ORIGINAL ARTICLE

CTLA-4 POLYMORPHISM ALONG WITH PROINFLAMMATORY CYTOKINES IN AUTOIMMUNE THYROIDITIS DISEASE

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ABSTRACT

The aim: Evaluating serum concentration of IL-17 and IL-23 in autoimmune thyroiditis patient and control group along with the role of CTLA-4 rs3087243 gene polymorphism.

Materials and methods: A case control study was conducted in 30 HT (Hashimoto's thyroiditis), 30 GD (Graves' disease) who attended the consultant clinic for thyroiditis in AL-Diwaniyah teaching hospital and in 30 people as control group. Blood samples were processed for measurement of serum IL-17 and IL-23 using ELISA test. The second part used for DNA extraction then CTLA-4 polymorphism was detected by Allele – specific PCR assay.

Results: The level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease, followed by control group and the difference was highly significant ($p < 0.001$; $p < 0.001$) respectively; however, the difference between patients Hashimoto's thyroiditis and patients with Graves' disease was not significant ($p > 0.05$; $p > 0.05$) respectively. There was no significant association between rs3087243 gene polymorphism and Hashimoto's thyroiditis ($p > 0.05$), no significant association between rs3087243 gene polymorphism and Graves' disease ($p > 0.05$). Moreover, there was no significant difference in rs3087243 genotypes frequencies between Hashimoto's thyroiditis and Graves' disease ($p > 0.05$).

Conclusions: Serum IL-17 and IL-23 level have been linked with autoimmune thyroiditis disease, while CTLA-4 rs3087243 polymorphism seem to have no role in disease susceptibility in Iraqi population.

KEY WORDS: Autoimmune thyroiditis, Hashimoto's thyroiditis (HT), Graves' disease, CTLA- 4 polymorphism rs3087243 gene, IL-17, IL-23

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INTRODUCTION

When the body's immune system assaults the thyroid gland, this is known as psychoactive drugs. Graves's disease (GD) and Hashimoto's thyroiditis (HT), both AITDs, are the most frequent causes of thyroid gland failure and no endemic goiter [1,2]. Self-thyroid antigen reactivity, which manifests as inflammatory or anti-receptor autoimmune disorders [3-4], has an impact on these structures. They occur by reaction to self-thyroid antigens and are produced by a complex interaction of environmental and genetic factors [2]. One of the most well-studied and researched AITD susceptibility genes include the HLA-DR gene cluster, as well as non-MHC genes, including CTLA-4, CD40, PTPN22, thymoglobulin, and TSH receptor genes [5-15]. Iodine, medications, sickness, smoking, stress, and genetic predisposition to AITD are all important environmental triggers of thyroid autoimmune disease, pointing to novel possible routes via which genetic-environmental interactions may contribute to thyroid autoimmunity [13]. Non-MHC proteins such as CTLA-4, CD40, PTPN22, thymoglobulin, and TSH receptor genes, as well as the HLA-DR gene locus [15], have all been discovered and described as significant AITD susceptibility genes. Iodine, medicines, illness, smoking, stress, and genetic susceptibility to AITD are all significant environmental triggers of thyroid autoimmune, leading to new potential pathways by which genetic-environmental interactions may contribute to thyroid autoimmunity [16].

THE AIM

Evaluating serum concentration of IL-17 and IL-23 in autoimmune thyroiditis patient and control group along with the role of CTLA-4 rs3087243 gene polymorphism.

MATERIALS AND METHODS

A case control study was conducted in AL-Diwaniyah province. Based on 30 patients with HT, equaling 5 males and 25 females, and 30 patients with GD (9 males and 21 females), who attended the consultant clinic for thyroiditis were taking part in this study. In addition to that, about 30 people (11 males and 19 females) apparently healthy volunteers were included as a control group. Blood samples were collected by venipuncture from 60 patients (30 for HT and 30 for GD) and 30 healthy controls, five milliliters of venous blood were drawn by disposable syringe under aseptic technique. Three ml of blood were put in gel tube and allowed to clot, then the serum was separated by centrifugation (1500 rpm for 5 minute). The serum has been collected in Eppendorf tube then stored at -20°C to be used for ELISA test to determine concentration of IL-17 and IL-23 in serum. Another two ml

Table I. Primers for CTLA-4genepolymorphism

Primer		Sequence (5'-3')	PCR product size
CTLA_4 (rs3087243)	F	CACCACTATTTGGGATATACC	216 bp
	R	AGCTCTATATTTCAGGAAGGC	

