Association of Human Leukocyte Antigens Class I & II with Graves' Disease in Iranian Population

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ABSTRACT

Background: Graves' disease (GD), a highly rampant autoimmune disorder of the thyroid gland, is responsible for 60-80% of the clinical cases of hyperthyroidism. Over the past decades, genetic association studies have identified several GD susceptibility loci in CTLA-4, TSHR and major histocompatibility complex regions. The information on the association between the human leukocyte antigens (HLA) and GD among Iranians is scarce. **Objective**: To identify HLA polymorphisms that might confer susceptibility or protect against GD. **Methods**: Eighty unrelated patients with a confirmed diagnosis of GD were included in the case group. The control group consisted of 180 unrelated healthy individuals with normal thyroid function tests. The polymerase chain reaction with sequence specific primers (PCR-SSP) method was used for HLA typing. **Results:** Frequencies of HLA-A*68 (15.6% vs. 4.2%, p=0.004) and B*08 (8.8% vs. 2.5, p=0.030) were significantly higher in patients with GD compared with healthy controls. No patients with GD had HLA-A*33, whereas it was found in 7.0% of the controls (p=0.011). HLA-DQB1*0201 was significantly less frequent among patients with GD (15.6% vs. 26.8%, p=0.040). Additionally, patients with GD were significantly less bound to have HLA-DQA1*0201 (6.2% vs. 15.1%, p=0.045). Concerning allelic distributions, no noticeable difference was found between GD patients with and without Graves' ophthalmopathy (p>0.05 in all cases). **Conclusion**: In the Iranian population, HLA-A*68 and -B*08 confer susceptibility to GD, whereas HLA-A*33, -DQB1*0201, and -DQA1*0201 appear to have protective roles.

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INTRODUCTION

Graves' disease (GD), a disorder of the thyroid gland is responsible for 60-80% of clinical cases of hyperthyroidism and is one of the most prevalent autoimmune diseases (1). The diagnosis of GD is made in the presence of a diffusely enlarged thyroid gland, thyrotoxicosis, and a possible involvement of the connective tissues around the orbits and in the skin (2). Aberrant production of activating antibodies that bind thyroid stimulating hormone receptor (TSHR), initiates the disease process which culminates in lymphocytic infiltrations of the thyroid gland by both B and T cells (3).

As in other autoimmune disorders, the etiology of autoimmune thyroid diseases including GD is multifactorial, influenced by the intricate interplay of genetic and environmental factors (4). The findings of a large twin study suggested that 79% of the susceptibility to GD can be ascribed to genetic factors (5). Indeed, genetic association research over the past decades has identified several GD susceptibility loci in CTLA-4, TSHR, and major histocompatibility complex (MHC) regions (6-8).

The MHC region, located on the short arm of the chromosome 6, harbors over 200 genes which encode class I and II human leukocyte antigens (HLA) (9). Polymorphism in HLA-DR3 was the first allele in the MHC region identified to confer susceptibility to GD (4). Since then, numerous additional polymorphisms in both classes of HLA have been linked to an augmented risk of GD. It should be noted that the MHC region is highly polymorphic and under immense levels of selection pressure (10,11). Consequently, HLA polymorphisms and their prevalence rates vary substantially across populations, elucidating why findings from association studies in select populations fail to be corroborated in other populations with different ethnic and genetic backgrounds. The analysis of genetic diversity among 11 ethnic groups in Iran, using HLA class II polymorphism, has shown that while all the included ethnicities are closely related, they are distinctly separate from other Asian and European populations (12). This underscores the need for population-specific studies on the association between HLA polymorphisms and GD. Accordingly, in the present study, the objective was to identify HLA polymorphism that might confer susceptibility or protect against GD in Iranian population. We further looked into the possible relationship between HLA alleles and the risk of Graves' ophthalmopathy among Iranian GD patients.

