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Association of HLA-DRB1*04 gene with Hashimoto's thyroiditis among Iraqi-Kurdish population in Erbil province

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Abstract

Background and objective: Hashimoto's thyroiditis is one of the common autoimmune thyroid diseases with increasing incidence in the general population. It has been suggested that a combination of genetic and environmental factors increase the risk of developing the disease. The current study aimed to find the association between HLA-DRB1*04 gene with Hashimoto's Thyroiditis among the Iraqi-Kurdish population in Erbil Province.

Methods: The case-control study was conducted on 45 untreated patients with Hashimoto's thyroiditis who have already been diagnosed and 45 control subjects. Blood specimens were taken from the subjects for gene detection purposes via conventional polymerase chain reaction. Sera specimens were used to run enzyme linked immunosorbent assay to measure the level of IFNγ.

Results: The age of subjects ranged from 32 to 63 years with no significant difference between mean ±SE of cases (46.822±1.087 years) with the mean ±SE (47.044 ±1.162 years) of the control subjects (P = 0.999). The mean ±SE of free T3 and T4 levels was lower in cases in comparison to those of controls, while the mean ±SE of TSH level in cases was significantly higher compared to those of controls. Statistically there was a highly significant difference in the mean ±SE of free T3, free T4 and TSH levels between cases and controls (P <0.001). Furthermore, the mean ±SE of anti-TPO, anti-Tg and IFNγ levels was higher in cases compared to those of control subjects. Statistical analysis shows a highly significant difference between mean ±SE of anti-TPO and anti-Tg antibody levels of cases compared to those of controls (P <0.001). As for IFNγ level, there was a significant difference between mean±SE of IFNγ levels in cases and controls (P = 0.021). The conventional polymerase chain reaction results showed that 39/45 (86.7%) of cases were tested positive for HLA-DRB1*04, while only 20/45 (44.04 %) of the control subjects tested positive for the gene, statistical analysis revealed a highly significant association between the existence of the gene with HT disease (P <0.001).

Conclusion: The study revealed a highly significant association between the existence of HLA-DRB1*04 gene and Hashimoto's thyroiditis disease among the Iraqi-Kurdish population in Erbil Province.

Keywords: Hashimoto's thyroiditis; HLA-DRB1*04; Hypothyroidism.

Introduction

Hashimoto's thyroiditis is an organ specific autoimmune disease belonging to the spectrum of autoimmune thyroid disease, which is characterized by the clinical manifestation of hypothyroidism.[1] It was first described in 1912 by Japanese physician Hakaru Hashimoto.[2] Hashimoto's thyroiditis occurs when immunocytes target thyrocytes resulting in a clinical syndrome.[3] The clinical manifestation of HT varies and it may present as a self-limiting disease to a more aggressive form of the disease manifested as hypothyroidism with or

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without as goiter.[4] In the early stages of the disease, lymphocytes infiltrate the thyroid tissue generating an inflammatory reaction.[5] As time passed chronic inflammation results in extensive damage to the thyroid tissue and ultimately the scarred tissue is replaced by connective tissue, thyroid dysfunction and hypothyroidism is resulted.[6]

Anti-thyroglobulin (TgAb) antibodies were first observed in the body of patients with Hashimoto's in 1956 by Doniach and Roitt.[7] In Hashimoto's thyroiditis, thyroglobulin molecules are one of the main targets for thyroid autoantibodies. The anti-thyroglobulin antibodies (TgAb) combine with thyroglobulin to form Ag–Ab complexes which are recognized by B and T cells. This is followed by their proliferation and differentiation and ultimately autoreactive immune response.[8,9] It has been known that TgAbs are incapable of triggering the complement system and therefore play a minor role in the process of HT pathogenies and thyroid tissue destruction.[10]

