Original Article

Evaluation of differences in HLA-DR4 gene and its subtypes prevalence among healthy people and RA patients in Isfahan province population

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Abstract

Background: There are a lot of evidences showing that genetic play an important role in RA disease. Inheritance of some subgroups of HLA-DR4 gene increases the propensity to RA disease. In this paper, the impact of HLA-DR4 gene and its subtypes or subgroups, be consistence on RA patient who lived in Isfahan province, has been evaluated.

Materials and Methods: In this survey, two groups of people (100 patients in case group and 100 normal persons in control group) have been selected. These two groups were similar in age and gender. Statistical population has been considered among people who visited Al Zahra rheumatology clinic. The participants were from Isfahan province and accepted to participate to the study voluntarily. The prevalence of HLA-DR4 and its 0401-0404 subtypes were evaluated between two groups; DNA was extracted from blood samples and studied using PCR SSCP method.

Results: It was found that 35% of RA patients had HLA-DR4 gene, of which 14 persons had 0401, 10 persons had 0404, and 11 persons had other subtypes, whereas 30 people in control group had HLA-DR4 gene, of which 10 people had 0401, 20 people had 0404, and nobody had other subtypes.

Conclusion: The observed differences between prevalence of HLA-DR4 gene between the case and control group were not statistically significant ($P = 0.45$; OR = 1.256; 95% CI = 0.69-2.27), but a relation was between HLA-DR4 0404 subtypes and RA ($P = 0.02$; OR = 0.44; 95% CI = 0.196-0.992).

Key Words: HLA-DR4 Gene, rheumatoid arthritis, 0401-0404 subtypes

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INTRODUCTION

Rheumatoid arthritis is a multi-system disease that is clarified with a symmetric polyarticular disease. It is the most common type of articular disease, which usually damages the joint and other synovial parts of body. The main symptom of RA is inflammation of synovial tissue and destruction of joint, but in spite of possibly destructive effects on human body, RA acuteness differs from one person to other. For example, some patients suffer from

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oligoarticular RA that is a self-limited disease. On the other hand, the most of patients suffer from a severe polyarticluar one. Generally, about 0.5-1% of adults suffer from RA, all over the world. The prevalence of RA is different due to geographic condition or belonging to a special race in a country. Genetic factors have influence on RA onset and its severity, but importance of these factors and their underlying mechanism is not clear. It is estimated that about one third of genetic risks of RA are located on major histocompatibility complex (MHC) and allelic variation in HLA-DR4, which is a serotype of Human Leukocyte Antigen (HLA)-DR gene, it encode chain molecule number 13 of MHC class II. The RA susceptibility mechanism of HLA-DR4 is unclear, but the considerable appeal is that the pathogenic alleles of HLA have a common amino acid sequence called shared epitop (SE) in location 70-74 of third hyper variable region of B chain of HLA-DR4 gene. The SE allele carriers are serum-positive for anti-CCP antibody, which is an indication of poor prognosis.[1] Certain shared epitope alleles, such as 0401, are correlated with disease onset and confer higher risk than other alleles, such as 0404, 0101, 1001, and 0901. Although a lot of study on HLA-DR4 and susceptibility to RA, there is not any convincing data because of different prevalence of alleles among different population. For example, the frequency of 0401 and 0404 alleles are found in about 50-70% of European people, but the most prevalent alleles in Asian are 0405 and 0901.

A survey by Da Rosa *et al*. showed that RA predisposing genes are related to MHC II (HLA-DR4).[2] These genes have influence on T-cell receptor and play an important role on presenting anti-genes for other cells. In other study, Lü *et al*. recorded that HLA-DR4 gene in Chinese RA patients is over-expressed and may have influence on RA pathogens.[3] They studied 24 RA patients and 12 healthy persons and observed that the prevalence of HLA-DR4 in normal group was about 20%, while it was about 60% in RA patients. Alvarez *et al*. showed that DR1, DR4, and DR10 have significant effects on RA outcome as well as on age of onset.[4] In other work, Lee *et al*. showed that genetic risk factors, especially PTPN22 and HLA-DR4 have influence on prognosis of RA disease.[5]

