

The Association of HLA-A, B and DRB1 with Buerger's Disease

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Abstract

Background: Thromboangiitis obliterans (TAO), also known as Burger's disease, is a devastating disease affecting the arteries and veins of the upper and lower distal limbs most commonly afflicting young male smokers of low socioeconomic status. The expression of human leukocyte antigen (HLA)-A, B and -DRB1 genes have been implicated in the pathogenesis of TAO. Our study aimed to examine the association of different HLA-A, B and -DRB1 genes in TAO patients in the Iranian population.

Methods: A case-control study examining 55 Iranian patients with TAO and 500 healthy subjects was performed in Imam Reza hospital, Mashhad, Iran. The prevalence of major histocompatibility complex (MHC) class I (-A, -B) and class II (-DRB) alleles were determined for each participant.

Results: Our results revealed the *HLA-A*03* (odds ratio [OR]=5.394), *HLA-A*24* (OR=5.143), *HLA-A*31* (OR=4.251), *HLA-A*11* (OR=3.034), *HLA-B*27* (OR=6.680), *HLA-B*15* (OR=3.959), *HLA-B*07* (OR=3.698), *HLA-B*51* (OR=3.370), *HLA-B*44* (OR=3.326), *HLA-DRB1*16* (OR=20.583), *HLA-DRB1*04* (OR=8.960), *HLA-DRB1*14* (OR=3.746), *HLA-DRB1*03* (OR=2.303), and *HLA-DRB1*15* (OR=2.111) alleles to occur at a significantly higher frequency in TAO patients compared to controls ($p < 0.05$). The *HLA-A*25*, *HLA-A*66*, *HLA-DRB1*08*, *HLA-DRB1*10*, and *HLA-DRB1*12* alleles resulted in infinite OR, and was associated with an increased risk of TAO. However, the alleles *HLA-A*30*, *HLA-B*08*, *HLA-B*45*, *HLA-B*46*, and *HLA-B*53* were associated with a protective role against TAO with an OR = 0.

Conclusions: This is the first study examining the *HLA* pattern in patients with Burger's disease in the Iranian population. Our findings have revealed an association between *HLA* class I and II alleles with TAO.

Keywords: Buerger's disease, *HLA*-DNA typing, MHC, Polymerase chain reaction, Thromboangiitis obliterans.

Introduction

Thromboangiitis obliterans (TAO), also referred to as Buerger's disease, is an episodic, sharply segmental, inflammatory and thrombotic-occlusive vascular disease with unknown etiology, most commonly affecting the small and medium-sized arteries and veins of the upper and lower distal

limbs. In the northeast of Iran, the prevalence of TAO was reported to occur in every 3 of 100,000 people. Among the different sexes, there are drastic differences in the prevalence of TAO in the Iranian population (98.7% males and 1.3% females), which is typical across many

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populations. TAO develops in patients from the age of 20 and older (average age: 40.7 ± 8.5 years) (1, 6). TAO is associated with devastating disease sequelae frequently leading to necessary amputation (1). Although the exact etiology of disease remains unclear, the development of TAO occurs most commonly in young male smokers (2). Some of the most prevalent regions with TAO include Asian and the Middle East. Furthermore, recent reports have indicated that Iran has an increased prevalence of TAO compared to western countries (3, 4). The primary risk factor for TAO is understood to be smoking (5). As the majority of patients with TAO have a history of smoking that results in the development of vasospasm and a hypercoagulable state (6). The occurrence and progression of TAO has been proved to be strongly associated with persistent smoking (7). A history of smoking along with the combination of infection, nutrition and the presence of autoimmunity are likely to be involved in the pathogenesis of TAO (8, 9). Patients who begin smoking early in adulthood mainly develop distal ischemia, ischemic ulcers, or gangrene (10).

