) was observed in patients than in the healthy group ($P = 0.003$), as shown in [Table 3]. The results of the study also found that the concentration of TLR-4 was higher in the serum of patients with AITD compared to healthy people, 113.15±8.71 compared to 19.56±2.76, where the difference was clear and significant ($P < 0.001$), as shown in [Table 4]. After comparing TLR4 (rs1927911) genotypes and TLR-4 levels in patient samples, our results showed that patients with TLR4 (rs1927911) C/T and CC genotypes had more TLR-4 increased compared to those with TLR4 (rs1927911) TT genotypes (both $P < 0.05$). However, the variation in TLR-4 levels was not large and significant between samples of patients with TLR4 (rs1927911) C/T and TLR4 (rs1927911) CC genotypes ($P > 0.05$), and the details in [Table 5].

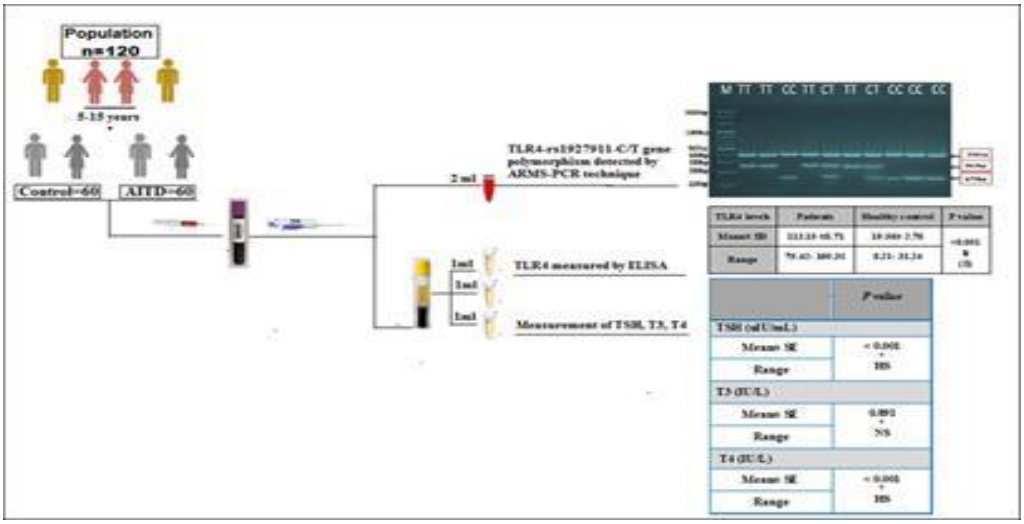

Fig 1: Study Design

Table 1: Demographic features of the study

Characteristic	Patients n = 60	Control n = 60	P
Age (years)			
Mean±SE	8.17±1.5	9.6±1.2	0.301
Range	1 - 12 years	1- 15 years	NS
Gender			
Male, *n* (%)	12 (20.0%)	20 (33.3%)	0.098
Female, *n* (%)	48 (80.0%)	40 (66.7%)	NS

n: number of cases; SE: standard Error of mean; NS: non-significant at P > 0.05.

Table 2: Thyroid hormones results for both groups.

Tests	Cases–Control Comparison		P value
	Patients n = 60	Control n = 60	
TSH (IU/mL)			
Mean±SE	4.81±0.61	1.71±0.32	< 0.001
Range	1.00- 9.00	0.31-3.40	HS
T3 (IU/L)			
Mean±SE	3.1±0.81	3.26±0.78	0.891
Range	1.45- 6.10	1.00-7.00	NS
T4 (IU/L)			
Mean±SE	86.82±17.1	34.45±7.23	< 0.001
Range	55.90 -105.51	25.10-42.64	HS

n: number of cases; SE: standard Error; HS: Highly significant at P ≤ 0.001; NS: non-significant at P > 0.05.

