CORRELATION OF SINGLE NUCLEOTIDE POLYMORPHISM OF THE *INTERLEUKIN-27* GENE IN AUTOIMMUNE THYROID DISEASE.

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ABSTRACT

The aim of this study was to investigate the association of IL-27 serum level and *IL-27* gene SNP rs153109 with the risk and clinical features of the Iraqi patients with AITD. The blood samples were collected from 120 AITD patients and 60 healthy controls. Serum levels of interleukin-27 were assessed using ELISA kit. The study results showed a higher significant difference of IL-27 in sera of AITD patients compered to controls (p<0.001). The allele frequency and genotypes in the patient and control groups were determined using High-resolution melting (HRM) to detect the genetic variation in *IL-27* gene SNP (rs153109). the result showed that CC and TC genotype of rs153109 had a statistically significant increased frequency in GD patients compared to controls (OR=19.4, P-value =0.0002), (OR=2.9, p-value =0.011). while the CC genotype of rs153109 was found to be more common in HT patients than control (OR = 6.8, value=0.017). In conclusion, the study suggested that genetic polymorphism of *IL-27* gene rs153109 may be associated with AITD risk among Iraqi population.

Key words: graves' disease, hashimoto's thyroiditis, SNP.

العلاقة بين تعدد أشكال النيوكليوتيدات المفردة لجين إنترلوكين-27 في مرض الغدة الدرقية المناعي الذاتي.

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المستخلص

تهدف الدراسة الحالية الى التحري عن إمكانية ارتباط مستوى مصل الانترلوكين- 27 وتعدد الاشكال الوراثية في جين انترلوكين -27 بخطر الإصابة بمرض الغدة الدرقية المناعي الذاتي للمرضي العراقيين. تم جمع عينات دم من 120 مريضا مصاب بالمرض و60 عينة من الافراد الاصحاء. تم تقييم مستوى مصل الانترلوكين -27 باستخدام تقنية الاليزا واظهرت النتائج فرقا معنويا في مصل المرضى مقارنة بالأصحاء (p<0.001). بينما تم تحديد تردد الاليل والانماط الجينية باستخدام تقنية (HRM) واظهرت النتائج ان النمط الوراثي CC وTCمن rs153109 كان له تردد متزايد ذو دلالة إحصائية في مرضى جريفز مقارنة بالأصحاء (OR=19.4, P-value =0.0002) (OR=2.9, p-value =0.011) بينما وجد ان النمط الجيني CC من rs153109 كان أكثر شيوعا في مرضى هاشيموتو مقارنة بالأصحاء (OR = 6.8, p value=0.017). لقد استنتجت الدراسة ان تعدد الاشكال الوراثي rs153109 مرتبط بخطر الإصابة بأمراض الغدة الدرقية المناعية الذاتية لدى العراقيين.

كلمات مفتاحية: مرض جريفز، التهاب الغدة الدرقية هاشيموتو, تعدد أشكال النوكليوتيدات المفردة.