In another study by Atouf *et al*. on 49 Moroccan normal people and 49 RA patients, it is demonstrated that there is a significant difference between two groups in prevalence of HLA-DR4 gene.[6] Kim *et al*. studied prevalence of HLA-DR4 gene in 95 South Korean patients and 118 healthy people. They concluded that this factor differ from 60% in patients group to 30% in normal group.[7] Nakai *et al*. studied two groups of Japanese people, and each group consisted of 63 persons. They observed that the prevalence of HLA-DR4 is about 41.8% in control group, while this factor increases to 71% in patients' group.[8] Kapitany *et al*. studied 83 RA patients and 55 normal people from Hungary. They evaluated HLA-DR4 prevalence and noticed this factor increases from 10.9% in normal people to 32.3% in RA patients.[9]

In Iran, some researchers also have investigated HLA-DR4 prevalence among rheumatoid arthritis patients. Adib *et al*. studied 30 RA patients and 80 normal persons and showed higher HLA-DR4 prevalence in RA patients.[10] Shekarabi *et al*. surveyed the prevalence of HLA-DR4 in Iranian people living in Tehran. Among 100 normal persons and 100 RA patients, it was observed that the prevalence of this factor in normal people is about 12%; while in patients group, it was between 50% to 75%.[11] Jamshidi *et al*. looked at 110 normal persons and 110 RA patients (89 females and 21 males) and observed that HLA-DR4 prevalence in normal people is about 19.6%, and this factor increases to 30.9% in patients group.[12]

In current research, the prevalence of HLA-DR4 gene and 0401 and 0404 subtypes in normal and RA patients from Isfahan and suburbs was studied. In addition, the influence of this gene and its subtypes in susceptibility to RA disease has been analyzed.

MATERIALS AND METHODS

This study is a case-control survey that was performed in 2012 at rheumatology clinic of Al Zahra hospital of Isfahan City, Iran. In this study, 100 patients (case group) and 100 healthy persons (control group) are participated voluntarily. The case group had been selected from RA patients who are living in Isfahan province who had visited the clinic to follow up their treatment process and to change their drugs dozen. All participants of this study filled informed consent. If any other rheumatoid disease was detected in each member of case group, he or she has been dismissed of study. The control group mutually matched to case group respecting to age and gender. Men are 22 percent of case group, and women include 75 percent of another one.

About 5 ml of each volunteers' blood was sampled, and then DNA was extracted via salting out method. In salting out method, first 0.5 ml of blood sample was mixed with Buffer A cell lysis and then the

mixture centrifuged in 5000 rpm for 4 min. The sediment is mixing with 0.5 ml cell lysis. The centrifuging and mixing are repeated until a clean pellet eventuated. About 0.3 ml nuclei lysis buffer was added to the pellet tube and mixed together well. Then, leave the tube for 15 to 20 minute till the nucleus lysis. After that, 0.1 ml saturated Nacl and 0.6 ml chloroform was added to the tube and shaken it hard. The tube was centrifuged in 5000 rpm for 4 minute until three phases form. About 0.3 ml of upper phase was mixed to 0.6 ml cold saturated ethanol (100%) and shaken until the DNA coils appear. Then, the tube was centrifuged in 10000 rpm in order to DNA coils sediment. Ethanol was discharged and after washing the tube, they dry in incubator at 56°C. Finally, according to DNA amount, sterilized water was added to the tube, and it was hold in 56°C for 5 minutes.