Thyroid peroxidase (TPO) is one of the principal enzymes involved in thyroid hormone synthesis, whose antigens are expressed at the apical part of the follicular cells, and it is one of the main targets for thyroid autoantibodies known as the anti-thyroid peroxidase antibodies (anti-TPOAbs) which belong to immunoglobulin class G.[11] The Ag-Ab complex formed from the interaction of TPO with anti-TPOAb enhances complement reactions resulting in the destruction of thyrocytes and a significant biological impact on the process of HT pathogenesis.[9]

The precise mechanisms by which the immune system loses its tolerance toward the thyroid gland are still being studied. Studies have reported there is an interaction among several genes and environmental factors that might potentially associate with the development of HT.[5]

Aim

This study aimed to investigate the frequency of HLA-DRB1*04 gene in patients with Hashimoto's thyroiditis in comparison to control subjects among Iraqi -Kurdish population in Erbil Province.

Methods

The current case-control study was conducted on a total of 45 untreated patients (5 males and 40 females) who have already been diagnosed with Hashimoto's thyroiditis by specialized physicians (endocrinologists), who attended the Razen clinical compound in Erbil Province. The patients were not on treatment and their ages ranged between (32-62) years. Following their permission, the patients were given a specially designed questionnaire form, and they were interviewed. Personal information and medical history including nationality, gender, age, residency, family history of thyroid disease, the sign and symptoms, and the results of thyroid function test along with anti-thyroid antibodies were recorded. Patients younger than 15 years old and pregnant females were excluded. The inclusion criterion for the participants was to be an adult Iraqi-Kurdish citizen in Erbil Province. The control subjects were included based on their clinical and laboratory investigations confirming that they were free from HT, and their information was recorded the same way as patients.

Specimen Collection

Peripheral blood specimens were collected following standard phlebotomy procedure. The blood samples were transferred in two tubes: an EDTA tube for DNA extraction and gene detection, and a gel tube, the gel tubes were used to produce sera by centrifugation at 3000 rpm for 15 minutes. The sera were transferred into 1.5 ml Eppendorf tubes and along with EDTA tubes were stored at -80ºC before their use to perform sandwich ELISA and conventional PCR.

Methods

Serum samples were added into the Eppendorf and placed into the sample rack, after that applicated the Cobas

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(Cobas Integra 400 Roche company) for measurement of TSH, free T4, free T3, anti -TPO and anti-Tg antibodies levels.

IFNγ levels were detected using Sandwich-ELISA as a method (Sunlog biotech ELISA kit), and the results were measured quantitatively using an ELISA reader at (450 nm) absorbance according to the manufacturer's instructions.

The normal range of IFNγ levels in the current investigation according to the manufacturer's instructions was 1.2 pg/ml - 80 pg/ml.

The whole blood samples were used for DNA extraction using a genomic DNA extraction Kit (BETA BAYERN genomic DNA extraction Kit-Germany), followed by amplification of the HLA-DRB1*04 gene by conventional polymerase chain reaction (PCR) using sequence specific forward and reverse primers as shown in Table 1. The primers were previously designed and used by other researchers.[12]

Polymerase chain reaction

The whole blood samples were used for DNA extraction using a genomic DNA extraction Kit (BETA BAYERN genomic DNA extraction Kit-Germany), followed by amplification of the HLA-DRB1*04 gene by conventional polymerase chain reaction (PCR) using sequence specific forward and reverse primers as shown in Table 1. The PCR reaction tubes were contained 12.5 µL PCR master mix (AMPLIQON Taq 2× master mix DENMARK), 0.3 µL of each forward and reverse primers, 2 µL template DNA, and 8.9 µL free-nuclease water. The PCR tubes were placed in thermal cycler, which was set at 35 cycles under the reaction condition of (94°C for 3 minutes for initial denaturation, 94°C for 30 seconds for denaturation, 58°C for 40 seconds for annealing, 72°C for 60 seconds for elongation step, and 72°C for 5 minutes for last elongation cycle). Following amplification, the PCR products were run on 2% agarose gel for analysis.

Ethical statement

The research proposal was submitted to the ethics committee of the College of Health Sciences /Hawler Medical University, and official permission was obtained from Razen clinical compound in Erbil City for the purpose of sample collection. The involved individuals were informed about the aim of the study and following their agreements and permission they were enrolled. The participants were ensured that their identities would be kept anonymous.