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INTRODUCTION

Autoimmune thyroid disease (AITD) is a family of pathological entities defined by an inflammatory response on the thyroid gland as well as primary dysthyroid. Grave's disease (GD) and Hashimoto's thyroiditis (HT) are the two most frequent thyroid autoimmune diseases. Both have a multifactorial etiology, which means they are caused by a combination of genetic and environmental factors (3). Hashimoto's thyroiditis is estimated to affect 2% of the world's population. Loss of self-tolerance to thyroid antigens is a common sign of HT. the significant characteristic of HT is circulating thyroid antibodies, thyroid lymphocyte infiltration and thyroid cell apoptosis, leading to follicle destruction and the cause of hypothyroidism (15). GD is an autoimmune disorder that affects the thyroid specifically with Anti-thyroid stimulation hormone (TSH) receptor (TSH-R) autoantibodies in circulation that can cause hyperthyroidism This induces follicular hypertrophy and hyperplasia, which leads to thyroid enlargement. GD is caused by a lack of immunological tolerance to thyroid antigens, particularly (TSH-R) (16). Numerous genetic and environmental factors have been proposed to contribute to the pathogenesis of both Hashimoto´s thyroiditis and Grave´s disease. In Hashimoto thyroiditis, genetic variables include immune-modulating genes such as CTLA-4, FOXP3, CD25, CD40, and the HLA-DR3, which is associated with the high risk of HT (24). On the other hand, excess iodine intake, selenium deficiency, and medication are considered as environmental factors that may contribute to the etiology of Hashimoto´s thyroiditis (19). Furthermore, several studies suggest the relationship between the degree of histological thyroiditis and the presence of lymphocytic infiltration and thyroid autoantibodies in the blood (9). In Grave´s disease, various genetic factors have been related to the etiology of the disease such as polymorphisms on HLA genes, thyroglobulin genes (TG) and thyroid stimulating hormone receptor genes (TSH-R) (11). Environmental variables include seasonal change (summer and spring), ionizing radiation, and smoking which have been suggested to contribute to the pathophysiology of Grave´s disease and other diseases (22, 27). Humoral and cellular immunity play a vital role in the development of AITD furthermore, Cytokines, which are molecules produced by both invading inflammatory cells and thyroid follicular cells (1). Although AITD is a multifactorial disease whose etiology has not been fully understood, evidence suggests that an imbalance of pro and anti-inflammatory cytokines, as well as the generation of abnormal autoantibodies, play a critical role in disease pathogenesis (7). An increasing number of research have suggested that pro-inflammatory cytokines, such as interleukin IL-27, IL-29, IL-35, and tumor necrosis factor TNF-α, may be associated with the development of GD (6, 18) while the overproduction of pro-inflammatory cytokines such IL-17, IL-6, IL-22 and TNF-α plays an essential role in the pathogenesis of Hashimoto's thyroiditis (HT) (23). Accordingly, a recent study revealed that newly diagnosed, untreated HT patients have higher serum levels of IL-22 than healthy controls (25). Interleukin-27 is heterodimeric cytokine comprising of Epstein–Barr virus (EBV)-induced gene 3 (EBI3)-soluble receptor subunit and non-covalently associated p28 helical subunit (14). The major sources of IL-27 are antigen-presenting cells (APC) including macrophage, monocytes, and dendritic cells. IL-27, which appeared to have both pro-inflammatory and anti-inflammatory properties although it was once believed that this cytokine contributed to the development of T helper (Th1) responses, it is now known that IL-27 can directly alter the effector functions of CD4+ and CD8+ T cells. These modifications eventually trigger the production of IL-10 and the development of specialized regulatory T (Treg)cell responses (18). The *IL-27* gene is located on chromosome 16p12.1-p11.2, And consists of five exon, this gene has several single nucleotide polymorphisms (SNPs) in main regions such as promoter regions or in regulatory sequences, influencing disease susceptibility and evolution (2). Single nucleotide polymorphisms (SNPs), one of the many different forms of mutations, are responsible for 90% of all human genetic variations . Among various polymorphisms, the majority of investigations have focused on

the rs153109 SNP and the associations of this SNP with many diseases for instance: Behcet's disease (BD) and systemic lupus erythematosus (SLE) (5,20).

MATERIALS AND METHODS

Patients and controls: A case control study was performed on 120 patients with auto immune thyroid disease and 60 individuals as healthy control (HC). Samples were collected from the specialized center for endocrinology and diabetes located in Baghdad, Iraq during the period from November 15, 2021, to March 20, 2022. the patients were divided into two groups according to the symptoms and thyroid function tests. The first group included 60 patients (18 males,42 females) with Hashimoto's disease and the second group referred to Graves' disease within 60 patients (31 males, 29 females). Information regarding age, gender, clinical sign. In addition to the laboratory tests which including, thyroid function TSH, T4, T3 levels and autoantibodies test anti-thyroid peroxidase (anti-TPO), anti-thyroglobulin (anti-TG), TSH-R levels. Inclusion criteria implicate patients with positive anti -TPO or anti -TG, normal or abnormal thyroid hormone levels for Hashimoto patients in addition to these criteria, the RTSH test was positive for Graves' disease group. individuals in the control group (20 males, 40 females) are defined by their, age, gender, thyroid function, autoantibodies test. the control group includes healthy subjects that have normal ranges of all the test above, as well as those who do not suffer from immune disease. The protocol of the study was approved by the Ethics Committee at the Iraqi Ministry of Health and Environment.