Genetic factors have been implicated in the etiology of TAO with increased prevalence among Israeli, Indian Japanese, Southeast Asian and Middle Eastern populations, while the prevalence of TAO in African-Americans is quite rare. Additional genetic factors have been suggested in the development of TAO, including major histocompatibility complex (MHC) haplotypes (11-13). Differences in *HLA* antigens can influence the T-cell response and the subsequent levels of cytokine production. The findings by Dellalibera-Joviliano *et al.* have indicated that TAO patients possess increased levels of proinflammatory cytokines (IL-1 β , TNF- α , IL-6), Th1 cytokines (IFN- γ , IL-12), Th2 cytokines (IL-4, IL-5, IL-13), and Th17 cytokine (IL-17, IL-23). The cytokine environment in peripheral lymphoid tissues and the vasculature greatly impacts the outcome of the initial events, leading to the development of autoimmune inflammation (14).

In Iran, and much of the Middle East, evidence regarding the role of genetic susceptibility to TAO is limited. This study is the first research effort to

evaluate the relationship of *HLA* in TAO patients in Iran, and to the best of our knowledge, is the largest *HLA* study carried out.

Materials and methods

Ethical approval

This study was approved by the Medical Research Committee affiliated with Mashhad University of Medical Sciences, Mashhad, Iran. Written informed consent was obtained from each participant. The study adhered to the principles outlined in the declaration of Helsinki.

Study design

A matched case control study was performed in Imam Reza hospital, Mashhad, Iran between 2014 and 2017 to examine the frequency of MHC *HLA* class I (-A, -B) and class II (-DRB) antigens in patients diagnosed with TAO in an Eastern Iranian sample population. A total of 100 participants were recruited, 80 of which had established TAO. Patients ranged from ages 20 to 70. Each TAO patient was subjected to angiography and met the Shionoya clinical criteria for TAO (15). Exclusion criteria included any history or evidence of atherosclerosis, abnormal angiograms, and unwillingness to provide informed consent. These criteria resulted in 25 TAO patients to withdraw from the study. Among the TAO patients and control subjects, the *HLA*-A, -B, and -DRB antigens were determined by Single Specific Primer (SSP) PCR. Control data was obtained from the published study by Esmaeili *et al.* (16), who recruited a sample of 500 controls from healthy individuals in Mashhad.

Sample preparation and DNA extraction

From each participant, 2mL of blood was collected in EDTA. Following centrifugation, the buffy coat was separated then stored at -80°C . For DNA extraction, the frozen sample was thawed and underwent DNA extraction using the HISTO TYPE SSP Kits (BAG Health care, Germany). DNA extraction as well as *HLA* analysis was carried out at the Bu Ali Research Institute, Mashhad, Iran. Briefly, DNA samples were amplified by PCR using the class I *HLA*-A, -B, and -DRB primer pairs. The reaction mixture underwent 32 amplification cycles, consisting of

initial denaturation at 96 °C (300 s), followed by 5 cycles, denaturation at 96 °C (20 s) and annealing at 68 °C (60 s), followed by 10 cycles, denaturation at 96 °C (20 s), annealing at 64 °C (50 s) and extension at 72 °C (45 s), followed by 15 cycles, denaturation at 96 °C (20 s), annealing at 61 °C (50 s) and extension at 72 °C (45 s), with a final extension at 72 °C (300 s). PCR products were analyzed using agarose-gel electrophoresis at 75 V.

Statistical analysis

The Chi-square test was used to examine the statistical significance between *HLA-A*, *-B*, and *-DRB* alleles. Logistic regression analysis was used to measure the odds ratios for the association of TAO with *HLA-A*, *-B*, and *-DR* alleles. Fisher's exact test was performed for small frequencies. A P-value below 0.05 was considered statistically significant.

Results

Table 1 shows the distribution of the *HLA-A* alleles among the patient and control groups. Of *HLA-A* alleles, the most frequent subtypes were *HLA-A*03* and *HLA-A*02* among patients and healthy controls, respectively. Among the patients, the *HLA-A*03* (OR = 5.394, CI = 2.937-9.906, P < 0.001), *HLA-A*24* (OR = 5.143, CI = 2.760-9.582, P < 0.001), *HLA-A*31* (OR = 4.251, CI = 1.563-11.560, P = 0.002), and *HLA-A*11* (OR = 3.034, CI = 1.574-5.848, P = 0.001) had the highest association with the TAO. The *HLA-A*25* and *HLA-A*66* had an infinite odds ratio, and was associated with an increased the risk of disease. The *HLA-A*30*, with an OR = 0, was determined to have a protective role against TAO.