MATERIALS AND METHODS

Patients. We enrolled patients with GD who referred to Thyroid Clinic of Vali-Asr Hospital (A teaching hospital affiliated with Tehran University of Medical Sciences) between January 2014 and January 2015. A total of 80 patients with a confirmed diagnosis of GD were included and served as the case group. Additionally, a group of 180 unrelated healthy individuals with normal thyroid function tests comprised the control group. The diagnosis of GD was made by an experienced endocrinologist (A.E) if all the following three criteria were met: (1) The presence of signs and symptoms of thyrotoxicosis (e.g. palpitation, diaphoresis, weight loss, tremor, irritability, sleep disturbances, and muscle weakness), (2) a physical examination indicative of a diffusely firm and enlarged thyroid gland. If the physical examination or imaging (ultrasonography or radionuclide scan) suggested an etiology other than GD (i.e. hot nodule or toxic multi-nodular goiter), the patient was excluded; (3) thyroid function test

showing elevated concentrations of T4 (>160 nmol/L) and/or T3 (>2.9 nmol/L) along with a suppressed concentration of thyroid stimulating hormone (TSH<0.30 mU/L). In doubtful cases, serum concentrations of anti-TSH receptor antibodies and anti-thyroid peroxidase antibodies (anti-TPO) were also measured. Patients were excluded if they were concomitantly diagnosed with another autoimmune condition (e.g. type 1 diabetes, vitiligo), or were pregnant or lactating. Graves' ophthalmopathy (GO) was diagnosed clinically using the American Thyroid Association NO SPECS criteria. Patients considered to have GO were in class 3 (proptosis), class 4 (involvement of extraocular muscles), class 5 (involvement of the cornea), and class 6 (involvement of the optic nerve causing vision loss).

Written informed consent was obtained from all participants prior to enrollment. All procedures dealing with human subjects were conducted in accordance with the latest revision of the Helsinki Declaration. Ethics committee of Tehran University of Medical Sciences also approved the study protocol.

HLA typing. 5 ml of venous blood was drawn from each patient and was sent to the Immunogenetics Lab in EDTA-vacutainers. Genomic DNA was extracted using the standard salting-out method. For HLA typing, the polymerase chain reaction with sequence specific primers (PCR-SSP) method was used. PCR-SSP kits low to intermediate resolution and Taq DNA polymerase were purchased from CTS (Heidelberg, Germany) and Roche (Basel, Switzerland), respectively. Primers with low and intermediate resolution were employed for amplification and typing of HLA class I alleles (24-A alleles and 48-B alleles mix), and class II (24-DRB1 alleles mix, -DRB3, -DRB4, -DRB5, 11-DQA1 alleles and 13-DQB1 alleles mix). The reaction mixtures were prepared in a final solution of 10 µl and amplified with an initial denaturation at 94°C (2 minutes), followed by 10 cycles of denaturation at 94°C (15 seconds), and annealing and extension at 65°C (60 seconds). Samples underwent an additional 20 cycles of denaturation at 94°C (10 seconds), annealing at 61°C (50 seconds), and extension at 72°C (30 seconds). The final PCR product was run on 2% agarose gel electrophoresis and then documented using an ultraviolet-transilluminator. The specific and internal control bands were interpreted according to the kit instructions manual.

Statistical Analysis. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 17.0 (SPSS Inc., Chicago, IL). Two-by-two contingency tables were created to elucidate the frequency of each allele between case and control groups and between GD patients with and without GO. The difference in the frequency of each allele was evaluated through the use of the Chi square (or Fisher's exact test where necessary). In each test, OR (95% CI) was calculated to specify GD (or GO). Accordingly, an OR<1.0 (provided that both upper and lower intervals are also <1.0) suggests the protective role of allele, whereas an OR>1.0 (provided that both upper and lower intervals are >1.0 as well) indicates that the allele in question increases the odds of GD (or GO). The threshold for statistical significance was determined as $p<0.05$ after adjusting p values using Benjamin-Hochberg method for multiple comparisons. Developed by Benjamini and Hochberg in 1995, this method is a conservative and robust technique for the adjustment of p value in multiple independent tests in a sample where the probability of type 1 error exponentially increases (13). The study population did not deviate from Hardy-Weinberg equilibrium in HLA class I and II loci**.**

RESULTS

Eighty patients with GD and 180 healthy controls were included in the final analysis.