Serum level of IL-27: Five milliliters of blood were obtained by venipuncture and distributed into gel clot tubes (3-5mL). The blood was held at 4°C to coagulate for about 25 minutes. The clotted blood was centrifuged at 3000 rpm for 15 min and the collected serum was frozen (-20 °C) after being distributed into a plane tube until used to assessment serum levels of (IL-27), which determined using enzyme-linked immunosorbent assay kits ELISA (Cat. No**.:** MBS702498 my bio source, China) and all the directions provided by the manufacturer were followed.

Genotyping of *IL-27 gene* rs153109 SNP by High-Resolution Melting (HRM) analysis: The genomic DNA was extracted from frozen blood by using Easy Pure® Genomic DNA Kit (TransGen, biotech. EE101-01) The purity and concentration of the sample are then assessed by using both a Nanodrop spectrophotometer 2000c (Thermo Fisher Sci. USA) and agarose gels . the primers sequences for SNP IL-27 gene rs153109 were designed according to their reference sequence (rs) in the database of NCBI, the University Code of Student Conduct (UCSC) and Primer 3plus, V4 programs were used to design the primers and synthesized by Alpha DNA Ltd (Canada) and kept lyophilized. The sequences of primers employed in this study experiments are displayed in Table (1). The detection of genetic variation in *IL-27* gene SNP (rs153109) was accomplished by using High-resolution melting (HRM) which is a closed-tube method, that measures the fluorescence reduction of intercalating dye during the dissociation of double- stranded DNA (4). qPCR-HRM was performed by using a Rotor Real-time PCR sys. (Qiagen software 2.3.1) The PCR reaction mix of 20 µL final volume was prepared to include 10 µL EasyTaq® PCR Super Mix (TransGen, biotech. EE101-01), 1µL forward primer (10 µM), 1µL reverse primer (10 µM), 3 µL of genomic DNA (100 ng/ µL) and 5 µL nuclease-free water. The PCR conditions were programmed as follows: initial denaturation at 94°C for 5 mins (One cycle), followed by 35 cycles of denaturation at 94°C for 5 sec, annealing at 56°C for 15 sec, and extension at 72°C for 20 sec, followed by one cycle of a final extension step at HRM analysis with ramping by 0.2sec for 1 degree from 55 to 95 °C. The resulting output of thermocycler of the three genotypes is shown in (Fig. 1)

Table 1. The study's designed primers for *IL-27* gene rs153109

Primer	Sequence (5′→3′ direction)	Product size (bp)	Ta(°C)
Forward	**GTCAGTGACCAGGATCGGG**	**84**	**56**
Reverse	**ATTCTTGGACCTGGTTGAGC**		

Statistical analysis: Allele and genotype frequencies were provided as numbers and percentages after testing for agreement with Hardy-Weinberg equilibrium (HWE), using Pearson's Chi -square test. Significant differences in allele and genotype frequencies between patients and controls were assessed using Fisher's exact test with two-tailed probability. The relationship between *IL-27* gene SNP and AITD was expressed in terms of odds ratio (OR) with a confidence interval (CI estimate at 95%) (https://www.medcalc.org/calc/odds_ratio.php). The statistical analysis was performed using IBM SPSS Statistics 28.0 (Armonk, NY: IBM Corp). The median value and Interquartile interval IQR range for the continuous variables with deviations from normal distribution (non-Parametric variables) were measured, and the Kruskal Wallis test was used to evaluate significant differences between medians. Receiver operating curve (ROC) analysis was used to determine the area under the curve (AUC), 95% confidence interval (CI), cut-off value, specificity, and sensitivity. The cut-off value was optimized using the Youden index. A P-value <0.05 was considered statistically significant.

