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Association Of Hashimoto'S Thyroiditis With IRGM Snps (Rs13361189 T/C, Rs4958847 G/A, And Rs10065172 C/T)In Iraqi Arab Patients.

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Abstract

Autoimmune thyroiditis including Hashimoto's thyroiditis and Graves' disease has recently been increased globally. The present study aimed to determine correlation between IRGM SNPs (rs13361189 T/C, rs4958847 G/A, and rs10065172 C/T) with the development of Hashimoto's disease in Iraqi Arab patients. For this purpose, 25 HT patients and 25 healthy subjects were enrolled in this investigation. All of them were subjected to the estimation of free thyroid hormones concentrations (ft3, ft4), TSH, thyroid autoantibodies (TPO, TG). In addition to the molecular detection of IRGM genotypes using Real Time PCR and RFLP techniques. The results found that the mutant alleles (CC and AA) were more prevalent in HT patients (20% and 16%, respectively) than in healthy controls (8% and 4%, respectively). The frequency of the heterozygote allele (TC) of the SNP (rs13361189) was higher in HT patients (48%) than in healthy subjects (40%), whereas the frequency of the heterozygote allele (GA) of the SNP (rs4958847) was equal in both groups. On the other hand, it is clear that all of the HT and healthy controls carried the (rs10065172) SNP's wild type (CC) allele. Moreover, a significant negative correlation was discovered between serum concentrations of thyroid antibodies and the IRGM SNPs (rs13361189 and rs4958847). The current study concluded that these IRGM SNPs are not associated with the development of Hashimoto's disease in Iraqi Arab patients.

Keywords: IRGM,Hashimoto's thyroiditis,SNPs, correlation.

Introduction:

Thyroid autoimmune diseases (AITDs) are many distinct clinical disorders, of which Hashimoto's hypothyroidism (HT) and Graves' hyperthyroidism are the most prominent. They reflect examples of

autoimmune organic-specific diseases (Casto et al.,2021).HT is highly abundant in female with an incidence ration of about (8:1).However, according to the positive results of laboratory test in women for occurrence of autoantibodies for thyroid, it appeared that about 10% of population are present with HT (Machała et al.,2019). In the pathogenesis, the thyroid antigens may be presented by dendritic cells as a foreign antigens to the T-cells leading to its proliferation and differentiation into thyroid-specific T-cells (Th1, Th2, and CD+8) producing different cytokines like IL-12, IL-17 and IFN-α which in turn mediate thyroid infiltration and cytotoxicity (Ramos-Leví, and Marazuela, 2016; Machała etal.,2019).Although the exact cause of AITD is unknown but the gene-environment interactions plays a critical role in the pathogenesis and progression (Wiersinga, 2016). However, studies have identified several genes that are associated with the development of AITD of these genes, the HLD-DR locus is the most frequent gene linked to the AITD susceptibility (Ramgopalet al.,2018). Other genes arethyrotropin receptor (TSHR) genes in addition tothose related to immunity likeCD40, protein tyrosine phosphatase-22 (PTPN22)(Wawrusiewicz-Kuryloneket al.,2019).

Immunity-related GTPase family M (IRGM) protein belongs to immunity-related GTPase (IRG) family of proteins, which was described first time in 1990s, and is further divided into five subfamilies namely IRGA, IRGB, IRGC, IRGD and IRGM based on homology of GTP-binding domain (Hunn et al.,2011). These genes have been shown to play a critical role in resistance to a range of intracellular pathogens that comprises Mycobacterium tuberculosis, Salmonella typhimurium, Listeria monocytogenes, Toxoplasma gondii, and Chlamydia trachomatis (Hu et al.,2020). Also, IRGM functions as an important regulator in inflammation, preventing undesired inflammation and protecting cells from oxidative stress by controlling apoptotic cell engulfment (Nath[a]et al.,2021). Recently,few researches have documented that polymorphisms of IRGM gene is linked to the development and progression of several autoimmune diseases including Graves' disease, Sjogren's syndrome and ankylosing spondylitis (Yao et al.,2018; Nath[b] et al.,2021). Moreover, there is no any Iraqi study has conducted to investigate the genetic variations of the IRGM gene among patients with AITD. In this context, the current study aimed to investigate the correlation between IRGM genotypes (rs10065172, rs4958847, and rs13361189) with Hashimoto's thyroiditis in Iraqi Arab patients.

