

Original article

Association of erythrocyte sedimentation rate and C-reactive protein and clinical findings with HLA-DQ8 allele in Rheumatoid Arthritis patients

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Abstract: *Background* — Rheumatoid arthritis (RA) is an inflammatory, autoimmune disease induced by certain auto-antigens. HLA-DRB1*0401 allele has a significant relationship with RA incident. Additionally, DQβ1*0301, *302(DQ8), *303, and *304 can increase RA risk especially in DQA1*0301 and *302 coincident. Recent studies suggest that distribution of this allele is different in various populations. *Material and Methods* — 70 patients and 70 healthy controls were analyzed for human leukocyte antigen (HLA) allele by specific primer-polymerase chain reaction (SSP-PCR) method. Patients were evaluated in terms of ESR and CRP. Data analysis was performed in SPSS V.17. *Results* — HLA-DQ8 allele was significantly more frequent in RA patients compared to control (P<0.0001). However, no significant relationship was observed between increased ESR (P=0.527), CRP (P=0.505), and mean counts of arthritic (P=0.691) and tender joints (P=0.669) among the patients who were carriers of HLA-DQ8. *Conclusion* — There is a significant association between RA and HLA-DQ8 allele, this allele can increase susceptibility to RA. These findings might relate to the ethnical variations of RA patients but we couldn't find a significant association between CRP and ESR with HLA-DQ8. We recommend to add specific inflammatory markers to CRP as well as assess ESR in larger sample sizes to obtain accurate results.

Keywords: C-reactive protein, erythrocyte sediment rate, HLA-DQ8, rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune, inflammatory disease that mostly affects diarthrodial joints and is characterized by synovial tissue inflammation, pannus formation, and bone erosion [1, 2]. Since the synovial joint is considered as the primary target organ in RA, no clear evidence proves whether macro-molecules in the cartilaginous tissue, bone, or synovium could act as a preferential auto-antigen in RA [3].

RA has unknown etiology, and scientists believe environmental and genetic factors are involved in pathogenesis of RA [4]. RA could be initiated with specific antigens in genetically susceptible individuals. Among genetic components, the major histocompatibility complex (MHC) might be the most significant predisposing factor in RA [5, 6]. However, MHC is not the only factor which can induce RA through mediating the leukocyte interactions [7, 8]. These antigens lead to T-cells activation. MHCs associate to specific molecules [9-11]. Recently, several non-MHC loci have been identified to contain the genes that are possibly involved in disease mechanisms [12, 13].

Several studies indicate complex of genes encode MHC proteins. Human leukocyte antigen (HLA) system could be involved in RA incident through certain HLA alleles [14-16]. Recently numbers of MHC allele subtypes are identified by polymerase chain reaction (PCR) method [17].

Despite noticeable variations in different ethnic groups, individuals carrying HLA-DRB1 alleles (*0101, *0401, *0404, and *0405) are at higher risk of developing RA (18-20). Polymorphisms are noticeable in antigen-binding sites of MHCs, while polymorphisms in HLA-DR are confined to DRβ chain (DRB1/3/4/5 genes), and the DRα chain is considered to be essentially monomorphic.

Despite some HLA disease associations, there is inadequate information regarding the nature of HLA-bound self-peptides, which of these HLAs are involved in the autoimmunity of RA. Restriction of the development of specific immune intervention strategies is aimed at the inhibition or prevention of such deleterious immune responses. Nevertheless, RA is arguably one of the best-described systems for comprehending the genetic associations between HLA-II alleles, autoimmunity, and presentation of self-peptides [21, 22].

Table 1. More details of the patients

Groups	Number	Mean Age	Arabic	Non-Arabic
Patient	70	43.84	66.2%	33.8%
Control	70	43.75	70.76%	29.23%

Table 2. List of the primers used

Primer	sequence	PCR product size (bp)
Control positive forward	5'CACTCTTCGAGCCTTCCTC-3'	354
Control positive reverse	5'AGTCCGCCTAGAAGCATTG-3'	354
0303 subtype forward	5'-GACGGAGCGCGTCGCTCT-3'	122
0303 subtype reverse	5'-CAGTACTCGGCGTCAGGCG-3'	122
0302 subtype forward	5'-GACGGAGCGCGTCGCTCT-3'	129
0302 subtype reverse	5'-CTGTCCAGTACTCGGCGG-3'	129

Table 3. Distribution of HLA-DQ8 allele between *RA patients and controls

Groups		DQ8		Total
		Positive	Negative	
Patients	Number	40	25	65
	Percent	61.5	38.5	100
	Percent of patients having the allele	72.7	33.3	50
	The percent of patients in the total population of the study	30.8	19.2	50
Control	Number	15	50	65
	Percent	23.1	76.9	100
	Percent of controls having the allele	27.3	66.7	50
	The percent of controls in the total population of the study	11.5	38.5	50
Total	Number	55	75	130
	Percent	42.3	55.7	100

Table 4. ESR change among the patients

Total	Up to 100/h	Less than 100 mm/h	0-20 mm/h	HLA-DQ8
61.5	3.1	36.9	21.5	Positive
38.5	1.5	20	16.9	Negative