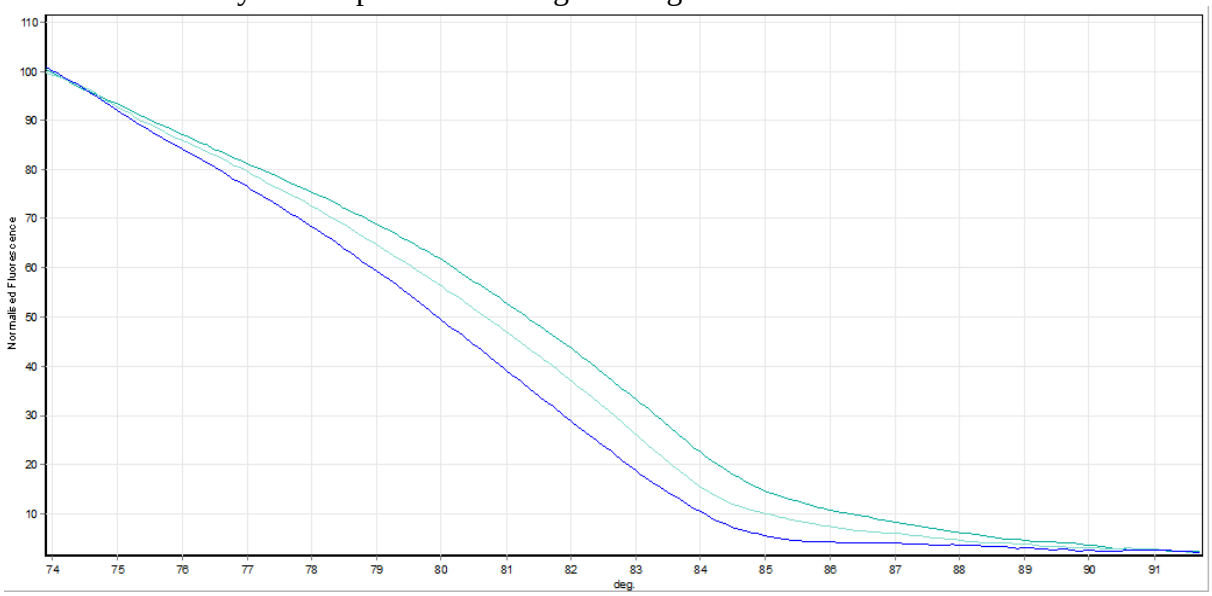

Figure 1. The result output of HRM for the three genotypes in SNP rs153109. Hetero TC: Mutant CC: Wild TT

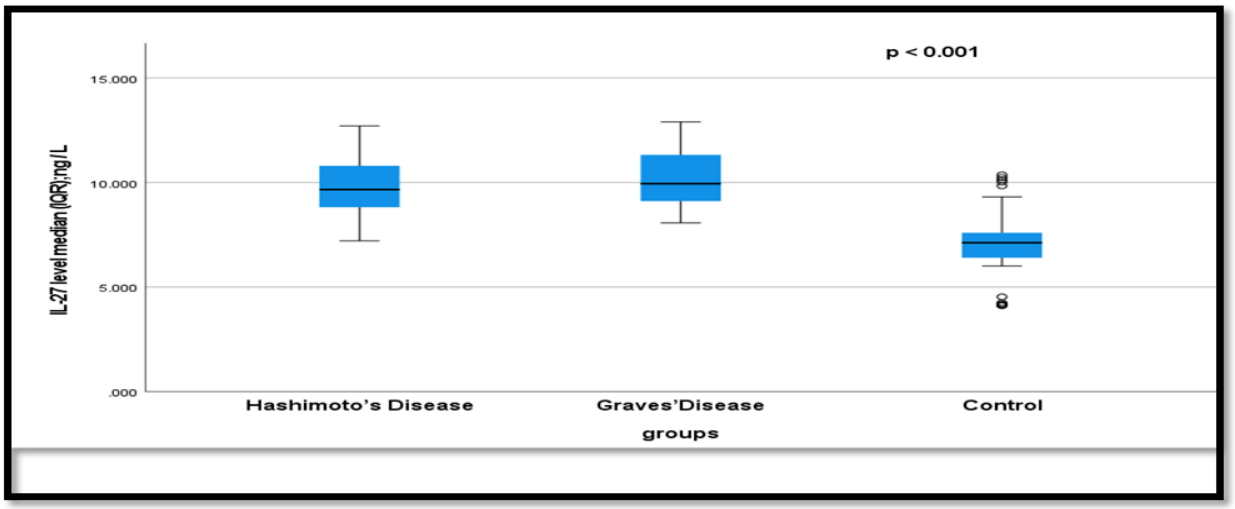

Fig.2. Boxplot presentation of IL-27 serum level in AITD patient's and controls. The horizontal line represents the median while interquartile interval range (vertical line). (p< 0.001).