Materials and Methods:

Study population and samples:

The current study involved (25) patients with Hashimoto's thyroiditis aging between (9-50 years old) and from both sexes. All of them have registered into specific center of diabetes and endocrine diseases in

Amar city, Iraq, during the period period Nov. 2019 to Nov. 2020.In addition, (25) apparently healthy persons from the matched age and sex were also involved and considered as a healthy control group. (5ml) of the venous blood were collected from patients and control and separated into two parts (EDTA and plane tube) for using in the serology and molecular procedure.

Estimation of thyroid hormones, TSH and thyroid antibodies:

Serum levels of the thyroid hormones free triiodothyronine (FT3), free thyroxine (FT4) and thyroid stimulating hormone (TSH) were determined in the same day of blood collection by using electrochemiluminescence immunoassay method (Cobas, comp. Penzberg, Germany). As indicated in manufacturer's instructions, the results have been expressed in IU/mL. Serum concentration of anti-Tg and anti-TPO were evaluated using chemiluminescent immunoassay (Mindray, China)As indicated in instructions of the manufacturer, the results have been recorded in IU/mL.

DNA extraction:

DNA was extracted from peripheral blood leukocytes of HT patients and healthy controls using a standardized salting-out procedureblood. DNA extraction kit(G-spin^{TM} Total DNA Extraction Mini Kit) which is specific for whole blood DNA extraction was used in the current study.

DNA amplification and genotyping:

The twoIRGM SNPs (rs10065172 C/T and rs4958847 G/A) of IRGM gene were genotyped using primers and probes specific for these SNPs. Reactions were conducted in 0.2 µl wells, in a total 25 ml volume using 2 ng of genomic DNA and a GoTaq® Probe qPCR Master Mix (Promega, USA). The wells were then positioned in a thermal cycler (Stratagene, USA) and heated for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The genotyping success rate was better than 95%, with a calculated error rate based on PCR duplicates of less than 1%. The IRGM SNP (rs13361189 T/C) was genotyped using restriction fragment length polymorphism RFLP) technique. Restriction endonuclease and buffer for the DNA manipulations were acquired from New England Biolab, USA (MluCI; catalogue number: R0538S). Restriction digests were prepared in a final volume of 40 µl, or multiples thereof, according to the appropriate purposes of digests. For preparative and analytical purposes, samples were prepared to meet the following criteria: 1-3 µg of PCR product was digested with a 1X appropriate restriction buffer and 10 units of the needed restriction enzyme. The digests then were incubated at 37°C (or enzyme appropriate temperature) for 3 hours and then subjected to electrophoresis to visualized the products.

Primer and probes used in the current study:

Table (1) showing the primers and probes used in the genotyping of the IRGM genotypes involved in the current study:

Table 1: Primers and probes used in the current study.

SNP	Primer	Sequence (5'-3')	Ta (°C)	Product size	Technique
rs13361189 T/C	**F**	5'-CTCGGTTGTCTCTAGCGTGC-3'	**56**	**365 bp**	**RFLP**
	R	5'GTTAACTTCCCTTTCTAAACTGTACC-3'			
rs10065172 C/T	**F**	5'-GTGCCTCCTATTTCTCTTC-3'	**56**	**132 bp**	**Real Time PCR**
	R	5'-CAACCATGATGAAGTCATAC-3'			
	Allele C	FAM-5'-ttccatcaggtagttctc-3'-BHQ1	**60**		
	Allele T	HEX-5'-ttccatcaagtagttctc-3'-BHQ1	**60**		
rs4958847 G/A	**F**	5'-ACTGGGAGAAGCTTTATAG-3'	**56**	**78 bp**	**Real Time PCR**
	R	5'-GAGCAATGAAATCTATTTTAGG-3'			
	Allele G	FAM-5'-tgcccaatatggctaaata-3'-BHQ1	**60**		
	Allele A	HEX-5'-tgcccaatatagctaaata-3'-BHQ1	**60**		

F: forward. R: reverse. Ta: melting temperature. bp: base pair

Statistical analysis:

Results of the current studywere analyzed using SPSS software package (performed by IBMCo.USA) as well asonlineMedCalc program (https://www.medcalc.org). Independent T Test, percentage, Pearson's correlation, and odd ratiotests were considered for the results analysis. A P-value of ≤0.05 was considered statistically significant.