Our findings show that the *HLA-B*35*, *HLA-B*51*, and *HLA-B*18* alleles were the most frequent alleles among TAO patients and healthy

Table 1. Frequencies of class I *HLA-A* alleles in patients with Buerger's disease and controls

HLA-A	Patient's alleles (n = 55)		Control's alleles (n = 500)		OR	CI* 95%	P-Value
	N	(%)	N	(%)			
HLA-A*01	11	20.0	58	11.6	1.905	0.932-3.895	0.073
HLA-A*02	17	30.9	105	21.0	1.683	0.913-3.101	0.092
HLA-A*03	22	40.0	55	11.0	5.394	2.937-9.906	<0.001
HLA-A*11	15	27.3	55	11.0	3.034	1.574-5.848	0.001
HLA-A*24	20	36.4	50	10.0	5.143	2.760-9.582	<0.001
HLA-A*25	2	3.6	0	0.0	∞	-	0.010
HLA-A*26	3	5.4	33	6.6	0.816	0.242-2.755	0.999
HLA-A*29	3	5.4	14	2.8	2.003	-0.557-7.198	0.232
HLA-A*30	0	0.0	27	5.4	0	-	0.097
HLA-A*31	6	10.9	14	2.8	4.251	1.563-11.560	0.002
HLA-A*32	4	7.3	16	3.2	2.373	0.764-7.367	0.126
HLA-A*33	1	1.8	14	2.8	0.643	0.083-4.984	0.999
HLA-A*66	2	3.6	0	0.0	∞	-	0.010
HLA-A*68	3	5.4	29	5.8	0.937	0.276-3.182	0.999
HLA-A*69	1	1.8	3	6.0	3.068	0.314-30.010	0.342

* CI: confidence interval, OR: odds ratio

controls (Table 2). Additionally, we found that TAO appears in the patients with *HLA-B*27* (OR = 6.680, CI = 2.282-19.552, p < 0.001), *HLA-B*15*

(OR = 3.959, CI = 1.469-10.669, p = 0.004), *HLA-B*07* (OR = 3.698, CI = 1.560-8.765, p = 0.002), *HLA-B*51* (OR = 3.370, CI = 1.710-6.641, p <

0.001), and *HLA-B*44* (OR = 3.326, CI = 1.345-8.226, $p = 0.006$). The frequency of *HLA-B*07* ($p = 0.002$), *HLA-B*15* ($p = 0.004$), *HLA-B*18* ($p = 0.032$), *HLA-B*27* ($p < 0.001$), *HLA-B*35* ($p = 0.003$), *HLA-B*38* ($p = 0.006$), *HLA-B*40* ($p = 0.036$), *HLA-B*44* ($p = 0.006$), and *HLA-B*51* ($p <$

0.001) in the patient group was significantly higher than in the control group. The *HLA-B*08*, *HLA-B*45*, *HLA-B*46*, and *HLA-B*53* alleles, with an OR = 0, appear to have a protective role against TAO.

