Lower Frequency of HLA-DRB1*01 in Southwestern Iranian Patients with Atherosclerosis

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ABSTRACT

Background: Human leukocyte antigen (HLA) complex is a gene family involved in antigen presentation associated with protection or susceptibility to inflammatory, infectious and autoimmune diseases. Atherosclerosis is a chronic inflammatory disease in which HLA molecules play a role in the initiation and development of the disease through presentation of self or foreign antigens to T cells. **Objective:** To investigate the association of HLA-DRB1 alleles with atherosclerosis in a sample of southwestern Iranians. **Methods:** We performed an analytical cross-sectional study involving 96 patients with atherosclerosis and 72 controls. HLA-DRB1 genotyping was performed by PCR-SSP method. **Results:** We observed a significantly lower frequency of DRB1*01 in patients with coronary artery atherosclerosis than in controls (4.68% vs. 13.1, P=0.0052, OR=3.09, CI 95%: 1.35-7.05). However, this allele showed a positive association with high blood pressure (P=0.009) in patients. Furthermore, DRB1*16 allele was associated with hyperlipidemia (P=0.008) in patients. **Conclusion:** Our results demonstrated that DRB1*01 may be a protective allele against atherosclerosis in individuals who live in southwest of Iran. The mechanism of this protection needs further investigation.

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Keywords: Atherosclerosis; Blood Pressure; HLA-DRB1; Hyperlipidemia; Inflammation

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INTRODUCTION

Human leukocyte antigens (HLAs) are highly polymorphic genes located on chromosome 6p21 (1). The HLA genes are classified into two major classes (i.e., HLA class I and class II) that are responsible for peptide presentation to CD8+ and CD4+ T cells, respectively, to elicit an effective immune response (2,3). Despite their fundamental role in the presentation of foreign antigens to T lymphocytes, certain HLA alleles are associated with specific diseases. More than 100 autoimmune diseases are reported to be linked to specific HLA alleles by presenting altered autoantigens or linkage to other causative genes (4,5). HLA association has also been reported in case of infectious diseases (6) and malignant disorders (7,8). Conversely, several alleles and haplotypes of HLA genes are protective and are associated with a lower susceptibility to different diseases (7,8).Atherosclerosis is one of the chronic inflammatory diseases in which HLA molecules and their genes should be taken into account. Atherosclerosis as a chronic inflammatory disease of arteries is considered as the leading cause of heart attack, peripheral artery disease, and carotid artery disease (9,10). Genetic factors and environmental factors have been shown to be involved in the initiation and progression of atherosclerosis (11-13). The contribution of genetic factors in atherosclerosis is about 30-60%. Besides, a large number of single nucleotide polymorphisms and more than 15 unique susceptibility loci are involved in atherosclerosis (14-16).Immune responses to several self-antigens such as oxidized LDL and heat shock proteins as well as some bacterial and viral antigens might be involved in the formation of atherosclerosis (17,18). A similar immunopathology between atherosclerosis and autoimmune diseases has also been suggested (18,19). More specifically, HLA-class II-induced T helper responses are major players in diseases (20,21). HLA-DR molecules are highly expressed by foam cells, dendritic cells, and vascular endothelial cells where self and/or non-self antigens can be presented to T cells and promote immune responses in atherosclerotic lesions (22-24).

In the present study, considering the scarcity of data regarding HLA alleles association with coronary heart diseases, we aimed to determine possible associations between HLA-DRB1 alleles and coronary artery atherosclerosis in southwestern Iranian patients.

MATERIALS AND METHODS

Subjects and Controls. In this analytical cross-sectional study, a total of 96 patients admitted to the catheterization laboratory of Namazi Hospital (Shiraz, Iran) from June 2015 to January 2016 and their disease was confirmed by angiography and participants were included after obtaining written informed consent. Angiography team consisted of two interventional cardiologist fellows as assistants and an interventional cardiologist (professor) as a supervisor in addition to a nurse and an interventional radiologist. In most patients, angiography was performed via the femoral approach (80%) and the remaining (20%) was done by the radial route. Inclusion criteria for patient enrollment were as follows: A) Equal or more than 50% stenosis in at least one of the coronary arteries; B) Lack of history of Atherosclerosis diagnosis and/or Myocardial Infarction or any cardiovascular intervention; and C) Being a native resident of Fars province. Patients with concurrent autoimmunity, chronic inflammatory diseases, infection, fever, and malignancy were excluded from the study. The protocol of this study was approved

by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz-Iran. Clinical data were collected at the time of sampling. The presence of hypertension was confirmed by studying patients' files and/or a questionnaire filled by the patients. Chronic (more than 3 measurements) systolic blood pressure (SBP) ≥130 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg as well as being applied in anti-hypertensive therapy were considered as the criterion for having hypertension (25).