Table 5. CRP changes among the patients

The present in the patients	CRP range
50.8%	Negative
18.5%	+1
12.3%	+2
18.5%	+3

Recent studies have investigated the inheritance pattern of different HLA alleles in various populations and its impact on the distribution of RA. However, none of these studies have been focused on the relationship between HLA-DQ8 alleles and related markers of disease activity biomarkers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), and arthritic and tender joints. We used CRP and ESR and HLA-DQ8 and arthritic tender joints in this study because HLA genes show a loss of sex- and age-dependent changes and this gene has the strongest association with the additional risk of RA [23]. CRP is a better measure of disease activity than ESR because it is much more specific in disease activity [24]. However, we used both of these test to achieve more confident results and have more markers in measuring disease activity. This study designed due to the lack of related research, this study designed to evaluate the associations of HLA-DQ8 allele with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) and clinical findings of RA focused on

arthritic and tender joints as markers to evaluate therapeutic response in RA patients in Ahvaz city, Iran because there is no enough study in this field.

Material and Methods

Patients

This analytic epidemiological trial was conducted on 70 patients aged above 16 years diagnosed with RA and 70 healthy (controls) at Imam Khomeini and Golestan hospitals in Ahvaz city, Iran. The control group consisted of healthy blood donors aged above 16 years who had no history of genetic or rheumatologic diseases. Inclusion criteria of the study were age of >16 years, scores of at least six in the EULAR 2010 criteria for RA diagnosis. Totally there were 49 female (75.5%) and 6 male (24.6%) participants in the study groups. Both study groups were matched in terms of age, gender, and ethnicity. Participants were selected via non-probability and convenience sampling. More details of the patients are described in Table 1. Sample size of the study was calculated at 70 subjects per each group using NCSS software for our statistical analysis.

Blood DNA extraction

DNeasy Blood & Tissue Kits of Qiagen which used for rapid purification of total DNA of frozen blood. The DNeasy membrane combines the binding properties of a silica-based membrane with simple microspin technology or with the QIAGEN 96-Well-Plate Centrifugation System. DNA adsorbs to the DNeasy membrane in the presence of high concentrations of chaotropic salt, which remove water from hydrated molecules in solution. Buffer conditions in DNeasy Blood used to enable specific adsorption of DNA to the silica membrane and optimal removal of contaminants and enzyme inhibitors. Purification requires no phenol or chloroform extraction or alcohol precipitation, and involves minimal handling. Silica-membrane technology, in convenient spin-column or 96-well formats, ensures fast and reproducible DNA purification, eliminating the need for organic extraction and alcohol precipitation. Samples are first lysed using proteinase K. Buffering conditions are adjusted to provide optimal DNA-binding conditions and the lysate is loaded onto the DNeasy Mini spin column or the DNeasy 96 plate. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water or buffer. The purification procedures using DNeasy Mini spin columns. The DNeasy 96 Blood & Tissue Kit used this kit prepare 96-well format using the QIAGEN 96-Well-Plate Centrifugation System. We used this kit because it was easy to use and the purification was high so we could gain high quality DNA.

Primer designing & polymerase chain reaction

First of all, we look up our gene in NCBI to choose our gene firstly then the primer3 software (Denmark), to design the suitable primer in other word we used our base pair information to build a set of primers within the range of our gene and we arranged our primers in Table 2.

Then we checked our primer in RepeatMasker web site to be sure it is efficient enough. We chose reaction temperature considered to the concentration of G and C nucleotide and the temperature meant to be 70°C.

Verification of HLA-DQ via single specific primer-polymerase chain reaction (SSP-PCR)

In order to eliminate false negative results in PCR we used positive control and PCR products β -actin 354bp as internal control and housekeeping genes in each PCR procedure and we present our control positive in *Table 2*.

C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)

We used Rheumajet CRP Latex Agglutination Test Kit for the detection of C-reactive protein. In this kit CRP latex reagent consists of latex particles coated with C-reactive protein. In the presence of C-polysaccharide of levels greater than 6mg/L in serum, the coated latex particles will agglutinate providing a visually observable reaction of antigen and antibody.

We used Erythrocyte sedimentation rate analysis model ESR20/ESR40 of BIOBASE brand to measure the ESR Measurement Range were described (1-140) mm/h for this device and this device Loading Capacity is 20/40 samples at same time and the reading time sat at 30 minutes.

Statistical analysis

Data analysis was performed in SPSS V.17 using Chi-square and independent T-test to determine the associations, and P-Value of less than 0.05 was considered significant based on the Fisher's exact test.

Ethical considerations

Written informed consent was obtained from all the participants prior to the study, and participation was free of charge. Moreover, participants were assured of confidentiality terms regarding their medical record information.

Results

Comparison of the frequency of HLA-DQ8 between RA patients and control subjects indicated that out of 65 RA patients, 40 cases (61.5%) were carriers of HLA-DQ8, while this allele was identified in 15 subjects (23.1%) of the control group. Totally, HLA-DQ8 was detected in 72.7% of the subjects in both study groups; whereas 27.3% of the subjects did not carry this allele. A statistically significant difference was observed between the study groups in this regard ($P < 0.0001$). Therefore, a significant association was deduced between the inheritance of HLA-DQ8 alleles and incidence of RA (*Table 3*).