Table 2. Frequencies of class I HLA-B alleles in the TAO patients and controls

HLA-B	Patient's alleles (n = 55)		Control's alleles (n = 500)		OR	CI* 95%	P-Value
	N	(%)	N	(%)			
	<i>HLA-B*07</i>	8	14.5	22			
<i>HLA-B*08</i>	0	0.0	15	3.0	0	-	0.205
<i>HLA-B*13</i>	3	5.5	30	6.0	0.904	0.267-3.064	0.583
<i>HLA-B*14</i>	1	1.8	20	4.0	0.444	0.058-3.377	0.365
<i>HLA-B*15</i>	6	10.9	15	2.9	3.959	1.469-10.669	0.004
<i>HLA-B*18</i>	8	14.5	33	6.5	2.409	1.052-5.516	0.032
<i>HLA-B*27</i>	6	10.9	9	1.8	6.680	2.282-19.552	<0.001
<i>HLA-B*35</i>	18	32.7	82	16.4	2.480	1.346-4.568	0.003
<i>HLA-B*37</i>	1	1.8	7	1.4	1.304	0.157-10.801	0.568
<i>HLA-B*38</i>	9	16.4	31	6.1	2.960	1.328-6.598	0.006
<i>HLA-B*39</i>	1	1.8	11	2.2	0.823	0.104-6.500	0.663
<i>HLA-B*40</i>	6	10.9	22	4.3	2.660	1.030-6.875	0.036
<i>HLA-B*41</i>	1	1.8	25	5.1	0.352	0.047-2.648	0.249
<i>HLA-B*44</i>	7	12.7	21	4.3	3.326	1.345-8.226	0.006
<i>HLA-B*45</i>	0	0.0	10	0.2	0	-	0.349
<i>HLA-B*46</i>	0	0.0	5	0.1	0	-	0.592
<i>HLA-B*49</i>	4	7.3	23	4.7	1.627	0.541-4.888	0.274
<i>HLA-B*50</i>	6	10.9	27	5.4	2.145	0.844-5.449	0.101
<i>HLA-B*51</i>	14	25.5	46	9.2	3.370	1.710-6.641	<0.001
<i>HLA-B*52</i>	5	9.1	19	3.8	2.532	0.906-7.072	0.067
<i>HLA-B*53</i>	0	0.0	7	1.4	0	-	0.480
<i>HLA-B*55</i>	2	3.6	18	3.6	1.010	0.228-4.475	0.608
<i>HLA-B*57</i>	1	1.8	12	2.3	0.753	0.096-5.904	0.625
<i>HLA-B*58</i>	1	1.8	10	0.2	0.907	0.114-7.225	0.701

*CI: confidence interval, OR: odds ratio

As for *HLA-DRB* alleles presented in Table 3, the most frequent alleles among TAO and control groups were *HLA-DRB1*04* and *HLA-DRB1*15*. It was more likely that TAO occurred in patients with *HLA-DRB1*16* (OR = 20.583, CI = 7.265-58.317, $p < 0.001$), *HLA-DRB1*04* (OR = 8.960, CI = 4.943-16.240, $p < 0.001$), *HLA-DRB1*14*

(OR = 3.746, CI = 1.283-10.940, $p = 0.010$), *HLA-DRB1*03* (OR = 2.303, CI = 1.086-4.884, $p = 0.026$), and *HLA-DRB1*15* (OR = 2.111, CI = 1.162-3.837, $p = 0.013$). The *HLA-DRB1*08*, *HLA-DRB1*10*, and *HLA-DRB1*12* resulted in an infinite odds ratio, indicating an increased risk for TAO in the presence of these alleles.

Table 3. Frequencies of class II HLA-DR alleles in the TAO patients and controls

HLA-DR	Patient's alleles (n=55)		Control's alleles (n=500)		OR	CI* 95%	P-Value
	N	(%)	N	(%)			
HLA-DRB1*01	3	5.4	38	7.6	0.701	0.209-2.352	0.787
HLA-DRB1*03	10	18.2	44	8.8	2.303	1.086-4.884	0.026
HLA-DRB1*04	31	56.4	63	12.6	8.960	4.943-16.240	<0.001
HLA-DRB1*07	8	14.5	75	15.0	0.965	0.438-2.123	0.929
HLA-DRB1*08	1	1.8	0	0.0	∞	-	0.099
HLA-DRB1*09	1	1.8	6	1.2	1.525	0.180-12.902	0.520
HLA-DRB1*10	3	5.4	0	0.0	∞	-	0.001
HLA-DRB1*11	8	14.5	75	15.0	0.965	0.438-2.123	0.929
HLA-DRB1*12	1	1.8	0	0.0	∞	-	0.099
HLA-DRB1*13	9	16.4	81	16.2	1.012	0.477-2.149	0.975
HLA-DRB1*14	5	9.1	13	2.6	3.746	1.283-10.940	0.010
HLA-DRB1*15	19	34.5	100	20.0	2.111	1.162-3.837	0.013
HLA-DRB1*16	11	20.0	6	1.2	20.583	7.265-58.317	<0.001