Table II. The restriction enzymes were used in RFLP-PCR assay with their company and country of origin

Target gene	Polymorphism	Restriction Enzymes	Company/Country
CTLA-4 gene(rs3087243)	G/A	NcoI	New England Biolabs. UK

Table III. Polymorphism Chain Reaction (PCR) Thermo cycler Conditions

PCR step	Temp	Time	Cycle repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30sec.	34cycle
Annealing	55°C	30sec	
Extension	72°C	30sec	
Final extension	72°C	5min	1cycle
Stop reaction	4°C	Forever	-

Table IV. Comparison of rs3087243genotype frequencies between control group and Hashimoto's thyroiditis

rs3750920 genotypes	Control n = 30	Hashimoto's thyroiditis n = 30	p
AA	8 (26.7 %)	7 (23.3 %)	0.850 C NS
A/G	14 (46.7 %)	13 (43.3 %)	
GG	8 (26.7 %)	10 (33.3 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table V. Comparison of rs3087243alleles frequencies between control group and Hashimoto's thyroiditis

rs3750920 alleles	Control n = 60	Hashimoto's thyroiditis n = 60	p
A	30 (50.0 %)	27 (45.0 %)	0.583 C NS
G	30 (50.0 %)	33 (55.0 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table VI. Comparison of rs3087243genotypes frequencies between control group and Graves' disease

rs3750920 genotypes	Control n = 30	Graves disease n = 30	p
AA	8 (26.7 %)	10 (33.3 %)	0.410 C NS
A/G	14 (46.7 %)	9 (30.0 %)	
GG	8 (26.7 %)	11 (36.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

of blood were collected in ethylinditetracitic acid (EDTA) tube and stored at -20°C for DNA extraction and detection of CTLA-4 polymorphism by Allele-specific PCR study design.

GENOMIC EXTRACTION

Genomic DNA from blood samples were extracted by using G-spin DNA extraction kit (Frozen Blood) INtRON, Korea; and done according to company instructions. The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO USA), which measured DNA concentration (ng/μL) and checked the DNA purity by reading the absorbance at (260 /280 nm).

STEM LOOP RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)-PCR

Restriction Fragment Length Polymorphism-PCR was performed for detection of CTLA-4 gene polymorphism (rs3087243) (G/A) in HT and GD patients and healthy control blood samples. This method was carried out, according to described one by López-Villalobos.

PRIMERS: PRIMERS FOR THE CTLA-4 POLYMORPHISM GENE

The RFLP-PCR primer for detection and genotyping of CTLA-4 (rs3087243) (G/A) gene polymorphism were

Table VII. Comparison of rs3087243alleles frequencies between control group and Graves' disease

rs3750920 alleles	Control n = 60	Graves disease n = 60	p
A	30 (50.0 %)	29 (48.3 %)	0.855 C NS
G	30 (50.0 %)	31 (51.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table VIII. Comparison of rs3087243genotypes frequencies between Hashimoto's thyroiditis and Graves' disease

rs3750920 genotypes	Hashimoto's thyroiditis n = 30	Graves disease n = 30	p
AA	7 (23.3 %)	10 (33.3 %)	0.521 C NS
A/G	13 (43.3 %)	9 (30.0 %)	
GG	10 (33.3 %)	11 (36.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table IX. Comparison of rs3087243alleles frequencies between Hashimoto's thyroiditis and Graves' disease

rs3750920 alleles	Hashimoto's thyroiditis n = 60	Graves disease n = 60	p
A	27 (45.0 %)	29 (48.3 %)	0.714 C NS
G	33 (55.0 %)	31 (51.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