All the subjects were included voluntarily, and they were given the right to decline participation in the study.

Statistical analysis

The data were analyzed using statistical package for the social sciences program software SPSS (version 25). The findings of the study were analyzed through means and standard error, independent samples t-test, and Chi-Square. A probability (*P*) value of less than 0.05 was considered statistically significant, and P value of less than 0.01 being considered highly significant**,** while *P* values greater than 0.05 was considered statistically insignificant.

Table 1 Sequence-specific primers used for HLA-DRB1*04 amplifications.[13]

Gene	Primer sequences		Product size: base pair (bp)
	Foreword 5′→3′	Reverse 5′→3′	
DRB1*04	GTTTCTTGGAGCAGGTTAAACA	CTGCACTGTGAAGCTCTCAC	260

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Results

This case-control study was done on 45 Iraqi Kurdish patients who were already diagnosed with HT and another group 45 healthy subjects as controls from Erbil Province. The age mean ±SE of the participants was (46.822±1.087 years) and (47.044±1.162 years) for the cases and controls respectively as shown in Table 4, statistical analysis showed no significant difference between age mean ± SE of the HT patients in comparison to those of the control group (P = 0.889).

Concerning gender, 11.1% (5/45) of subjects from both case and control groups were males, and 88.89% (40/45) of them were females as shown in Table 2.

As for residency, the majority of HT patients were from the city center representing (80%) of the HT cases, the rest (20%) residing in the surrounding of Erbil city. Statistical analysis showed no significant association between where the patients reside with the HT development (P = 0.327). When it comes to family history, HT patients had a greater percentage of relatives with thyroid diseases compared to the control subjects, but still the statistical analysis showed no significant assiociation between family history and the disease development (P = 0.090).

Age distribution of cases and controls with the mean age

Table 3 reveals that the age mean ±SE years of cases and controls were (46.822±1.087 years) and (47.044±1.162 years) respectively, with the minimum and maximum age being 32 years for both groups and (62) and (63) years for cases and controls respectively. Statistically, there was no significant difference between the age distribution of the two groups (P = 0.999).

Table 2 Baseline demographic characteristics among HT patients and controls.

Characteristics	HT group No. 45	Control group No. 45	*P* value
Age /years (Mean ±SE)	46.8±1.087	47.0 ±1.162	0.999
Gender No. (%)			
Male	5 (11.1%)	5 (11.1%)	1.000
Female	40 (88.9%)	40 (88.9%)	
Residency No. (%)			
City Centre	36(80.0%)	32 (71.1%)	0.327
Surrounding	9 (20.0%)	13 (28.9%)	
Family history No. (%)			
Yes	24(53.3%)	16 (35.6%)	0.090
No	21(46.7%)	29 (64.4%)	

Table 3 Age distribution of cases and controls.

	Age distribution							
	Group	Frequency	Mean	Std. Deviation	Std. Error Mean	Min	Max	*P* value
Age (year)	Case	45	46.822	7.293	1.087	32	62	0.999
	Control	45	47.044	7.798	1.162	32	63	

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The differences in serum levels of thyroid hormones FT3, FT4, and TSH

Table 4 shows that the mean ±SE of free T3 and T4 levels was lower in cases in comparison to those of controls, while the mean ±SE of TSH level in cases was significantly higher compared to those of controls. Statistically there was a highly significant difference in the mean ± SE of free T3, free T4 and TSH levels between cases and controls (P <0.001).

The differences in serum levels of anti-TPO, anti-Tg antibodies, and IFNγ

Table 5 shows that the mean ±SE of anti-TPO, anti-Tg and IFNγ levels was higher in cases compared to those of control subjects. Statistical analysis shows a highly significant difference between mean ±SE of anti-TPO and anti-Tg antibody levels of cases compared to those of controls (P <0.001). Moreover, there was also a significant difference between mean ±SE of IFNγ levels in cases and controls (P = 0.021).