RESULTS AND DISCUSSION

Serum level of IL-27: Cytokines play a significant role in the course of autoimmune diseases by participating in the induction and effector phases of all inflammatory and immunological responses. Excess decreased, or incorrect cytokine responses all have a critical role in the development of autoimmune inflammation (10). Among these cytokines is IL-27, the analysis of the median level of the

IL-27 cytokine in the studied individuals revealed a significant difference between patients and controls. the level of IL-27 was significantly higher in patients compared to healthy controls, with a slight increase in GD patients compared to HT patients (HT 9.66 [IQR: 2.09], GD. 9.94[IQR: 2.23] and HC 7.12 [IQR: 1.22] ng/L; p- value < 0.001) as showed in (Fig.2). It has been shown that IL-27 has both pro and anti-inflammatory properties, among its pro-inflammatory properties, suppression of Th17 cells, limiting the generation of pro-inflammatory cytokines by CD4 T cells like IFN and antagonistic influence on IL-6 activity (21). These findings point showed that IL-27 having a potentially harmful effect that may contribute in the development of autoimmune thyroid disease. These results agree and support the pro-inflammatory properties of IL-27 as revealed in previous studies on autoimmune rheumatic diseases such as rheumatoid arthritis and Ankylosing Spondylitis (13, 19). In contrast, a recent study showed lower IL-27 serum levels in Graves' disease patients, indicating a potential anti-inflammatory effect of these cytokines as demonstrated in prior studies on several autoimmune (17). ROC curve analysis indicates the IL-27 predictive significance value (AUC = 0.811; 95% CI = 0.756–0.865; p < 0.001; Youden index = 0.49; cut-off value = 9.10 ng/L; sensitivity = 76.7.1%; specificity = 71.9%) (Fig.3). Based on the data obtained, the ROC analysis confirmed that IL-27 occupied a significant AUC (diagnostic value) in AITD; as a result, the potential of IL-27 in the progression of AITD is increased. Consistent with these findings, previous studies demonstrated the significance of IL-27 in the pathogenesis of AITD (12). It is widely believed that relationships between genes and the environment contribute in the pathophysiology of AITD. the polymorphisms of *IL27* gene have been properly studied in several autoimmune disorder. Therefore, it is plausible to hypothesize that *IL27* gene SNPs