Results:

The clinical characteristics of the study groups are presented in the table (2). Females with HT were significantly higher than males (P value = 0.001), however, the mean age between patients and healthy controls was insignificant. Also, HT patients showed significant increase in the levels of TSH, anti-TPO and anti-Tg, and significant decrease in the levels of FT3 as well as FT4 compared to the healthy controls.

Table 2: Clinical features of HT patients and healthy controls.

Characters	HT patients	Healthy controls	P value
Male	9 (18%)	25(50%)	0.001**
Female	41(82%)	25(50%)	
Age (Mean ±S.D)	37.00±12.56	35.23±10.40	0.236
FT3 Mean ±S.D	4.76±1.50 (pmol/L)	7.84±4.39 (pmol/L)	0.031*
FT4 (Mean ±S.D)	12.07±4.27 (pmol/L)	15.57±3.58 (pmol/L)	0.056*
TSH (Mean ±S.D)	18.53±17.60 (µIU/ml)	2.45±1.50 (µIU/ml)	0.000**
Anti-TPO (Mean ±S.D)	424.79 ± 381.90 (IU/L)	1.92 ± 1.08 (IU/L)	0.000**
Anti-Tg (Mean ±S.D)	56.76 ± 15.41 (IU/L)	3.59 ± 5.07 (IU/L)	0.000**

****results are significant at 0.01 level. *results are significant at 0.05 level. S.D: standard deviation**

The current study found the three alleles for each of the two SNPs (rs13361189 and rs4958847), but only one allele (CC the wild type) was found for the SNP (rs10065172). The distribution of the aforementioned alleles in HT patients and healthy controls is presented in the table (3). The results of the table (3) also show that mutant alleles (CC and AA) were more prevalent in HT patients (20% and 16%, respectively) than in healthy controls (8% and 4% respectively). The frequency of the heterozygote allele (TC) of the SNP (rs13361189) was higher in HT patients (48%) than in healthy subjects (40%), whereas the frequency of the heterozygote allele (GA) of the SNP (rs4958847) was equal in both groups. On the other hand, it is clear that all of the HT and healthy controls carried the (rs10065172) SNP's wild type (CC) allele. All of the above findings are insignificant suggesting that these SNPs are not associated with the development of HT disease in the current study.

Table 3: Distribution of the alleles of IRGM SNPs in HT patients and healthy controls.

IRGM SNP	Allele	HT patients No (%)	Healthy control No (%)	P value	O.D	95% CI
rs13361189	TT	8(32%)	13(52%)			
	TC	12(48%)	10(40%)	0.569	1.3846	0.4515 to 4.2465
	CC	5(20%)	2(8%)			

	T allele	20 (80%)	23(92%)	0.2358	0.3478	0.0607 to 1.9934
	C allele	5 (20%)	2(8%)			
rs4958847	GG	14(56%)	17(68%)			
	GA	7(28%)	7(28%)	1.00	1.00	0.2909 to 3.4373
	AA	4(16%)	1(4%)			
	G allele	21(84%)	24(96%)	0.1891	0.2188	0.0226 to 2.1137
	A allele	4(16%)	1(4%)			
rs10065172	CC	25 (100%)	25 (100%)			
	CT	0(0%)	0(0%)			
	TT	0(0%)	0(0%)			
	C allele	25 (100%)	25 (100%)	1.00	1.00	0.0191 to 52.3653
	T allele	0(0%)	0(0%)			

TT: wild type. TC: heterozygote. CC: mutant allele. OD: odd ratio. 95% CI: confidence intervals.

The effects of the studied IRGM SNPs (rs13361189) and (rs4958847) on thyroid autoantibody concentrations are shown in tables (4-7). Anti-TPO (table 3-33) and anti-Tg (table 3-34) levels were found to be insignificantly lower in HT patients who had the mutant (CC) and heterozygote (TC) alleles of the (rs13361189) SNP compared to those who carried the wild type (TT) allele. Anti-TPO antibodies were also considerably greater (P value = 0.003) in patients with the (TC) allele than in individuals with the (CC) genotype. The (TT) allele resulted in higher anti-TPO concentrations in healthy controls than the (TC) allele (P value = 0.036) or the (CC) allele (P value = 0.024).