Table 4 The differences in serum levels of thyroid hormones FT3, FT4, TSH.

Test	Case Mean ±SE	Control Mean ±SE	*P* value
Free T3	2.516±0.039	6.015±0.909	<0.001
Free T4	8.668±0.214	15.537±0.335	< 0.001
TSH	5.845±0.106	2.573±0.157	< 0.001

Table 5 The differences in serum levels of anti-thyroid antibodies and IFNγ.

Test	Cases Mean ±SE	Control Mean ±SE	*P* value
Anti-TPO	64.311±2.101	13.844±0.872	< 0.001
Anti-Tg	144.622±2.061	19.111±0.966	< 0.001
IFNγ	98.288±4.176	78.299±7.384	0.021

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Frequency of HLA-DRB1*04 gene among case and control groups

Figure 1 shows the PCR products of HT patients run on (2%) Agarose gel electrophoresis. The bright bands show 216 bp PCR product size assigned as positive results for the HLA-DRB1*04 gene in HT patients.

Figure 2 shows that 39/45 (86.7%) of cases were tested positive for HLA-DRB1*04 gene, while only 6/45 (13.3%) of them showed negative results for the target gene's existence. Regarding the controls, 20/45 (44.40%) of them tested positive for HLA-DRB1*04 gene, while the other 25/45 (55.6%) of them showed negative results for the existence of the gene. Statistical analysis revealed a highly significant association between the existence of the gene and the HT disease (P <0.001).

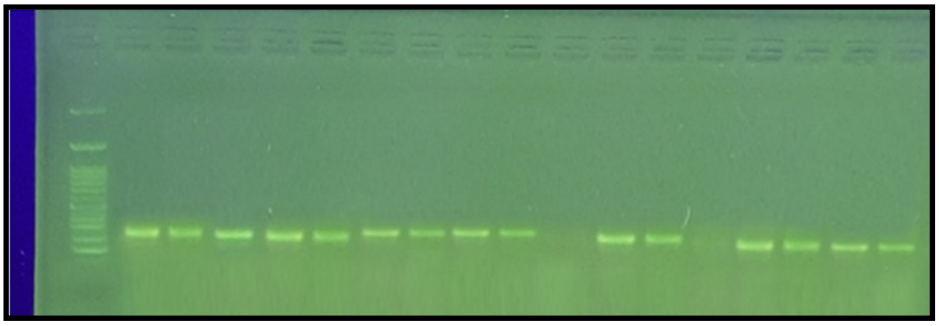

Figure 1 An image of a gel post electrophoresis, the bright bands of 216 base pair PCR product size assigned as positive results for the HLA-DRB1*04 gene in 17 case subjects.