A new guideline created by National Cholesterol Education Panel's (NCEP) for Adult Treatment Program-3 (ATP-III) was used for classification of schemes and treatment levels of hyperlipidemia. Based on these criteria, we registered hyperlipidemia according to the latest blood lipid measurements in patients' files (26). Control group consisted of 72 healthy individuals among which 70 were blood donors who referred to Fars Blood Transfusion Center and two of them were laboratory personnel. The genetic background of controls was considered to be associated with their place of birth (in Fars province) and the random selection was tested by Hardy-Weinberg equilibrium. They were normal healthy individuals with no history of the acute/chronic disease.

Samplings and DNA Extraction. DNA was extracted from 200 µl of peripheral blood using a column Based DNA extraction commercial kit according to the manufacturer's instructions (Genet Bio, Korea). The quality of DNA was measured using a spectrophotometer (NanoDrop spectrophotometer 2000c, 2048-element linear silicon CCD array, Thermo Fisher Scientific, USA) for more assurance.

HLA DRB1 Typing. HLA-DRB1 alleles were determined using polymerase chain reaction with sequence-specific primer (PCR-SSP) method using a commercial kit (Texas Biogen, Morgan DR typing kit) based on the manufacturer's instructions. Briefly, to make the PCR master mix, 250 µl master buffer (containing 0.15 mM dNTP, 1.5 mM MgCL2, 50 mM KCl, 15.75 mM Tris-HCl, 0.5% glycerol and cresol red) was mixed with 24 µl ddH2O, 24 µl DNA (10-50 ng/µl) and 2 µl Taq polymerase (5 U/µl, Bioflux biotech) and dispensed 12 µl into 24 wells of the Typing Tray. Typing Tray was covered with strip caps and placed on a thermal cycler (Prime thermal cycler, Techne Flexigene, 0.2 ml block, UK) with PCR Touchdown program based on the manufacturer's instructions. PCR products were visualized by electrophoresis on 2.5% agarose gel after staining with safe stain (Sinaclon, Iran). HLA-DRB1 alleles were assigned using TBG software (TBG SSPal ver. 2.5) and rechecked manually using the typing worksheet.

Statistical Analysis. Discontinuous variants (sex, angiography characteristic, and smoking status) were reported by n (%) and continuous variants (age, BMI, and lipid levels) reported by mean (mean ± SD).The frequency of DRB1 alleles and genotypes was determined by direct counting. Deviation from Hardy-Weinberg equilibrium was determined using Arlequin 3.1 software. Data were analyzed using SPSS (ver. 18, SPSS Inc, Chicago, IL, USA) and EPi Info (ver. 6). Chi-square test or Fisher's exact test was used for comparing the allele or genotype frequencies between patients and controls. The odds ratio (OR) was calculated with 95% confidence intervals (CI=95%) using MedCalc ver. 15.8 and $P<0.05$ was considered statistically significant.

RESULTS

In this study, distribution of HLA-DRB1 alleles was investigated in patients with atherosclerosis and was compared with that of the controls. The characteristics of our patients are summarized in Table 1. The patients' group included 59 (61.5%) males and

37 (38.5%) females whereas the control group was comprised of 49 (68.1%) males and 23(31.9%) females. The mean age of patients and controls was 59.65 ±10.84 (range 37-89 years) and 48.22 ± 8.9 (range 28-72 years), respectively.

Table 1. Demographic, clinical and paraclinical characteristics of atherosclerotic patients.