Table 4: Effects of the rs13361189 T/C SNPs on the concentrations of anti-TPO in patients and healthy controls.

Hormones	**IRGM rs13361189 T/C**								
	Anti-TPO (IU/ml /L) Mean ± S.D								
	TT	**TC**	**P value**	**TT**	**CC**	**P value**	**TC**	**CC**	**P value**
HT Patients	426.78 ±390.49	423.48 ±410.49	0.323	426.78 ±390.49	236.82 ±200.36	0.116	423.48 ±410.49	236.82 ±200.36	0.003**

Healthy Control	2.31 ±1.14	2.30 ±1.33	0.036*	2.31 ±1.14	1.500 ±0.002	0.024*	2.30 ±1.33	1.500 ±0.002	0.380

****Results are significant at 0.01 level. *Results are significant at 0.05 level. S.D: standard deviation.**

Table 5: Effects of the rs13361189 T/C SNPs on the concentrations of anti-Tg in patients and healthy controls.

Hormones	**IRGM rs13361189 T/C**								
	Anti-Tg (IU/ml /L) Mean ± S.D								
	TT	**TC**	**P value**	**TT**	**CC**	**P value**	**TC**	**CC**	**P value**
HT Patients	79.77 ±50.31	42.14 ±40.98	0.046*	79.77 ±50.31	3.56 ±2.85	0.104	42.14 ±40.98	3.56 ±2.85	0.050*
Healthy controls	7.64 ±6.91	1.69 ±1.07	0.029*	7.64 ±6.91	0.50 ±0.001	0.292	1.69 ±1.07	0.50 ±0.001	0.219

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***Results are significant at 0.05 level. S.D: standard deviation.**

In HT patients who carry the mutant allele (AA), anti-TPO concentrations are significantly lower than in those who possess the wild type (GG) allele (P value = 0.014) or the homozygote (GA) allele (P value = 0.000). In comparison to the homozygote (GG) allele, the latter was similarly associated with a non-significant drop in anti-TPO levels (see table 6). Tables (7) reveal that the (rs4958847) SNP has similar effects on anti-Tg antibodies in HT patients and healthy controls. This is the first study in Iraq to explore at the impact of IRGM SNPs on anti-TPO and anti-Tg serum concentrations. All of the evidence in the current study suggested that these SNPs have no impact on the progression of HT illness in Iraqi Arab patients.

Table 6: Effects of the rs4958847 G/A SNPs on the concentrations of anti-TPO in patients and healthy controls.

Hormones	**rs4958847 G/A**								
	Anti-TPO (IU/ml) Mean ± S.D								
	GG	**GA**	**P value**	**GG**	**AA**	**P value**	**GA**	**AA**	**P value**
HT Patients	510.85	439.13	0.088	439.13	91.09	0.014**	510.85	91.09	0.000**

	±475.61	±346.94		±346.94	±69.81		±475.61	±69.81	
Healthy controls	2.81 ±1.18	1.51 ±0.68	0.020*	1.51 ±0.68	3.89 ±0.02	0.405	2.81 ±1.18	3.89 ±0.02	0.033*

****Results are significant at 0.01 level. *Results are significant at 0.05 level. S.D: standard deviation.**

Table 7: Effects of the rs4958847 G/A SNPs on the concentrations of anti-Tg in patients and healthy controls.

Hormones	**rs4958847 G/A**								
	Anti-Tg (IU/ml) Mean ± S.D								
	GG	**GA**	**P value**	**GG**	**AA**	**P value**	**GA**	**AA**	**P value**
HT Patients	30.66 ±25.58	104.17 ±100.22	0.017**	30.66 ±25.58	0.80 ±0.001	0.052*	104.17 ±100.22	0.80 ±0.001	0.112
Healthy controls	1.80 ±1.68	18.16 ±17.66	0.000**	1.80 ±1.68	0.50 ±0.001	0.472	18.16 ±17.66	0.50 ±0.001	0.036*

****Results are significant at 0.01 level. *Results are significant at 0.05 level. S.D: standard deviation.**

The results of the table (8) describe Pearson's correlation test between the thyroid autoantibodies and IRGM SNPs (rs13361189 T/C and rs4958847 G/A). The (rs13361189) SNP clearly has a negative insignificant correlation with anti-TPO (R=-0.196, P=0.171) and anti-Tg (R=-0.052, P=0.650). A negative significant correlation test result was also recorded between the second SNP (rs4958847) and anti-TPO antibodies (R=-0.296, P=0.023). However, the correlation was insignificant between the (rs4958847) SNP and anti-Tg (R=-0.230, P=0.876).