Table 1. Frequency of HLA class I alleles in patients with Graves' disease and healthy controls.

	Graves' Disease		Healthy Controls		Odds ratio	
	(2N=160 alleles)		(2N=358 alleles)		(95% CI)	Pc
	N	%	N	%		
HLA-A*						
01	20	12.50	22	6.14	2.18 (1.15-4.12)	0.056*
02	30	18.76	63	17.59	1.08(0.69-1.75)	0.892
03	21	13.12	58	16.2	0.78 (0.46-1.34)	0.712
11	16	10.00	28	7.82	1.31 (0.69-2.49)	0.712
23	0	0	6	1.67	---	0.712
24	23	14.38	44	12.29	1.20 (0.70-2.06)	0.514
26	8	5.00	22	6.14	0.80 (0.35-1.85)	0.808
29	7	4.38	6	1.67	2.68 (0.89-8.12)	0.394
30	2	1.25	33	9.21	0.12 (0.03-0.53)	0.004*
31	2	1.25	4	1.11	1.12 (0.20-6.18)	0.970
32	3	1.87	25	6.98	0.25 (0.08-0.86)	0.480
33	0	0	25	6.98	---	0.011*
36	0	0	2	0.55	---	0.970
66	3	1.87	3	0.83	2.26 (0.45-11.33)	0.712
68	25	15.62	15	4.18	4.23 (2.17-8.28)	0.004*
69	0	0	2	0.55	---	0.970
HLA-B						
07	10	6.25	7	1.95	3.34 (1.25-8.95)	0.165
08	14	8.81	9	2.51	3.72(1.57-8.78)	0.030*
13	5	3.12	18	5.02	0.61(0.22-1.67)	0.980
14	5	3.12	12	3.35	0.93(0.32-2.69)	0.890
15	6	3.75	11	3.07	1.24(0.45-3.42)	0.890
18	9	3.75	11	3.07	1.90 (0.77-4.69)	0.825
27	1	0.62	7	1.95	0.31 (0.04-2.58)	0.890
35	23	14.41	73	20.39	0.65 (0.39-1.09)	0.825
37	1	0.62	3	0.83	0.74 (0.08-7.21)	0.980
38	6	3.75	17	4.74	0.78(0.30-2.02)	0.980
39	0	0	3	0.83	---	0.980
40	6	3.75	22	6.14	0.59 (0.24-1.50)	0.953
41	6	3.75	8	2.23	1.70 (0.58-5.00)	0.980
44	5	3.12	7	1.95	1.62(0.50-5.18)	0.980
45	0	0	3	0.83	---	0.980
47	1	0.62	4	1.11	0.56(0.06-5.02)	0.980
48	1	0.62	0	0	---	0.980
49	3	1.87	6	1.67	1.12 (0.28-4.54)	0.980
50	11	6.87	20	5.58	1.25(0.58-2.67)	0.890
51	33	20.61	54	15.08	1.46 (0.90-2.36)	0.825
52	5	3.12	19	5.3	0.58(0.21-1.57)	0.953
53	4	2.55	12	3.35	0.74(0.23-2.33)	0.980
54	1	0.62	2	0.55	1.12(0.10-12.44)	0.980
55	1	0.62	8	2.23	0.27 (0.03-2.22)	0.953
57	0	0	5	1.39	---	0.980
58	2	1.25	13	3.63	0.34(0.07-1.51)	0.825
73	0	0	2	0.55	---	0.980

(*) Statistically significant associations are marked with an asterisk.

The average age of the sample was 38 ± 10 years, comparable between the case and control groups (p>0.05). The proportion of women in the case and control groups were 56.3% and 34.6%, respectively (p=0.001). Frequencies of HLA-A and –B alleles in the case and control groups are presented in Table 1.

Table 2. Frequency of HLA class II alleles in patients with Graves' disease and healthy controls.