Among the studied RA patients, rate of ESR was high in 40 cases (61.5%), while it was relatively low in 25 cases (38.5%). In female patients, ESR rate was higher than the values obtained from $+10 \text{ age}/2$, and for male patients, it was considered more than the $\text{age}/2$ as increased. In this study, we considered the CRP increase equal to or higher than $+1$ in both genders, and CRP was reported to increase in 32 cases (49.2%). Mean counts of the tender and arthritic joints during the examination were 4.5077 in patients and 4.2462 in control group respectively. The summary of ESR results is mentioned in *Table 4*.

Among the studied participants, (65%) had the HLA-DQ8 allele, and 40% (with or without alleles) had high ESR. However, no significant association was observed between the increased level of ESR and presence of the alleles ($P = 0.527$).

According to our findings, 21 patients who were carriers of the HLA-DQ8 allele (52.5%) had CRP of equal to or greater than $+1$, and 32.4% of the RA patients with and without the allele were positive for CRP. The CRP results are mentioned in *Table 5*.

However, the association between CRP and HLA-DQ8 allele was not statistically significant ($P = 0.505$).

Discussion

RA is a progressive, autoimmune, inflammatory disease, which causes the destruction of joint cartilage and bones in its advanced stages [25]. Advanced or untreated RA could lead to disability of the patients along with extra-articular manifestations [26].

In the literature, several studies have confirmed the association between the incidence of RA and specific antibodies (e.g., anti-Citrullinated peptide antibodies) in patients who are positive for HLA-DRB1, as well as the majority of the patients with HLA-DR4, HLA-DR1 or both these alleles [27-29]. Recent studies indicate that DQ alleles are significantly associated to RA incident [30, 31]. Due to the relationship between DR β alleles and various alleles belonging to the DQ β 1- β 4 family, especially DQ7 and DQ8, it remains unclear whether these alleles increase susceptibility to RA independently or in combination with other alleles [32, 33]. This denotes the importance of the prognostic types of these alleles.

Among the studied subjects, females had higher rate of age distribution, which is can be responsible for development of RA [34]. In one study, Drosos et al. (1995) evaluated the clinical, serological and genetic considerations of RA in Greece [35]. According to their findings, there was no significant relationship between DQ alleles and the incidence of RA, which is inconsistent with the results of the present study. This could be due to the differences population of RA in the region of the study. In another research, Massardo (1990) reported no association between the presence of DQ8 alleles and RA incidence, while they stated that DR9 and DR4 serotypes were significantly more frequent in patients compared to the controls [36]. These reports could be due to the significant variations in the inheritance pattern of the alleles in population associated with the development of RA is different.

In one study in this regard, De Veris (2002) reported a significant association between HLA-DQ8 and DQ5 in patients with RA, which is in line with the results of the current study [37]. In Pakistan, Ali et al. (2006) evaluated the frequency of HLA-DQ β 1 in patients with RA [38] and reported no statistically significant difference between the patients and control group, which is inconsistent with our findings. This could be due to the variations in the ethnicity of the studied subjects. In accordance with the aforementioned studies, Wagner (2003) and Turesson (2005) claimed that HLA-DQ and other serotypes had no impact on the severity and progression of RA [39, 40].

In Iran, the only study focused on the assessment of the relationship between HLA-DQ8 and RA incidence was performed by Tavakol Afshari et al. (2006). According to their findings, RA patients were positive for this allele based on the genetic examination [41]. Correspondingly, they reported that DQ5 and DQ8 were the most dominant HLA-DQB1 alleles associated with higher susceptibility to RA. Moreover, they stated these alleles were possibly correlated with the increased risk of RA and therefore, could be used as molecular markers for the diagnosis this disease.

According to the results obtained by Laivoranta-Nyman et al. (2004), DQ8 carriers were at a higher risk of developing RA [42]. Among these cases, the association between shared epitope haplotypes and RA incidence was confirmed. These findings are in line with the results of the present study as HLA-DQ8 allele had a higher prevalence among the RA patients compared to control subjects in our sample population.

Conclusion

According to the results of the present study, there was a significant association between the incidence of RA and presence of the HLA-DQ8 allele which might increase the susceptibility of the patients to RA. However, no significant difference was observed between control group and RA patients in terms of the counts of tender and arthritic joints and raised levels of acute inflammation markers, ESR, and CRP. In conclusion, it is recommended that future studies in this regard be performed on larger sample sizes using more specific inflammation markers in order to find significant correlations between these alleles and development of RA.

Limitations

We had imitations such as excluding five patients from the study due to diagnoses of other than RA disease, participants with history of genetic, rheumatologic, and autoimmune diseases and those aged less than 16 years were excluded from the study. More specific inflammatory markers should be added to the study to make it much more specific and CRP and ESR should be assessed in larger sample sizes to obtain accurate results.

Authors' Contributions

All authors contributed equally to this work.

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Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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