* CI: confidence interval, OR: odds ratio

Discussion

Burger's disease, or TAO, has been well documented to mainly exist among young males under the age of 50. As this disease is extremely rare in females, there were no woman included in the TAO patient group. However, some reports have indicated TAO to occur among women, which may be related to the increase in the use of tobacco (17-20).

The HLA has been associated with several different diseases, of which, the profile of these alleles within the diseased individuals drastically varies among different populations (21). Therefore, it is important to investigate the relationship of different HLA alleles and disease among various racial groups (22). Several studies have shown there to be a statistically significant influence of specific HLA alleles and certain diseases. Thus, HLA typing can be used as a screening tool to determine the susceptibility of an individual to certain autoimmune diseases. Additionally, HLA typing can be used to help determine prognosis, as it may be involved in the development and progression of the disease (23). Despite the prevalence of TAO among smokers, the etiological role of tobacco in this medical condition remains unknown (24), as the disease is also reported in ex-smokers (25) and smokeless-tobacco users (26). As a result, recent studies have been examining additional factors, in particular, genetic predisposition and autoimmune disturbances (27-29).

Recent efforts have been shifted to DNA typing to identify the polymorphisms of the *HLA* antigens more precisely than previous serological typing. No specific *HLA* patterns have been suggested for Burger's disease in Iran. Using the PCR-SSP method, our findings have determined the most frequent subtypes of HLA to be *HLA-A*03* and *HLA-A*02* among

In a study by Jaini et al., indian patients with TAO *HLA*- presented a high frequency of the *HLA-A*02*, *HLA-A*11*, and *HLA-A*24* alleles, however, these findings were not statistically significant. But in our research, there was a statistically significant association of *HLA-A*31*, *HLA-A*03*, *HLA-A*24*, and *HLA-A*11* alleles in the Iranian population sampled. Conversely, Jain et al. found no significant correlation between antigenic frequencies of *HLA-A* and disease susceptibility (30). Among the English and Swiss population, the *HLA-A*09* antigen was reported to occur at a higher frequency in patients with TAO (31, 32). However, this trend was not observed when examining the *HLA* pattern of northeastern Iranians with TAO. These findings were corroborated in work by Zervas et al. (27). These differences in *HLA* alleles may be related to the heterogeneity of the population and TAO pathogenesis.

The DNA typing analysis of the present study confirmed an increased frequency of *HLA-B*27*, *HLA-B*15*, *HLA-B*07*, *HLA-B*51* and *HLA-B*44* alleles in TAO patients and a strong association with the disease ($OR > 3$, $p < 0.05$) in our sampled Iranian population. The *HLA-B*27* indicated the highest risk of TAO, with an $OR = 6.680$ (2.282-19.552). In a study by Puechal *et al.*, a few cases of undifferentiated *HLA-B*27* spondylarthropathies were documented in 83 patients with Buerger's disease who had a history of osteoarticular involvement (33). Additionally, our study showed *HLA-B*18*, *HLA-B*35*, *HLA-B*38*, and *HLA-B*40* ($2 < OR < 3$, $p < 0.05$) to be significantly increased in TAO patients. Jaini *et al.* investigated 21 Asian Indian patients from a lower socioeconomic background. All were males and heavy smokers (10–40 cigarettes per day). On the basis of their serological typing, it was found that *HLA-B*40* was involved in defining the peptide presenting MHC phenotype in TAO (30). These findings were consistent with our results of the 55 Iranian TAO patients. Conversely, using PCR SSOP, Aerbajinai *et al.* determined a high frequency of *HLA-B*54* among Japanese patients, which was not similar to our findings in the Iranian population (29). Through serological typing, Numano *et al.* reported similar findings (34). The increased frequency of *HLA-B*5* (27, 31) and *HLA-B*7* (27, 32) has been found in the literature on TAO. Moerloose *et al.* showed a decreased frequency of *HLA-B*12* in 46 TAO patients from Switzerland (32), while in 28 TAO patients from England, the *HLA-B*12* allele appeared to have a protective role against TAO (*i.e.*, its frequency was zero) (31). The *HLA-B*8* presented both positive (35) and negative (27) associations with the disease among 20 Austrian and 20 Greek patients, respectively. In the study by Chen *et al.*, 131 Japanese TAO patients had an increased prevalence of *HLA-B*5401* compared to controls, however this difference was not statistically significant (36). Yasuda *et al.* found a the relationship with *HLA-B*52* and TAO patients (37). Susceptibility to TAO has been proposed to be partially regulated by genes responsible for innate and adaptive immunity (20). The presence or absence of some *HLA-B* subtypes has given rise to controversy. For example, the *HLA-B*07* allele