	Graves' disease		Healthy Controls			
	(2N=160 alleles)		(2N=358 alleles)		Odds ratio	Pc
	N	%	N	%	(95% CI)	
HLA-DRB1*						
01	11	6.87	28	7.82	0.87 (0.42-1.79)	0.960
03	18	11.21	39	10.89	1.04(0.57-1.87)	0.960
04	17	10.62	36	10.05	1.06 (0.58-1.96)	0.960
07	11	6.87	55	15.36	0.41(0.21-0.80)	0.112
08	4	2.51	6	1.67	1.50 (0.42-5.41)	0.960
09	4	2.51	3	0.83	3.03 (0.67-13.72)	0.840
10	4	2.51	9	2.51	0.99 (0.30-3.28)	0.960
11	36	22.5	72	20.11	1.15 (0.73-1.81)	0.960
12	1	0.62	0	0	---	0.960
13	17	10.62	35	9.81	1.09(0.59-2.02)	0.770
14	8	5.00	15	4.18	1.20 (0.50-2.90)	0.960
15	12	7.51	36	10.05	0.72 (0.37-1.43)	0.944
16	17	10.62	24	6.71	1.65 (0.86-3.17)	0.677
DRB3	80	50	161	45	1.22 (0.84-1.78)	0.925
DRB4	31	19.4	94	26.3	0.67 (0.43-1.07)	0.677
DRB5	29	18.1	60	16.8	1.07(0.66-1.75)	0.770
HLA-DQB1*						
01:01	2	1.25	0	0	---	0.364
02:01	25	15.62	96	26.81	0.50 (0.31-0.82)	0.040*
03:01	43	26.87	77	21.50	1.34 (0.87-2.06)	0.483
03:02	16	10.01	34	9.49	1.06(0.57-1.98)	0.920
04:01	4	2.51	6	1.67	1.50 (0.42-5.41)	0.899
05:01	38	23.75	77	21.50	1.14(0.73-1.77)	0.899
06:01	32	20.01	66	18.43	1.11(0.69-1.77)	0.899
06:04	0	0	2	0.55	---	0.920
HLA-DQA1*						
01:01	9	3.75	27	7.54	0.73 (0.33-1.59)	0.802
01:02	26	16.25	60	16.75	0.96 (0.58-1.59)	0.960
01:03	23	14.37	35	9.77	1.55(0.88-2.72)	0.562
01:04	12	7.51	25	6.98	1.08 (0.53-2.21)	0.960
02:01	10	6.25	54	15.08	0.37 (0.19-0.76)	0.045*
03:01	19	11.87	36	10.05	1.20 (0.69-2.17)	0.802
04:01	4	2.51	6	1.67	1.50 (0.42-5.41)	0.802
05:01	56	35.01	115	32.12	1.14(0.77-1.69)	0.802
05:02	1	0.62	0	0	---	0.960

(*) Statistically significant associations are marked with an asterisk.

The frequency of HLA-A*68 was significantly higher in patients with GD compared with healthy controls (15.6% vs. 4.2%, p=0.004). HLA-B*08 frequency was also

significantly higher among patients with GD (8.8% vs. 2.5%, p=0.030). On the other hand, no patient with GD possessed HLA-A*33, whereas it was found in 7.0% of the healthy controls (p=0.011), suggesting that its presence might be associated with a decreased likelihood of having GD.

Table 2 compares the frequencies of HLA class II alleles. HLA-DQB1*0201 was recognizably less frequent among patients with GD (15.6% vs. 26.8%, p=0.040). Furthermore, GD patients were significantly less likely to have HLA-DQA1*0201 (6.2% vs. 15.1%, p=0.045), once more suggesting that this allele might confer protection against GD (Table 2).

Further investigated in this research was the association of HLA polymorphisms with the risk of developing GO among patients with GD (Tables 3 and 4). Forty-five patients with GD were diagnosed with GO, yielding a prevalence rate of 56.2%. No significant results were obtained from the comparison of allele frequencies between GD patients with and without GO (p>0.05 for all tests). HLA class I and II alleles conferred neither susceptibility to, nor protection against GO**.**

DISCUSSION

In the present case-control study, using PCR-SSP method, we aimed at investigating the association of HLA class I and II polymorphisms with GD and GO in a sample of Iranian individuals. It was observed that the frequencies of the five alleles (three class I and two class II alleles) were significantly different between the case and control groups. Frequencies of HLA-A*68 and HLA-B*08 were noticeably higher among patients with GD, compared with healthy controls, hence associated with an increased risk of GD. On the other hand, frequencies of HLA-A*33, HLA-DQB1*0201, and HLA-DQA1*0201 were significantly higher in the control population, indicating that these alleles might bear protection against autoimmune hyperthyroidism.