Variables	**patients**	**(n=96)**
Male		59 (61.5%)
Female		37 (38.5%)
Age range (yrs)		37-89
Age (yrs) (mean±SD)		59.65 ± 10.84
Body mass index (BMI) Kg/m^2		25.3 ± 4.2
Clinical and laboratory data		**Number (%)**
Hypertension		31 (32.3%)
Hyperlipidemia		37 (38.5%)
Triglyceride (mg/dl) (mean±SD)		188.6±82.6
Cholesterol (mg/dl) (mean±SD)		174.3±68.7
High density lipoprotein (mg/dl) (mean±SD)		41.7±13.4
Low density lipoprotein (mg/dl) (mean±SD)		115.1±16.9
Smoking status		**Number (%)**
Current		25 (26%)
Ex-smoker		17 (17.7%)
Never-smoker		54 (56.3%)
Angiographic characteristics		**Number (%)**
Single vessel disease		34 (35.4%)
Two vessel disease		29 (30.2%)
Three vessel disease		33 (34.4%)
Type 2 diabetes		**Number (%)**
Positive		7(7.3%)
Negative		89(92.7%)

Allele frequencies of DRB1 in 96 patients were compared to 72 healthy controls and summed up in Table 2. As shown, the frequency of DRB1*01 allele was significantly decreased in patients compared to the controls (4.69 vs. 13.2 %, P=0.0052, OR=3.09). There was no DRB1*09 allele in patients and DRB1*12 allele in controls. DRB1*11 was the most frequent allele in both patients and controls with the frequencies of 23.96% and 22.22%, respectively. Other frequent alleles in patients were DRB1*15 (15.1%), *03 (13.55%) and *16 (11.46%), while in healthy controls, more frequent alleles were *01 (13.2%), *07 (11.81%), *03 (11.11%), and *15 (10.42%).

Association of HLA alleles with clinical features of the patients is presented in Table 3. As shown, the prevalence of DRB1*01 allele in hypertensive patients (blood pressure >13/9 mmHg) was higher compared to the non-hypertensive patients (P=0.009). Moreover, the prevalence of DRB1*16 allele in patients with hyperlipidemia was higher compared to the non-hyperlipidemic patients (P=0.008).

However, we did not find any relationship between the severity of disease and any allele.

Table 2. HLA-DRB1 allele frequencies in southwestern Iranian patients with atherosclerosis compared to healthy controls.

HLA-DRB1Alleles	Patients (n=96)	Healthy Controls (n=72)	P-value	OR (CI 95%)
HLA-DRB1*01	9 (4.69%)	19 (13.2%)	**0.0052***	3.091(1.354-7.053)
HLA-DRB1*03	26 (13.55%)	16 (11.11%)	0.49	0.793 (0.408-1.541)
HLA-DRB1*04	17 (8.85%)	9 (6.25%)	0.37	0.686 (0.297-1.587)
HLA-DRB1*07	17 (8.85%)	17 (11.81%)	0.37	1.378 (0.677-2.803)
HLA-DRB1*08	2 (1.04%)	2 (1.39%)	>0.99	1.338 (0.186-9.614)
HLA-DRB1*09	0 (0%)	1 (0.69%)	0.42	1.007 (0.993-1.021)
HLA-DRB1*10	4 (2.08%)	2 (1.39%)	0.7	0.662 (0.12-3.65)
HLA-DRB1*11	46 (23.96%)	32 (22.22%)	0.7	0.907 (0.542-1.516)
HLA-DRB1*12	2 (1.04%)	0 (0%)	0.5	0.99 (0.975-1.004)
HLA-DRB1*13	9 (4.69%)	10 (6.94%)	0.37	1.517 (0. 6-3.83)
HLA-DRB1*14	9 (4.69%)	7 (4.86%)	0.94	1.309 (0.378-2.859)
HLA-DRB1*15	29 (15.1%)	15 (10.42%)	0.2	0.654 (0.336-1.271)
HLA-DRB1*16	22 (11.46%)	14 (9.72%)	0.61	0.832 (0.410-1.689)

Chi-square test or Fisher's exact test (Expected cells value less than 5) was used for comparison. Hardy-Weinberg equilibrium was determined using Arlequin 3.1 software. * = P-value<0.05.