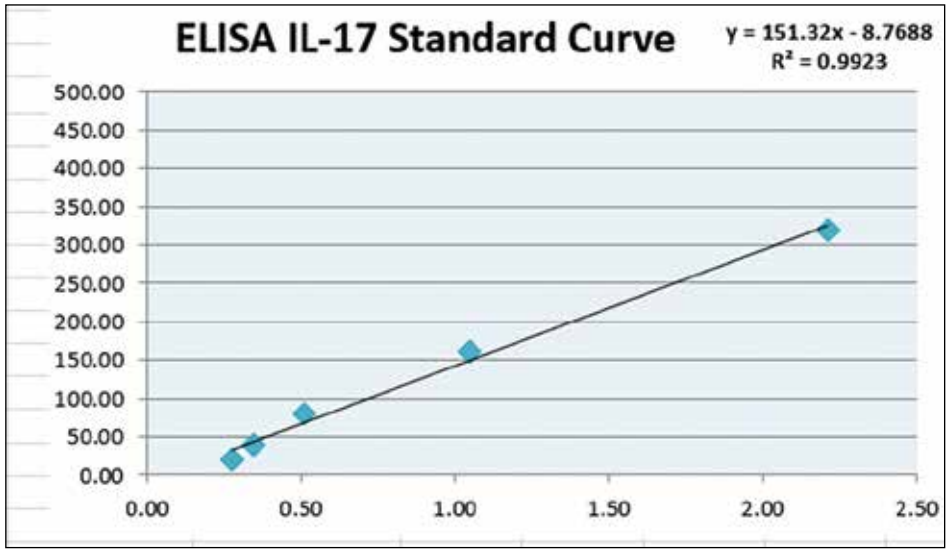

Fig. 1. Standard curve of Interleukin-17

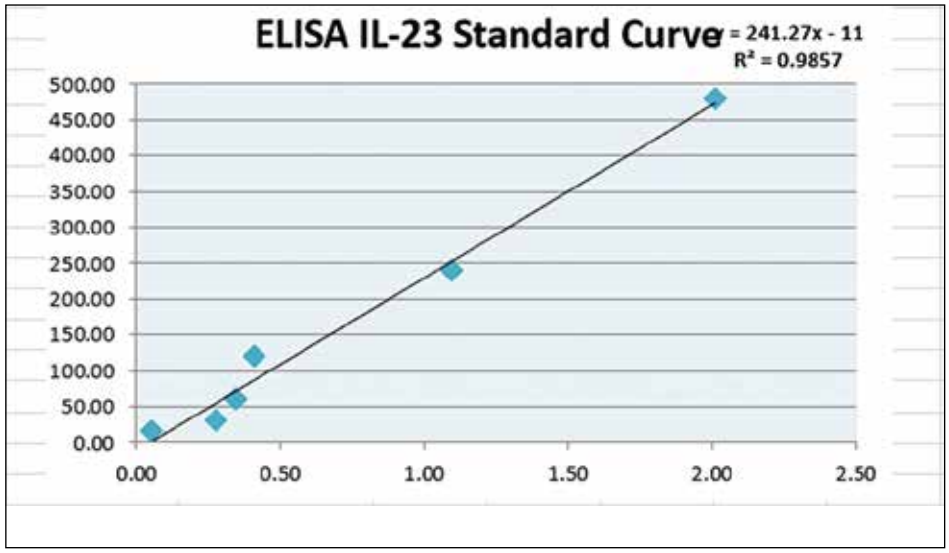

Fig. 2. Standard curve of Interleukin-23
Thermo cycler Conditions of PCR

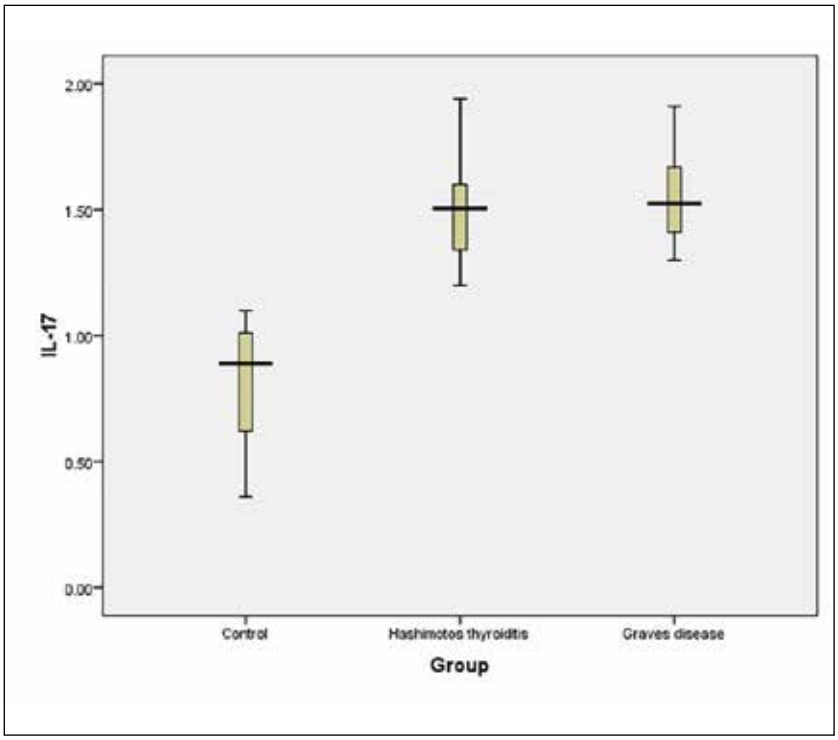

Fig. 3. Box plot showing comparison of serum interleukin-17 among patients with Hashimoto's thyroiditis, Graves' disease and control group