Table 8: Pearson's correlation test between thyroid antibodies and IRGM rs13361189 T/C and rs4958847 G/A SNPs.

Thyroid autoantibodies				
IRGM SNPs	**Anti-TPO**		**Anti-Tg**	
	R	**P value**	**R**	**P value**
rs13361189 T/C	-0.196	0.171	-0.052	0.722
rs4958847 G/A	-0.296*	0.039	-0.023	0.876

Correlations are significant at 0.05 level.

Discussion:

To our knowledge, this is the first study in Iraq to investigate the correlation between IRGM genotypes and HT disease. The results of this study are consistent with the results of Yao et al, who concluded that three SNPs of the IRGM gene are not associated with HT but rather they are strongly associated with the development of GD (Yao et al.,2018). Genetic studies have revealed that IRGM genetic polymorphisms are associated autoimmune diseases other than AITD. Glas et al, discovered that IRGM SNPs (s rs13371189, rs10065172, and rs1000113) increases susceptibility to Crohn's disease in German population (Glas et al.,2013). Pranculienė and colleges, stated that variants of IRGM (rs4958847) as well as NOD2 (rs2066847) were responsible for increased risk to inflammatory bowel disease (Pranculienė et al.,2016). Moreover, polymorphism IRGM have been linked to be a risk factor for gastric carcinoma and systemic lupus erythematosus (SLE), however, the role of IRGM in these diseases remains to be fully depicted (Petkova et al.,2013).

Genetic variations of the IRGM gene, on the other hand, have been linked to bacterial and viral infection. In this context, Song et al. discovered that IRGM rs10065172 was linked to active tuberculosis in a Korean population (Song et al.,2014). In a Chinese population, Lu et al. discovered that the IRGM SNPs rs10065172 and rs13361189 were protective factors against latent TB progression (Lu et al.,2016). The IRGM rs13361189 polymorphism was discovered to be linked to leprosy by influencing the production of IL-4, IL-6, and INF-γ (Yang et al.,2014). IRGM genotypes together with ATG16L1 played a minor role in the impact on the susceptibility to both mucosal and systemic Candida infections (Rosentul et al.,2014). Furthermore, most recently its concluded that IRGM (rs4958847) and (rs13361189) SNPs plays a relevant role in H. pylori-related inflammation, as, the presence of the GG genotype might lead to decreased inflammation in the gastric mucosa during H. pylori infection and thus, a lower risk of gastric cancer (Goswami, 2019).

Otherwise, genetic studies have evaluated the association between HT disease and certain genetic polymorphisms other than IRGM. The SNP (rs2276886) inside the CXCL9 gene increases susceptibility to HT illness in both Japanese and Chinese populations (Akahane et al., 2016; Mo et al.,2019). In the Chinese Han population, the rs153109 of the IL-27 gene was strongly connected with the development of GD, while the rs17855750 of the same gene was significantly associated with the development of HT (He et al.,2019). Furthermore, the thyroglobulin gene SNP (rs180195) was identified to predispose individuals to AITD (Lahooti et al.,2017), while Kherrour and colleagues revealed that

variants of the inositol hexaphosphate kinase 3 gene reduced the HT risk in Algerian people (Kherrour et al.,2020). Similarly, the genotype (rs822339) frequency of the PD-L1 gene was not different in patients with AITD compared with healthy controls (Yoon et al.,2021).

The disparities in the outcomes of the genetic research cited above could be attributable to changes in the number of study populations, environmental factors, nutrition habits, and investigation methodologies, as well as the sociodemographic characteristics of the individuals included in each study. The small number of patients and healthy controls in this study, however, is a serious disadvantage. As a result, studies with large number of participant are recommended.

Conclusion:

The current study concluded that there is negative correlation between IRGM SNPs (rs13361189 T/C, rs4958847 G/A, and rs10065172 C/T). Due to the negative results of Pearson's correlation test, it can be concluded that the existence of these SNPs in the genome may act as a protective factors against the development of Hashimoto's thyroiditis in Iraqi Arab patients.

Conflict of Interest:

All authors declare that there is no conflict of interest.

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