Table 3: Frequency of TLR4 (rs1927911) genotype in the two study groups.

Mode	TLR4-rs1927911	Patients n = 60	Control n = 60	P	OR	95% CI
Co-dominant	CC	14 (23.3%)	7 (11.7%)	0.039	2.84	1.02-7.84
	C/T	15 (25.0%)	9 (15.0%)	0.069	2.37	0.91 -6.09
	TT	31 (51.7%)	44(73.3%)	Reference		
Dominant	CC+C/T	29 (48.3%)	16 (26.7%)	0.014	Reference	
	TT	31 (51.7%)	44 (73.3%)	S	0.388	0.18-0.83
Recessive	CC	14 (23.3%)	7 (11.7%)	0.092	2.30	0.85-6.20
	C/T+TT	46 (76.7%)	53 (88.3%)	NS	Reference	
Alleles	C	43 (35.8%)	23 (19.2%)	0.003	2.35	1.31-4.24
	T	77 (64.2%)	97(80.8%)	S	Reference	

NS: non-significant at P > 0.05, S: significant at P ≤ 0.05

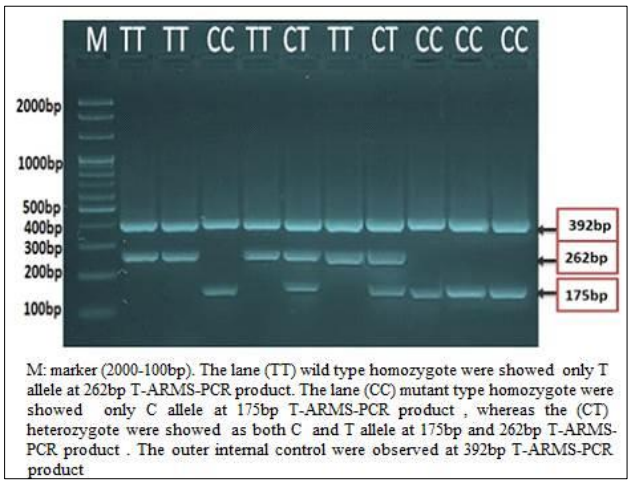

Fig 2: Agarose gel electrophoresis image that showed the product analysis of for rs1927911 TLR4 gene polymorphism

Table 4: The compared between the study groups regarding TLR4 levels.

TLR4 levels	Patients	Healthy control	P value
Mean±SD	113.15±8.71	19.56±2.76	<0.001
Range	75.42-160.31	8.21-31.24	S

S: significant at $P \leq 0.05$

Table 5: Association between TLR4-rs1927911-C/T genotype and serum TLR4 levels in patient with Autoimmune thyroid disease

Serum levels	TLR4-rs1927911-C/T genotype			
	TT genotype	CT genotype	CC genotype	P
TLR4 levels				
Mean±SE	108.43±9.33	112.56±5.03	121.2±9.19	P=0.108
Range	75.42 – 132.5	89.34 – 151.23	88.32-160.31	NS