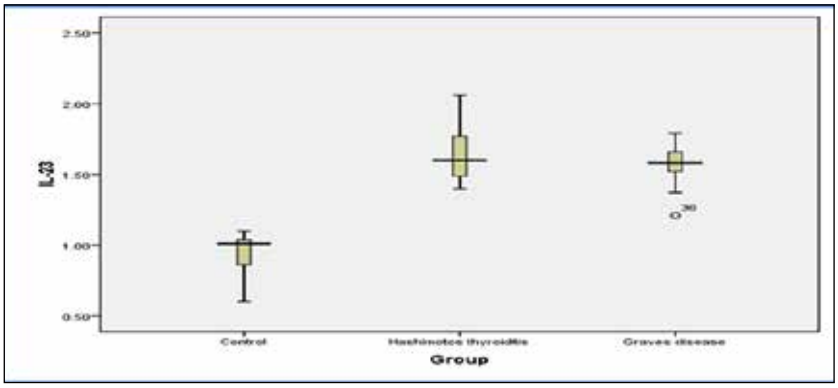

Fig. 4. Box plot showing comparison of serum interleukin-23 among patients with Hashimoto's thyroiditis, Graves' disease and control group

designed by López-Villalobos [1]. These primers were provided from (Macrogen company, Korea) as indicated in following tables (I-II).

RESTRICTION ENZYME

Table II. The restriction enzymes were used in RFLP-PCR assay with their company and country of origin

ELISA TEST

The quantitative sandwich enzyme immunoassay methods were used in this test. An antibody specific for IL-17 and IL-23 has been pre-coated on the micro-ELISA plate. The antigen is then attached to the immobilized capture antibody, and the standard and samples are pipetted into the wells, with any IL-17 and IL-23 present being bound by the immobilized antibody, following a basic washing technique to remove any loose substances. To the wells, a biotin-conjugated antibody specific for IL-17 AND IL-23 is applied. After washing, Avidin conjugated Horse radish peroxidase (HRP) was added to each microplate well, incubated, and washed to eliminate any unbound Avidin-enzyme reagent. Finally, a substrate solution specific to the enzyme in the well was added. The amount of IL-17 AND IL-23 bound in the initial stage is exactly proportional to the color intensity generated, when a stop solution is added to the enzyme-substrate reaction, the color changes to yellow. At a wavelength of 450nm, the optical density (OD) is measured spectro-photometrically. The OD value is proportional to the concentration of IL-17 and IL-23; therefore, we compared the OD of the samples to the standard curve to compute the concentration of IL-17 and IL-23 in the sample.

CALCULATION OF RESULTS

The ELISA results were: calculation depending on the average of the duplicate readings for each standard and samples optical density. Then, we created a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and drew a best fit curve through the points on the graph in excel office program, figures (1-2).

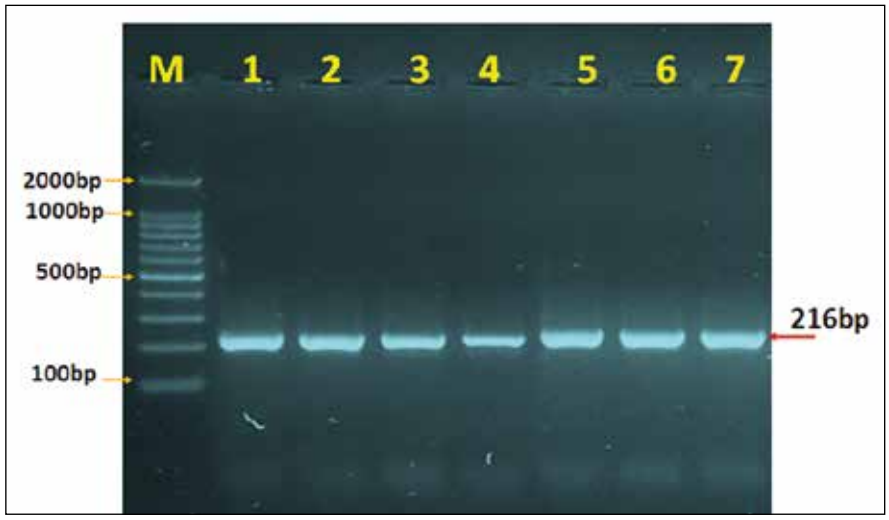

Fig. 5. Agarose gel electrophoresis image that showed the PCR product analysis of CTLA4 gene from patient and healthy control blood samples. Where M: marker (2000-100bp), lane (1-7) positive PCR amplification at 216bp PCR product size