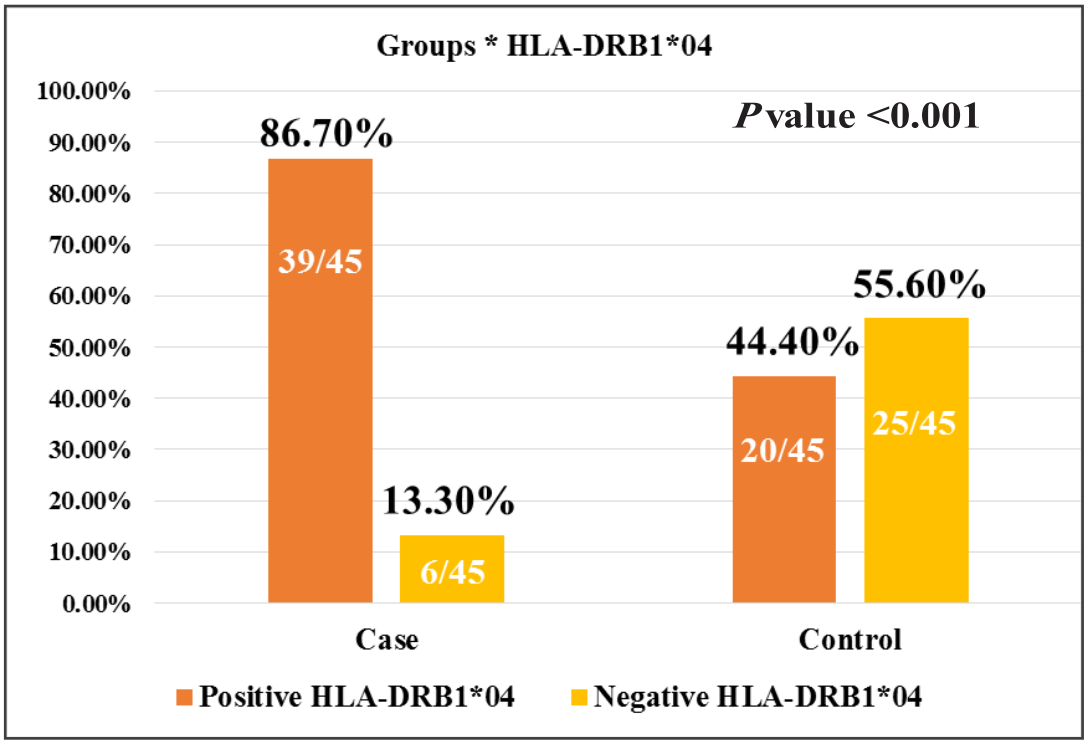

Figure 2 Frequency of HLA-DRB1*04 gene among case and control groups.

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Discussion

Hashimoto's thyroiditis which is characterized by the clinical manifestation of hypothyroidism, is an organ specific autoimmune disease belonging to the spectrum of autoimmune thyroid disease.[2] It is believed to be caused by a combination of genetic and environmental factors.[5]

Studies have observed a remarkable rise in thyroid diseases in Iraq over the last few decades, among these diseases, Hashimoto's thyroiditis was shown to be considerably increased. Al-Hashimi AM who had conducted a cohort study on 79 Iraqi citizens with goiter showed that approximately 6.3% of the affected individuals were actually having HT.[13]

Studies have demonstrated that the risk of HT development raises with age, a study done by Pyzik et al.[14] shows that the highest rate of the disease was detected between the age of 45 to 65 years which is in accordance with our results.

Concerning gender, females made up the vast majority of the HT patients in our study. It has been known that females are more likely to develop autoimmune disorders which could be due to the extra X chromosome which harbors a variety of genes linked to the immune system, and hormonal influences.[15,16] A study done by Siriweera and Ratnatunga[17] reveals that (91.12%) of a total 349 HT cases were females, and this finding confirms our results which show that (88.8%) of HT cases were female.

As for the residency of the subjects, the results revealed that the majority of the patients were from the city center with no significant association between the patient's residence with the HT development (P = 0.327). This might be caused by increased exposure to various household and industrial chemicals along with air pollution in urban areas. A case control study done by de Freitas et al.[18] concludes that the prevalence and risk of HT was significantly greater among individuals residing in petrochemical complex areas compared to those in control areas.

It has been suggested that HT like other autoimmune diseases run in family as genetic susceptibility. Although a greater percentage of cases did have family history of thyroid diseases compared to control subjects but still the statistical analysis shows no significant association between family history and the disease development (P = 0.090). This could be due to the small sample size of the current study, or possibly the included cases were unaware of having the familial history for thyroid diseases. In contrast to our results, a study by Kust and Matesa [19] revealed that subjects who have family history of the thyroid diseases are more likely to get the HT compared to those with no family history, with a significant difference of (P = 0.026).

Regarding the thyroid function test, there were highly significant differences between the mean ±SE of free T3, free T4 and TSH levels of patients in comparison to the healthy subjects (P <0.001). Our results are similar to the findings of Kawasaki et al.[20] which revealed highly significant differences (P <0.01) in the mean ±SE of FT3, FT4 and TSH levels between the cases and control.