Then, the samples were stored in -20°C temperature to perform the polymerase chain reaction (PCR) operation. PCR was performed using DR4-specific primers, DRBAMP-4 (5-GTTTCTTGGAGCAGGTTAAAC) and DRBAMP-B (5'-CCGCTGCACTGTGAAGCTCT). It was done by primary denaturation at 95°C for 15 min, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 61°C, and then 45 seconds at 74.0°C. After that, we used single strand conformational polymorphism (SSCP) method. Amount of 3 µl of PCR amplified product was mixed with 20 µl loading buffer (95% formamide, 20 mM EDTA/pH 8.0, 0.05% bromphenol blue, 0.05% xylene cyanol); then, heat denaturation was performed at 95°C for 5 minutes. After that, 3 µl of this mixture was applied on an 8% polyacrylamide gel (acrylamide: Bis = 49: 1 with 5% glycerol). Electrophoresis was performed at 25°C to 30°C for 3.5 to 4 hours at 30 mA in 45 mM Tris/pH7.6, 45 mM boric acid, 1 mM EDTA (0.5x TBE). After staining with ethidium bromide (1 µg/ml H2O) for 15 min, gels were examined under UV illumination and documented by photography.

Amount 8 µl of PCR-amplified sample was incubated with 5 U of restriction endonuclease (Sac II) in 20 µl of appropriate buffer at 37°C overnight. After digestion, 10 µl of the mixture was run on a 8% polyacrylamide gel. Hae III digested fX174 was used as a molecular weight marker.

The genome analyzed data were processed in a chi-square statistical analysis to find any significant relationship between gene and disease. In this analysis, significant level was predefined at *P* value £ 0.05. Another test called t-independent was used to investigate the some other factor such as age and gender in selecting case and control group members.

RESULTS

According to group member selection process, both the case and control group have same range of young and old people. The youngest one was 19-years-old, and the oldest member was 81-years-old. The average and deviation of age in case group was 47.6 ± 15.2 while it was 46.9 ± 14.8 for control group. The *P*-value of independent-t statistical test was calculated as 0.74, which did not show any significant difference between these groups' age distribution. The effect of slight difference in sexual distribution of case and control group has been considered by chi-square statistical criteria. The result declares that there is not any significant difference, and *P* value was calculated as 0.62. Therefore, the study population has been selected properly [Table 1].

According to the results of genotyping, which a sample of them is shown in Figure 1, among member of case control, 35 persons had HLA-DR4, of which 14 persons of them had 0401 subtype, 10 persons had 0404 subtype, and 11 persons had other HLA-DR4 subtypes. On the other hand, 30 persons of control group had this gene. Among them, 20 persons had 0401 subtype, 10 persons had 0404 subtype, and nobody had another subtypes of HLA-DDR4. The chi-square criteria showed that there is not any important relationship between RA disease and possessing HLA-DR4 gene. The *P* value was calculated as 0.45 (OR = 1.256; 95% C I = 0.69-2.27), which is greater than meaning value 0.05. But, this criterion showed a relation between

Table 1: Distribution of age and gender in study population

		Case group	Control group	*P* value
Age	Mean±S.D.	47±15.2	46±14.8	0.74
Gender	Male	22%	25%	0.62
	Female	78%	75%	

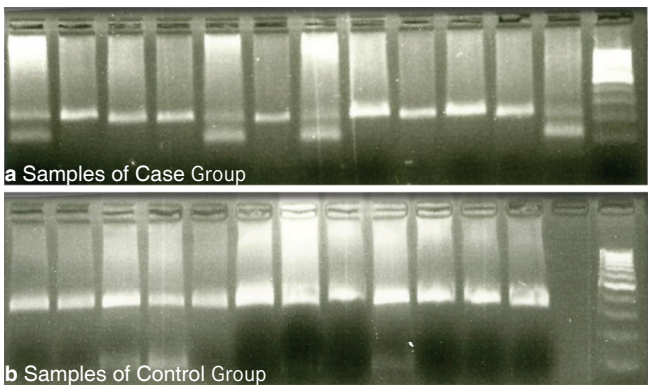

Figure 1: Representative gel of PCR-RFLP analysis

Table 2: Results of genome analysis about HLA-DR4 and its subtypes in study population

		Case group	Control group	*P* value
HLA-DR4, Negative		65	70	0.45 (OR = 1.256; 95% CI = 0.69-2.27)
HLA-DR4, Positive and its Subtypes	Total	35	30	
	0401	14	10	0401: 0.38 (OR = 1.46; 95% CI = 0.62-3.47)
	0404	10	20	0404:0.02 (OR = 0.44; 95% CI = 0.196-0.992)
	Others	11	0	

HLA-DRB1*0404 subtypes and RA, the *P* value was 0.02 (OR = 0.444; 95% CI = 0.196-0.992).