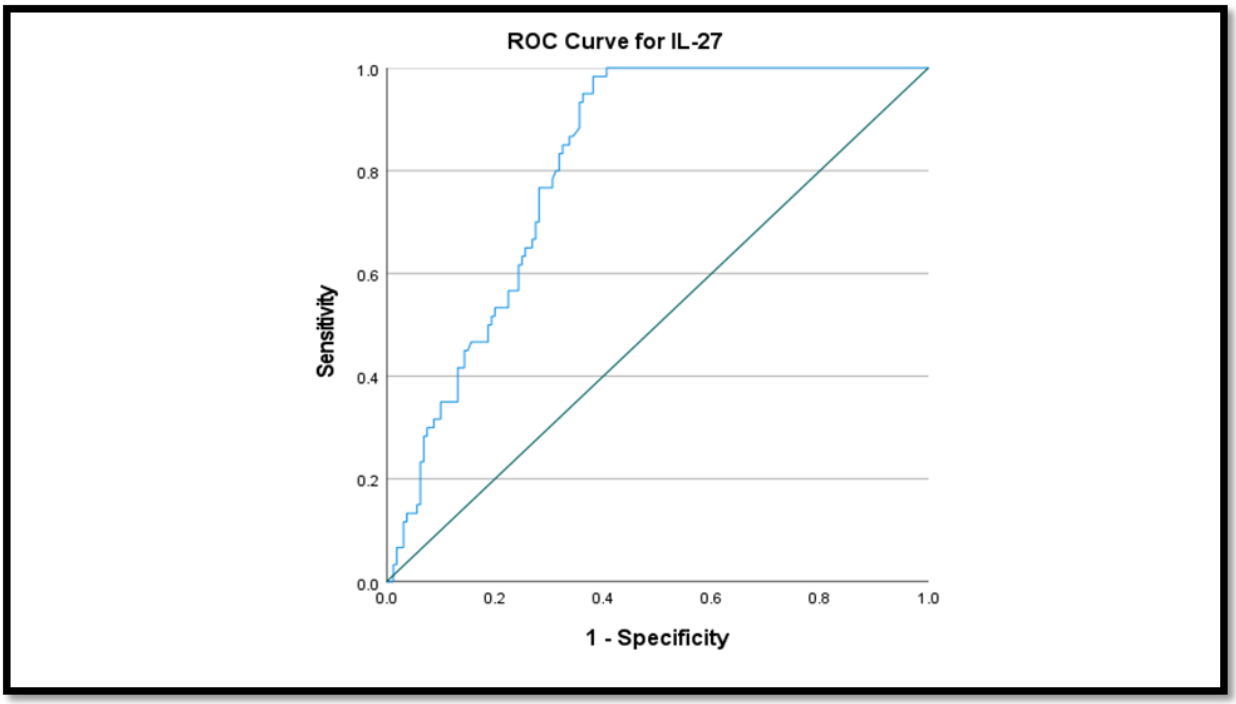

Fig. 3. Receiver operating curve (ROC) analysis of IL-27 (AUC = 0.811; 95% CI = 0.756–0.865; p < 0.001; Youden index = 0.49; cut-off value = 9.10 ng/L; sensitivity = 76.7.1%; specificity = 71.9%). in AITD patients as compared to controls

Association between *IL-27* rs153109 and AITD : The distributions of genotypes for *IL-27* rs153109 showed compatibility with the assumptions of the Hardy–Weinberg equilibrium (HWE) in the control group. as there was no significant variation between observed and expected genotype frequencies. (Table-2) may be associated with susceptibility to AITD (26). In this current study, the SNP of *IL-27* gene rs153109 were analyzed in AITD patients,

Table 2. Comparison of the genotype and allele frequencies of *IL27 gene* polymorphism (rs153109) between patient Graves group and control group and between patients Hashimoto group and control group

	Hashimoto disease n (60)		P value	Graves' disease n (60)		P value	Healthy control n (60)		P value
genotype	Observed n (%)	Expected n (%)		Observed n (%)	Expected n (%)		Observed n (%)	Expected n (%)	
TT	30 (50.0)	26.67 (44.44)		19 (31.6)	15.5 (25.84)		41 (68.3)	40.84 (68.06)	
TC	20 (33.5)	26.67 (44.44)	<0.052	23 (38.3)	29.99 (49.99)	<0.071	17 (28.3)	17.32 (28.87)	<0.884
CC	10 (16.5)	6.67 (11.11)		18 (30.0)	14.5 (24.17)		2 (3.3)	1.84 (3.06)	

the result showed that CC genotype of rs153109 had a statistically significant increased frequency in GD patients compared to controls (30.0% vs 3.3 Freq, OR=19.4 ,95% CI=0.4-9.2, P-value =0.0002). the TC genotype of rs153109 also revealed an increased frequency in GD patients compared to controls. (38.3% vs 28.3 Freq, OR=2.9,95% CI=0.6-4.9, P-value = 0.011). According to the dominant model, Individuals with the *IL-27* rs153109 variant (TC+CC) genotype had a significantly higher risk of GD (68.4% vs. 31.6 Freq, OR = 4.6, 95 % CI= 2.0–10.0, p-value =0.0001). Allele C also showed high frequency in GD patients compared to controls (49.2% vs 17.5 Freq, OR= 2.6, 95%CI= 1.2-5.4, p-value=0.01) (Table -3).