HLA-DQA1*0201, it was found, emerged as a protective allele for GD. Possessed by 15.1% of the controls; this allele was found merely in 6.2% of the patients. In line with our findings, Wong surawat *et al.* (2006) also demonstrated that HLA-DQA1*0201 might confer protection against GD. In their assessment of Thai population, HLA-class II allele frequencies were compared across 124 patients with GD and 124 healthy controls. HLA-DQA1*0201 was identified in 2.0% and 7.7% of the case and control groups, respectively (OR, 95% CI: 0.25, 0.08-0.72) (14). Other alleles reported herein have not been previously linked to GD in association studies. Iran, geographically located at the heart of the Silk Road, has been a gateway to many immigrant populations from Asia and Europe throughout history, resulting in an appreciable admixture level of genetic pool between the population of Iran and Eurasia (15,16). Nevertheless, it has been shown that while the population of Iran might be ethnically diverse and differences of moderate size are observed across ethnic groups, such diversity does not lead to marked genetic heterogeneity (12). Although immigration, interbreeding, and selection pressure, due to infectious agents, have entailed the diversification of highly polymorphous genes, Iranian ethnicities still share significant similarities as far as the polymorphisms of the MHC region genes are concerned. Therefore, the genetic signature of the Iranian population remains unique (12), highlighting the need for population-specific studies for the evaluation of the role of HLA polymorphisms in

autoimmune diseases, and explaining why in the present report, no previously-identified polymorphisms were found to predispose to, or protect against GD.

In the current study, no significant associations were observed between HLA polymorphisms and GO, which stands contrary to several studies that have shown significant links between polymorphisms in both class I and II alleles and susceptibility to GO (17). To curb the effect of type 1 error (i.e. to erringly identify the chance differences between the case and control groups as true positive), the Benjamini-Hochberg procedure is employed for p value adjustments in multiple comparisons. However, strict adjustment of p values with the aim of decreasing type 1 error inevitably culminates in an increase in the rate of type 2 error (i.e. adoption of the null hypothesis of no difference where there exists a true difference in allele frequencies between the two groups). If we were to abandon the adjustment procedure, the difference in the frequencies of six alleles would pass the threshold for statistical significance (data not shown). It appears that our study was not robust enough to identify true differences of small size between the case and control groups; consequently, the lack of significant findings in the subset of patients with and without GO should be interpreted with caution. Studies of larger sample size are clearly needed to further investigate the relationship between HLA polymorphisms and GO in the Iranian population. A major hindrance pertinent to all studies (the present is no exception) evaluating the associations between HLA allele polymorphisms and GD is the robust linkage disequilibrium between HLA alleles and undefined alleles of neighboring loci which may exert the primary effect (18,19). For instance, in a case-control study of 871 patients with GD and 621 controls, Simmonds *et al.* (2005) demonstrated that although allelic polymorphisms in three HLA class II loci (-DRB1, -DQB1, and -DQA1) were associated with GD, the association seemed to be explained by either DRB1 or DQA1, but not by DQB1 (20). Their analysis indicated that strong associations might merely reflect a strong level of linkage disequilibrium rather than a causal relationship (20). To surmount such a limitation, additional functional studies are required to scrutinize the associations between different alleles and GD and to determine the existence of a causal link between allelic polymorphisms and the development of thyroid autoimmunity. Despite such limitations, our study was the first to investigate the association between HLA polymorphisms and GD in an Iranian population. We conclude that HLA-A*68 and -B*08 confer susceptibility to GD whereas HLA-A*33, -DQB1*0201, and -DQA1*0201 appear to have a protective role.

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