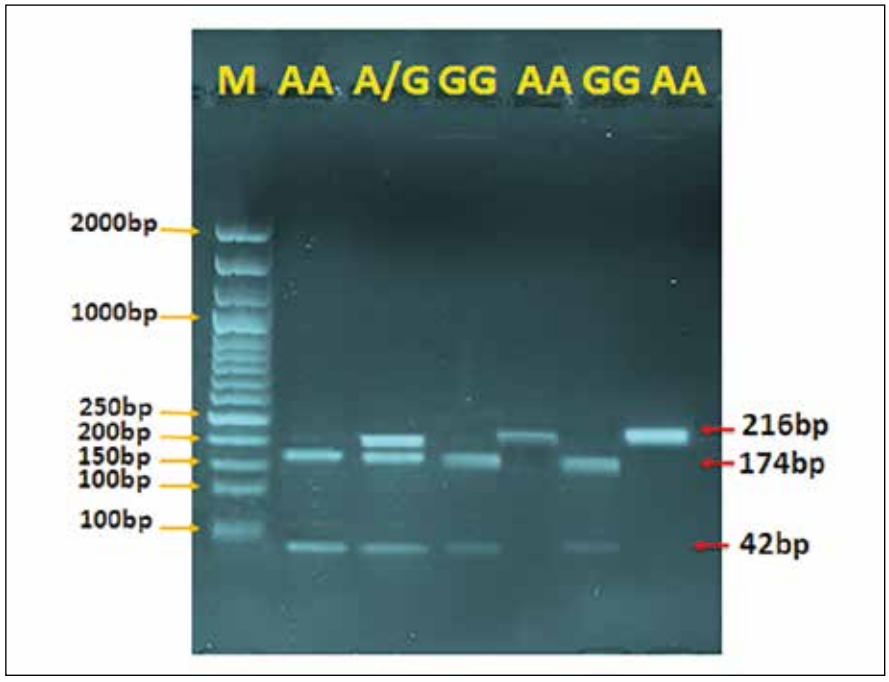

Fig. 6. Agarose gel electrophoresis image that showed the RFLP-PCR product analysis of CTLA4 (rs087243) gene polymorphism from patient and healthy control blood samples by using NcoI restriction enzyme. Where M: marker (2000-50bp), lane (AA) wild type homozygote, the PCR product was undigested by restriction enzyme and still 216bp band, the lane (GG) mutant type homozygote that showed product was digested by restriction enzyme into 174bp and 42bp band, and the lane (A/G) heterozygote, the product was digested by restriction enzyme into 216bp, 174bp, and 42bp bands.

PCR thermo cycler conditions were done for each gene in dependent as following table III.

STATISTICAL ANALYSIS

All data were normally distributed and recorded in Microsoft Excel spread sheet, statistical analysis carried out with SPSS version 0.17 software, numeric data were presented as mean, standard deviation, median and Interquartile range (IQR), while nominal data were expressed as number and percentage. Independent sample T test was used to compare mean value between two groups, while Mann Whitney U test was used to compare median value between two groups, the level of significance considered when p-value was less than 0.05.

RESULTS

The level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease and followed by control group and the difference was highly significant ($p< 0.001$; $p< 0.001$) respectively; however, the difference between patients Hashimoto's thyroiditis and patients with Graves' disease was not significant ($p > 0.05$; $p > 0.05$) respectively, figures (3-4). There was no significant association between rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis ($p> 0.05$), as shown in tables (IV-V). In addition, there was no significant association between rs3087243 gene and allele polymorphism and Graves' disease ($p> 0.05$), as shown in table VI and VII. Moreover, there was no significant difference in rs3087243 genotypes and alleles frequencies between Hashimoto's thyroiditis and Graves' disease ($p> 0.05$).