DISCUSSION

Our results indicated that the HLA-DRB1*01 frequency was lower in patients compared to controls and this allele was associated with protection against atherosclerosis. The lower frequency of this allele among our patients may put them at a higher risk of susceptibility to atherosclerosis (in this case 3 times more than controls). A few studies that have investigated the association of HLA genes, especially HLA-DRB1, with the coronary artery disease have indicated both susceptibility and/or protective effect of these molecules in atherosclerosis (24,27-33). For instance, different alleles of DRB1 including *07 in Sweden and *12 in China were shown to have a protective role against coronary artery disease (CAD) (29,30). In contrast to our results, the DRB1*01 allele was reported to be associated with an increased risk of CAD in Finland (32) and a higher susceptibility to acute coronary disease (ACS) in China (27). However, a weak association was observed between this allele and acute myocardial infarction in Sweden (30).The apparent discrepancy between our results and other investigations may be attributed to different rates of genetic admixture among ethnic groups in Iran compared to other populations. A previous study on the genetic proximity of Iranians to the people of other countries based on HLA class II alleles showed that Iranians are close to southwest Asians, well separated from the southeast Asians, and slightly far from some European populations (34). Accordingly, the difference in DRB1 allelic distribution between Iranians and Swedish, Finish, and Chinese is not surprising. DRB1 allelic distribution was reported to be different in two Chinese populations with varied genetic background and DRB1*01 was reported as the predisposing allele to CAD just in one of these populations (27,29). Linkage of certain DRB1 alleles to causative genes in the vicinity of this locus might be considered as the second reason for the inconsistency of our results with some other reports.

Table 3. The relationship of DRB1 alleles with clinical features of patients with atherosclerosis.

HLA-DRB1 alleles	Hypertension n(%)			Hyperlipidemia n(%)				Angiography status n(%)			
	Yes (n=63)	NO (n=129)	P-value	Yes (n=79)	NO (n=103)	Missing (n=10)	P-value	SVD (n=63)	2VD (n=59)	3VD (n=70)	P-value
HLA-DRB1*01	7(11.1%)	2(1.55%)	**0.009***	2(2.53%)	6(5.83%)	1	0.76	3(4.76%)	3(5.08%)	3(4.29%)	0.97
HLA-DRB1*03	6(9.53%)	20(15.5%)	0.27	7(8.87%)	16(15.54%)	3	0.39	10(15.88%)	8(13.6%)	8(11.43%)	0.9
HLA-DRB1*04	4(6.35%)	13(10.07%)	0.59	7(8.87%)	10 (9.7%)	0	0.95	9(14.28%)	3(5.08%)	5(7.14%)	0.25
HLA-DRB1*07	6(9.53%)	11(8.53%)	0.78	9(11.39%)	7(6.8%)	1	0.12	4(6.35%)	6(10.17%)	7(10 %)	0.56
HLA-DRB1*08	0(0%)	2(1.55%)	0.82	0(0%)	2(1.94%)	0	0.69	0(0%)	1(1.69%)	1(1.43%)	0.57
HLA-DRB1*09	0(0%)	0(0%)	NA	0(0%)	0(0%)	0	NA	0(0%)	0(0%)	0(0%)	NA
HLA-DRB1*10	2(3.17%)	2(1.55%)	0.82	1(1.26%)	3(2.91%)	0	0.96	1(1.59%)	2(3.39%)	1(1.43%)	0.68
HLA-DRB1*11	18(28.57%)	28(21.7%)	0.25	17(21.52%)	26(25.24%)	3	0.86	15(23.8%)	15(25.42%)	16(22.86%)	0.88
HLA-DRB1*12	1(1.59%)	1(0.78%)	0.82	2(2.53%)	0(0%)	0	0.28	0(%)	2(3.39%)	0(0%)	0.19
HLA-DRB1*13	2(3.17%)	7(5.43%)	0.5	4(5.06%)	5(4.85%)	0	0.97	2(3.17%)	3(5.08)	4(5.71%)	0.67
HLA-DRB1*14	1(1.59%)	8(6.2%)	0.3	2(2.53%)	7(6.8%)	0	0.49	2(3.17%)	5(8.47%)	2(2.85%)	0.23
HLA-DRB1*15	8(12.7%)	21(16.28%)	0.55	12(15.19%)	16(15.54%)	1	0.6	11(17.47%)	6(10.17%)	12(17.14%)	0.45
HLA-DRB1*16	8(12.7%)	14(10.86%)	0.66	16(20.25%)	5(4.85%)	**1**	**0.008***	6(9.53%)	5(8.47%)	11(15.72%)	0.41

NA= not applicable, SVD= Single vessel disease; 2VD= Two vessel disease; 3VD=Three vessel disease. Chi-square test or Fisher's exact test (Expected cells value less than 5) was used for comparison. * = P-value <0.05.