Studies have reported that more than 90% of patients with HT develop anti-TPO antibodies, and to a lesser extent anti-Tg antibodies (found in 80% of HT cases), which predominantly belong to class G immunoglobulin.[11] The anti-thyroid antibody tests of the HT patients showed a significant increase in the levels of anti-TPO and anti-Tg antibodies with highly significant differences between the mean ±SE in the levels of anti-Tg and anti-TPO antibodies of the patients as compared to the control subjects

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(*P* <0.001). Our results are similar to the findings of a study by Abdullah et al.[21] which revealed a highly significant difference between the mean±SE in the levels of anti-thyroid antibodies of cases compared to those of normal controls (*P* <0.001).

It has been reported that IFNγ along with other inflammatory mediators plays a vital role in the initiation and progression of HT by increasing the expression of class II MHC on thyrocytes.[22] It promotes the development of anti-thyroid antibodies and induces apoptosis of thyrocytes mediated by the cellular immune system (Th1 cells).[23]

The results of this study show that the serum level of IFNγ was higher in HT patients compared to control subjects with a significant difference in the mean ±SE of IFNγ level of the cases in comparison to those of the control subjects (*P* value = 0.021). Our results are similar toa study done by Bossowski et al.[23] which showed a considerable increase in the levels of IFNγ among HT patient compared to those of control subjects with significant difference between mean ±SE of IFNγ levels of both groups (*P* <0.001).

When it comes to risk factors of autoimmune thyroid diseases, very few etiological studies have been conducted on the genes associated with Hashimoto's thyroiditis, partially because of the traditional hypothesis that states HT is genetically identical to Graves' disease.[24]

The HLA-DRB1 genes belong to MHC class II, which involves in antigen presentation by encoding proteins expressed on the surface of antigen presenting cells. These proteins bind to foreign peptides and present them to other immunocytes for recognition, leading to immunological reactions.[25] In autoimmune thyroid diseases HLAs which presents self-antigens to immunocytes inducing immunological responses against host cells.[26] It has been reported that certain HLA alleles were over-expressed and could exhibit a great affinity toward self-antigens found in thyroid gland leading to autoimmune reactions against thyroid gland.[27] HLA-DRB1*04 has been suggested to be associated with a number of different autoimmune diseases including HT.[25,26] Since this complex genetic locus is highly polymorphic in human genome, it has revealed some ethnic and racial differences in genetic association researches.[28] This study aimed to investigate the association of HLA-DRB1*04 with HT among Kurds.

Our results from Figure (2) shows that the frequency of the HLA-DRB1*04 gene was significantly higher in HT patients compared to the control subjects (*P* <0.001). This finding is confirmed by the results of a study conducted by Kokaraki et al.[29] In Greece which reported that the prevalence of HLA-DRB1*04 was significantly greater in HT patients in comparison to control subjects (*P* <0.0001). A similar study by Ramgopal et al.[30] showed that HLA-DRB1*04 synergically with several other genes was significantly associated with HT patients (*P* = 0.008), while DRB1*10 and DRB1*03 seemed to play protective roles. Another study done by Choe et al.[31] reported HLADR-B1*04, HLA-DRB1*46, HLA-Cw*01 to be associated with HT in Korean population, while HLADR-B1*01 presented a protective factor for the disease.

In Caucasians, various HLA alleles have been reported to be associated with HT, including HLA-DRB1*04-DQB1*0301 haplotypes.[32] HLA-DR4 (HLA-DRB1*04, HLA-DQB1*03, HLA-DQA1*03) haplotypes (*P* = 0.002), while HLA-DRB1*13 (HLA-DRB1*13, HLA -DQB1*06, HLA-DQA1*01) have a showed protective role (*P* = 0.001).[33] In Japanese population, HLA-A*02:07 and

HLA-DRB1*04 were reported to be genetic risk factors to HT.[35]

Conclusion

Based on our findings, individuals with HLA-DRB1*04 gene in their genome are more likely to develop Hashimoto's thyroiditis.

Funding

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Competing interests

The authors declare that they have no competing interests.

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