GENETIC ANALYSIS

There was no significant association between rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis ($p> 0.05$), as shown in tables (IV-VI). In addition, there

was no significant association between rs3087243 gene and allele polymorphism and Graves' disease (p> 0.05), as shown in tables (VII-VIII). Moreover, there was no significant difference in rs3087243 genotypes and alleles frequencies between Hashimoto's thyroiditis and Graves' disease (p> 0.05), as shown in tables IV and V.

DISCUSSION

In the present study, the level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease and followed by control group. The difference was highly significant (p< 0.001). In the study of Gerenova [2], serum levels of both cytokines, IL-17 and IL-23, were significantly higher in patients with Hashimoto's thyroiditis in comparison with healthy control subjects; therefore, the results of the current study are in line with that of [2]. Our results are also comparable to the results of Degertekin [3], who found that serum level of IL-17 was significantly higher in patients with Hashimoto's thyroiditis in comparison with healthy control subjects. According to Kim et al in 2012 [4], serum IL-23 was significantly higher in patients with Graves' disease in comparison to control subjects, thus our results are in line with [4].

Moreover, the serum level of IL-23 has been shown to be significantly higher in newly diagnosed patients with Graves' disease and patients with active Graves' disease in comparison with patients with inactive Graves' disease [5]. The significantly higher level of serum IL-17 and serum IL-23 level in the current study in patients with Hashimoto's thyroiditis and Graves' disease supports the suggestion that IL-23/IL-17 axis plays an important role in the immune pathogenesis of these thyroid autoimmune disorders. Th17 cells, a newly discovered CD4+ T cell subset, and distinguished from the Th1 and Th2 cells, mainly produce IL-17 which acts in vitro and in vivo as a potent inflammatory cytokine [6]. It's functions reflected in the ability of the collective mobilization, recruitment and activation of Neutrophils by the effectors they secreted[7,8]. Th17 cells and IL-17 play an important role in various autoimmune diseases. Previous findings[9,10] and the latest study from Nanba [11] have shown that Th17 cells and IL-17 were related to the pathogenesis of Graves' Disease, Hashimoto's thyroiditis, and Graves' ophthalmopathy. A number of studies have shown that IL-23 is required for full acquisition of the pathogenic function and maintenance of effector Th17 cells [12,13]. IL-23 promotes the secretion of inflammatory factors, cytokines and Chemokines via binding to IL-23 receptor. The combination of IL-23 and IL-23 receptor may activate STAT3 signal, induced memory T-cells to differentiate into Th17 cells and affected the expression of IL-17 by increasing the expression of RORγt, and, ultimately, promoted inflammation and autoimmune diseases. IL-23/IL-17 axis mostly is composed of IL-23, IL-23 receptor, Th17 cells and IL-17. It's a critical pathway in activation and maintenance of Th17 cells. Studies found that the IL-23/IL-17 pathway is involved in the pathogenesis of autoimmune disease including Graves' disease [14]. Thus, our findings of high serum IL-17 and IL-23 are in line with Zheng et al., 2013 in that the IL-23/IL-17 pathway is involved in the pathogenesis of Graves' disease. The exact etiology of AITDs remains unknown, but it is believed that they are caused by an interactive combination of susceptibility genes and environmental triggers.

Recently, with the advent of new genomic tools and the accomplishment of the human genome, several susceptibility genes have been identified in AITDs, including protein tyrosine Phosphatase- 22 (PTPN 22), thymoglobulin gene (TG) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [15-17]. These susceptibility genes implicate autoimmunity in the pathogenesis of AITDs. Among them, CTLA-4 has been recently associated with functional relevance and with susceptibility to a variety of AITDs [18,19]. Our findings are in line with the observation of several previous authors who found no significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis [20] and are in controversy to the results of other authors who found significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis [21]. In addition, our findings are in line with the observation of several previous authors who found no significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Graves' disease [22,23] and are in controversy to the results of other authors who found significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Graves' disease [20-25]. Therefore, the association between CTLA- 4 rs3087243 gene polymorphism and thyroid autoimmune diseases is still controversial and needs further research work in order to reach a clear consensus.

CONCLUSIONS

Both of the pro-inflammatory cytokines (IL-17 and IL-23) serum level have an impact on disease development in patient with autoimmune thyroiditis disease, in addition CTLA-4 rs3087243 polymorphism seem to have no role in disease susceptibility in Iraqi population.

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Conflict of interest:

The Authors declare no conflict of interest.

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A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article