HLA-DRB1*01 has been reported to be associated with CAD only in combination with other genes in HLA region and in the form of haplotypes. In this regard, studies in China, Sweden, and Finland reported that DRB1*01 is associated with CAD only in connection with other genes like C4A, C4B, and DQA1*01-DQB1*05 but the exact contribution of this allele in susceptibility or protection to CAD is still unknown (27,30,31). Cardiovascular diseases are a heterogeneous group of heart and blood vessels disorders including both acute and chronic diseases with or without myocardial infarction that have relatively independent clinical pathology from each other. The type of cardiovascular disease, as well as inclusion criteria for patients' allocation in different studies, might be considered as another source of inconsistency in results. Studies in Indian patients with two different cardiovascular diseases showed that different alleles were involved in different etiopathology. Interestingly, the late stage of atherosclerosis had a great impact on the association of some HLA alleles in comparison to the early stage of the disease (28). Moreover, other criteria such as gender, concurrent diabetes and/or blood pressure, smoking status, and nutrition might be important in this regard. Several studies have mentioned the gender-based association of certain HLA alleles with CAD; therefore, the male/female ratio may affect the results with unknown mechanisms (28,29,31). Other important factors that must be considered are the methodology of HLA genotyping, i.e. high, intermediate, and low resolution. Another important consideration that should be enrolled in the interpretation of different studies is related to differential significance and hierarchy of predominant HLA molecules in antigen presentation in a certain population. Considering the inflammatory status of atherosclerosis, the HLA-DRB1*01 allele may be selected in a normal population for the presentation of foreign antigenic peptides and immune response against infectious diseases throughout evolution (17). Reduced frequency of this allele in patients may predispose them to atherosclerosis as DRB1*01 was previously reported as a protective allele against infectious disease (35,36). Interestingly, the presence of this allele in our patients was associated with increased blood pressure. Investigations have demonstrated that immune system especially T cell subsets are involved in hypertension and certain HLA phenotypes are associated with increased blood pressure and severe complications (37,38). The higher prevalence of this allele in a normal population may suggest a selective advantage of this allele or neighboring alleles; otherwise, it would be accompanied by an increased risk of hypertension. Our results also suggested that HLA-DRB1*16 was associated with hyperlipidemia. Various studies presented the association of this allele with multiple autoimmune diseases such as Graves' disease, myasthenia gravis, and rheumatic heart disease (39,40). Previous works have demonstrated that CD4+ T cells of arterial plaque recognize modified self-antigen in association with HLA-DR molecules (41). In addition, many auto-antigens including those involved in lipids and cholesterol metabolism have been suspected to trigger an immune response in atherosclerosis. Antigens such as cholesteryl ester transfer protein and Apo B-100 containing lipoproteins can elicit proatherogenic T and B cell immune responses in the setting of an inflammatory state, make deregulating lipid metabolism, and induce hyperlipidemia (42). In this regard, we found that DRB1*16 was associated with hyperlipidemia in our patients. Although studies on HLA-DRB1 alleles in cardiovascular disease are scare, a previous study did not find any association between alleles of HLA-DRB1 and lipoprotein (a) (43). One limitation of our study was the small sample size of patient and control groups, which decreases the power of our study and makes finding rare alleles

more difficult. In addition, lack of information about the blood pressure, smoking status, and lipid profile of control group hampered comparison of risk factors between cases and controls. Also, it would be more accurate if we could have had a complete gender/age matching of the cases and controls. Another limitation of this study would be the ethical prohibition of performing angiography procedure in healthy individuals, which is the gold standard of confirming the lack or presence of atherosclerotic plaque in an individual.

In conclusion, our findings demonstrated that HLA-DRB1*01 could be a protective allele against atherosclerosis in our studied population. Recognition of predisposing alleles including HLA- class II will not only help in the prediction of the risk of disease in our population but also can be helpful in designing antigenic peptides to be used in atherosclerosis vaccines. The discovery of antigens, which can be presented by these alleles to T cells and involved in the atheroprevention, is currently the subject of intense scientific investigations.

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