is a dominant loci in Iran, while *HLA-B*05*, *HLA-B*12* and *HLA-B*54* were absent in our Iranian population of patients and controls. The presence of *HLA-B*52* was increased among TAO patients within our study, however, this increase was not statistically significant (9.1% *vs* 3.8%, $p = 0.067$). Interestingly, *HLA-B*08* was observed to be a protective HLA allele for TAO. For the first time, a particular *HLA* pattern of northeastern Iranians with TAO demonstrated a protective influence for the alleles *HLA-B*45*, *HLA-B*46*, and *HLA-B*53*. Conflicting evidence regarding the protective or detrimental nature of *HLA* has been observed throughout different regions (9, 27, 29, 30, 38), which may be a result of race and methodological sensitivity differences (19).

Population-based genetic approaches have indicated an increase in the frequencies of different *HLA* class II genes in TAO patients in comparison to healthy individuals (29, 32, 34, 38, 39). In our study, *HLA-DRB1*04* and *HLA-DRB1*15* showed the highest frequency in the patients and controls. The patients with *HLA-DRB1*16*, *HLA-DRB1*04*, *HLA-DRB1*14*, *HLA-DRB1*03*, and *HLA-DRB1*15* had a significantly higher risk for TAO. The *HLA-DRB1*08*, *HLA-DRB1*10*, and *HLA-DRB1*12* resulted in an infinite odds ratio. No study has yet to examine the exact same subtypes. However, Aerbajinai *et al.*, using PCR-RFLP typing, demonstrated that the frequencies of *HLA* class II genes, *DRB1*0405*, *DQB1*0401*, *DQAI*03*, and *DPB1*0501* were significantly increased in 36 Japanese patients (29). Jiani *et al.* reported that 75% of TAO patients in India carried either the *HLA-DRB1*1501*, *1502 or *1602 allele (30). Based on the existing evidence across the globe, Vijayakumar *et al.* concluded that those with TAO have an inconsistent pattern in *HLA* haplotypes and there is a large amount of heterogeneity among patients (40).

In conclusion, in patients with Buerger's disease in the Iranian population, the *HLA*-associated disease susceptibility gene was predominantly associated with four *HLA-A* subtypes (*i.e.*, *HLA-A*03*, *HLA-A*24*, *HLA-A*31*, and *HLA-A*11*), five *HLA-B* subtypes (*i.e.*, *HLA-B*27*, *HLA-B*15*, *HLA-B*07*, *HLA-B*51* and *HLA-B*44*), and five *HLA-DRB* subtypes (*i.e.*, *HLA-DRB1*16*, *HLA-DRB1*04*,

*HLA-DRB1*14*, *HLA-DRB1*03* and *HLA-DRB1*15*). The presence of antigens *HLA-A*25*, *HLA-A*66*, *HLA-DRB1*08*, *HLA-DRB1*10*, and *HLA-DRB1*12* gave an infinite odds ratio for TAO risk. These findings further emphasize the possibility that there may be an *HLA*-associated susceptibility gene(s) in TAO patients that acts in

tandem with a several environmental and immunological factors in the expression of this disease.

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