It was supposed that HLA-DR4 subtypes play a role in onset of RA. This study declared that possessing HLA-DRB1*0404 genotype increases possibility to RA onset respect to the people who do not have this genotype. Table 2 summarizes these results with statistical analysis. On the other hand, there is not any relation between HLA-DRB1*0401 and RA disease. Statistical results show *P* value equal to 0.38 (OR = 1.46; 95% CI = 0.62-3.47).

The mean onset time of disease in case group was 40.7 ± 15.7, and statistical independent-t test did not show any relation between possessing HLA-DR4 and onset time of disease. As shown in Table 3, the calculated value of *P* value would be 0.273.

Using chi-square criterion to investigate the role of sexuality in RA and its relation to HLA-DR4 gene showed that there is not any significant relation between gender and this gene in case group, instead an important association can be seen in control group [Table 4]. The prevalence of HLA-DR4 in female was higher than in male.

Table 3: Relation between HLA-DR4 and onset time of disease

	HLA-DR4, Positive	HLA-DR4, Negative	*P* value
Onset time of disease	38.09±17.1	41.8±15.5	0.273

Table 4: Result of genome analysis about HLA-DR4 and its subtypes in study population

Group	Male	Female	*P* Value
Case	28.6%	71.4%	0.09
Control	26.7%	73.3%	0.03

DISCUSSION AND CONCLUSION

In this paper, the prevalence of HLA-DR4 gene and two of its most common subtypes (0401 and 0404) have been investigated among RA patients who live in Isfahan city and suburbs comparing to healthy population. The volunteer participants are categorized in case (patients) and control (healthy) group. Healthy people were selected so that they match with case group with respect to age and gender. The selection process was validated by independent-t statistical criterion.

The chi-square criterion did not show any significant relationship between prevalence of HLA-DR4 and RA onset in case and control group. Although this study did not show any relevancy, Da Rosa *et al*. found an important relationship.[2] Also Lu *et al*. showed that HLA-DR4 inheritance play a role in RA incidence in Chinese patients and Alvarez *et al*. reached to the same results in Indian population.[3,4] Similarly, Kim *et al*. found a significant relation between HLA-DR4 and RA in Korean population.[7] Kapitany *et al*. confirmed this relation in Hungary.[9] Atouf *et al*. observed correlation of HLA-DR4 and RA disease in Moroccans.[6] In Iran, Adib *et al*., and in a distinct study, Shekarabi *et al*. found an important relation in this regards.[10,11]

Interestingly, unlike above studies and like present study, Jamshidi *et al*. did not find any relation between HLA-DR4 gene and RA disease in Tehran population.[12] Hameed *et al*. also rejected accompaniment of this gene and RA in Pakistan.[13]

Applying this criterion to investigate the role of HLA-DR4 subtypes showed that they have an important relation with RA disease. HLA-DR4 0404 subtype have a positive effect on RA onset, and HLA-DR4 0401 does not have any relation with disease. In other word, people who have 0404 subjected to a higher RA risk than the people who do not have it. Jamshidi *et al*. showed a relation between RA disease and HLA-DR4 0404 and 0408 subtypes.[12] They claimed that other subtypes, such as 0401, are irrelevant.

Tan Pl *et al*.[14] studied New Zealand Indo-European RA patients, and they showed a relationship between 0404 subtype and RA. Thomson *et al*.[15] also showed similar results.

In this study, independent-t statistical criterion did not show any significant difference in RA onset time between patients who have HLA-DR4 and the others. But, Jamshidi *et al*.[12] found a relationship between

HLA-DR4 and the onset age. Chi-square showed that HLA-DR4 prevalence has a significant difference with respect to the gender in control group, but it did not differ in patient one. Jamshidi *et al*.[12] also showed the same result.

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