Table 3. Observed and expected number and percentage frequencies of *IL-27 gene* rs153109 genotype and their HWE in patients and controls

IL27 polymorphism rs153109	Frequencies (%) Control group (n=60)	Patients Graves Group (n=60)	P value	Odd ratio (95% CI)	Frequencies (%) Control group (n=60)	Patients Hashimoto Group (n=60)	P value	Odd ratio (95% CI)
Codominant								
TT	68.4% (n=41)	31.6 % (n=19)	---	1.00 (Reference)	68.4% (n=41)	50.0 % (n=30)	---	1.00 (Reference)
TC	28.3 % (n=17)	38.3 % (n=23)	0.011	2.9 (0.6-4.9)	28.3 % (n=17)	33.5 % (n=20)	0.24	1.6 (0.7-3.7)
CC	3.3% (n=2)	30.0 % (n=18)	0.0002	19.4 (0.4-9.2)	3.3 % (n=2)	16.5 % (n=10)	0.017	6.8 (1.3-33.4)
Dominant								
TT	68.4% (n=41)	31.6 % (n=19)	---	1.00 (Reference)	68.4% (n=41)	50.0 % (n=30)	---	1.00 (Reference)
TC+CC	31.6 % (n=19)	68.4 % (n=41)	0.0001	4.6 (2.0-10.0)	31.6 % (n=19)	50.0 % (n=30)	0.04	1.1(0.4-3.0)
Recessive								
TT+TC	96.7 % (n=58)	70.0 % (n=42)	---	1.00 (Reference)	96.7 % (n=58)	83.5 % (n=50)	---	1.00 (Reference)
CC	3.3 % (n=2)	30.0 % (n=18)	0.002	10.2 (0.2-4.6)	3.3 % (n=2)	16.5 % (n=10)	0.05	4.8(0.9-23.08)
Allele								
T	82.5 % (n=99)	50.8 % (n=61)	---	1.00 (Reference)	82.5 % (n=99)	66.7 % (80)	---	1.00 (Reference)
C	17.5 % (n=21)	49.2 % (n=59)	0.01	2.6 (1.2-5.4)	17.5 % (n=21)	33.3 % (40)	0.005	2.3(1.2-4.3)

On the other hand, the CC genotype of rs153109 revealed significantly higher frequency in HT patients than controls (16.5% vs. 3.30 Freq, OR = 6.8, 95 % CI = 1.3–33.4, p-value =0.017). but TC genotype of rs153109 showed no significant differences between HT patients and controls (33.5% vs. 28.3 Freq, OR= 1.6, 95% CI = 0.7–3.7, p-value = 0.24) According to the dominant model, Individuals with the *IL-27* rs153109 variant (TC+CC) genotype had a significantly higher risk of HT (50.0% vs 31.6 Freq, OR=1.1, 95% CI= 0.4-3.0, p-value =0.04). Additionally, Allele C of rs153109 gene polymorphism showed a significant association with the risk of HT disease (33.3% vs 17.5 Freq, OR = 2.3, 95 % CI = 1.2–4.3, p- value =0.005) (Table 3) One study has reported the association between the *IL-27* rs153109 and AITD in the Chinese Han population which found a significant correlation between the *IL-27* SNP and AITD, which is compatible with our findings and provide genetics evidence for the role of *IL27* in the pathogenesis of AITD (8).

Table 4. Comparison of serum IL-27 level in different genotypes

Genotype	TT	CT	CC	P value
Hashimoto's disease	**9.57**	**10.053**	**10.26**	**0.415**
Graves' disease	**9.67**	**10.40**	**9.14**	**0.785**
Healthy control	**6.87**	**7.11**	**7.49**	**0.805**

Impact of genotype on *IL-27* level

In order to investigate the relationship between genotype and phenotype by study the effect of *IL-27* rs153109 genotype on the serum level of IL-27 in AITD patients and control .as shown in Table 4, the results revealed there was no significant association between the genotype of rs153109 and serum IL-27 levels. Perhaps, the current study has been the first that investigated the relationship between genotype of rs153109 and serum level of IL-27 in AITD. however, another investigation has clarified this association in terms of bladder cancer (28).

CONCLUSION

the study supports the concept that the *IL-27* rs153109 genotype contributes to genetic predisposition to AITD and supports the critical role of IL27 cytokine level in AITD pathogenesis.

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