NS: non-significant

4. Discussion

Our results showed that there was no statistically significant difference regarding the age variable for the two study groups, and this is consistent with a study conducted by Calcaterra *et al* [10]. Also, with regard to gender, there was no noticeable difference between the two groups, but the difference was large between females and males, as the incidence of the disease in females was higher than in males, and this is consistent with the findings of the study by Whitaker *et al* [11] which is that AITD is more prevalent in females and is less common in males by a ratio of 7:3 and this result matches what was reached by Calcaterra *et al* [10], where they showed that the difference between the genders has a clear role in immune thyroid diseases, as females have more than males, and this includes the study group of children, as females increased by a ratio of 1:2 this percentage is due to many factors, including genetic, hormonal, immune response, and organ weakness. Females produce humoral and cellular immune responses that are strong and active enough after antigenic challenge compared to males. Perhaps the increase in these responses in females causes an increase in the incidence of some disorders that may be of autoimmune origin. However, the biological nature of bisexuality when studying autoimmune diseases is ambiguous, while previous studies in this regard have shown that inactivation of the X chromosome is an active participant in the pathogenesis of the high incidence of females suffering from AITD [12,13]. As for thyroid hormones, the results showed that there was a significant variance amidst the two study groups, as the TSH and T4 hormones increased in the serum of patients compared to healthy people. This confirms that TSH is considered the best initial test to measure the disease, as the higher it is, the higher it leads to the evaluation of the free thyroid hormone fT4 in the blood serum, which proves that the child has hypothyroidism, diagnosed in the serum of those with high concentrations of Tg Abs and/or TPO Abs [14]. It is recommended to examine T4 and TSH for children and monitor patients every 6 to 12 months [15]. rs1927911is located in intron 1 of the TRL4 gene. It has not been determined whether this site is functional or not, and it is possible that intron differences affect the appropriate mechanism for transcribing or splicing the messenger RNA. The current study also showed statistical variation in the hereditary form of TLR4 gene patterns in people with AITD compared with healthy controls, it also showed that polymorphism TLR4 (rs1927911) it was more frequent in patients compared to healthy and frequencies TLR4(rs1927911) C/T and CC genotypes higher in patients with autoimmune thyroid disease compared to the control group. The disease-responsive gene has been identified as the TLR4 rs1927911 C allele. Where this can be explained polymorphism TLR4 rs1927911C/T can cause autoimmune thyroid diseases through its effect on immune recognition, as the C allele can modify immune responses, which leads to acute and chronic inflammation and participates in the autoimmune process. It is considered Polymorphisms are a contributing factor in the development of autoimmune thyroid disease, although AITD is complex and has multiple influencing factors. This is consistent with what Toussi *et al* [16] found in this regard, they concluded that TLR4 rs1927911 CC and CT revealed an important preventive genotype for TAO disease when comparing results with healthy controls. TLR4 works via MyD88 and MyD88 to enhance the production of interleukin IL-12, the secretion of IFN, as well

as the secretion of a significant number of Th1-type cellular and humoral immune responses. Kutikhin *et al* [17] also found that dysregulation of TLR4 signaling as a result of SNPs can cause differences in association and homeostasis between them in both pro-inflammatory and anti-inflammatory cytokines, and ultimately modify the danger of persistent inflammatory. This study faced limitations, including the small study sample and its focus on a population area around the globe. Most importantly, there is difficulty in obtaining adequate monitoring of children for ethical reasons, as well as difficulty in obtaining samples from sick children.

5. Conclusion

From the above conclude that the role genetic is considered the effective variable in the pathogenesis of AITD, as C allele increased frequency in the patient group may be a strong link to the increased incidence and severity of the disease, and this varies between the individual population groups of the patient samples, that is, the race factor is included as a potential variable in subsequent studies. Since this study could be unique in this regard, it can serve as a reference for a complementary study to understand the immune pathways through which TRL4 influences AITD by utilizing the data and results of this study as the basic building block for understanding the physiological mechanism of AITD.

Abbreviations

TLR4	Toll-like receptor 4
ARM	Amplification-refractory mutation system
PCR	Polymerase Chain Reaction
ELISA	Enzyme-linked Immunosorbent Assay
AIT	Autoimmune thyroid disease
HD	Hashimoto disease
GD	Graves' disease
HLA	Human leukocyte antigen
EDTA	Ethylenediaminetetraacetate
SD	Standard deviations
TSH	Thyroid-stimulating hormone
T4	Thyroxine, or tetraiodothyronine
T3	Triiodothyronine
fT4	Free thyroid hormone
Tg Abs	Thyroglobulin antibodies
TPO Abs	Thyroid Peroxidase Antibodies
RNA	Ribonucleic Acid
My D88	Myeloid differentiation primary response 88
IFNs	Interferons

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