Association of *Mycoplasma pneumoniae* and (rs9271366) HLA-DRB1gene Polymorphism with the Immune Susceptibility to Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic, inflammatory disease that affects the immune system. The primary cause of RA is unknown, but there is evidence that genetic and environmental factors also contribute to the development of the disease. **Objectives:** This study aimed to illustrate the relationship between the RA and *Mycoplasma pneumoniae*. **Materials and Methods:** The work was performed on 50 RA patients of various ages, ranging from 25 to 75 years of age, who were treated at the rheumatology clinic of the city of Medical Marjan between February 2022 to October 2022.Blood samples were used for DNA extraction. HLA-DRB1, IL-6 and mycoplasma were detected by enzyme-linked immunosorbent assay (ELISA). **Results:** The results showed that compared with RA patients not infected with *Mycoplasma pneumoniae* and healthy subjects, serum IL-6 concentration was significantly increased in RA patients infected with *Mycoplasma pneumoniae*. The results showed that serum concentrations of HLADRB1 were significantly elevated in RA patients infected with *M. pneumoniae* compared with RA patients not infected with *M. pneumoniae* and healthy subjects. **Conclusions:** In HLA-DRB1 the SNP rs9271366 was significantly associated with RA and G allele represent as dominant pathogenic allele in which the individual that carry GG and AG genotype have more susceptibility to mycoplasma infection than subjects that carry AA genotype ($P = 0.036$).

Keywords: HLA-RB1, IL-6,mycoplasma, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases that affect the body's immune system, the primary symptom of which is inflammation in the joints and their destruction, this causes significant disability and premature death. People who are afflicted with RA account for 0.5%–1.0% of the total adult population worldwide.[1] RA pathogenicity is mostly complicated and have heterogeneous manifestations. Autoantibodies are considered as characteristic of seropositive RA, they are biomarkers of a more erosion disorder. Several changes may occur before clinical presentation of disease, this stage is probably last several years characterized by the appearance of autoantibodies without signs of joint inflammation.[2] Genetics and environmental factors play an important role in the development of RA, and some microorganisms have been identified as selective factors causing RA. The microbiome in different organs of the body, such as the eye, mouth, and gut, has been associated with RA, and the relationship between RA etiology and the microbiome remains unclear. However, the microbial flora that inhabits the human body has a marked effect on the immune system and thus it has been implicated in many autoimmune diseases.[3] Evolutionary maintenance of resistance and genetic diversity. The exponential growth of genomic data has had a profound impact on human genetics and the way in which genetic variation in HLA

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genes is analyzed and interpreted:[4] e.g. type 1 diabetes, RA, and psoriasis, responses to infections (e.g. dengue infection, HIV, sequelae of HIV), neuropsychiatric disorders (e.g. narcolepsy, schizophrenia) and certain cancers (e.g. B. Hodgkin lymphoma). In this context, the current study examined the association between the three most common HLA-SNPs worldwide and the occurrence and detection of RA.[5] *Mycoplasma pneumoniae,* the smallest self-replicating biological system, is a common cause of upper and lower respiratory tract infections and the pathogen of a variety of pulmonary and extrapulmonary manifestations.[6] *M. pneumoniae* is a fastidious bacterium. Most of them require cholesterol, a unique trait among prokaryotes. Because of the fastidious nature of *M. pneumoniae*, culture methods are relatively insensitive, time-consuming, expensive, and labor-intensive, and are only successful in 30-60% of serologically diagnosed cases.[7]Periodontitis (PD) is presently regarded as a risk factor for RA. Porphyromonas gingival is bacteria were the main cause of PD, this bacterium can be expressed of peptidyl arginine deiminase (PAD), this enzyme participated to the development of RA during the production of anti-cyclic citrullinated peptide antibodies (ACCPA) by stimulating citrullination that consider key to initiate of RA.[8] RA effectiveness may be more prevalent in women than men, may be a result of female hormones, but the function of these hormones in the progression of RA still indefinite. RA prevalence in female is more than male, in rate of up 4 until the 50 age, but less of 2 after 60 ages. Furthermore, the top of RA prevalence occurs during the age of 50 years.[9,10] Many inflammatory mediators involve TNF-α, and interleukins such as (IL- 1, IL-6, and IL-17) are released via flow way at arthritis site of the joint, which create an environment to the cartilages and bone erosion based on that possibility of treatment for RA patients involve monoclonal antibodies incorporation proteins against these molecules[11]. This work aimed to study the relation between immune makers (HLA-DRB1,antimycoplasma pneumonia IgG-Ab and IL6), genetic polymorphism rs9271366 HLA-DRB1 gene with the susceptibility to RA.

Materials and Methods

Study design

Fifty RA patients (12 males and 38 females) aged 25 to 75 years were included in this case-control study. Clinically diagnosed patients have to rely on specialists when they come to the rheumatology clinic in the medical city of Marjan. The samples collection time is from February 2022 to October 2022. All patients were free of any other chronic diseases. Control subjects included an apparently healthy group randomly selected from relatives of patients who had never had RA and other chronic diseases. Forty subjects (13 males and 27 females) ranging in age from 25 to 75 years were included, age and sex approximately matched to the RA group.

Subject samples

Whole blood and serum were collected from each subjects involved in this study. Serum samples were used for detection of immunological markers including Anti-Mycoplasma pneumonia-IgG antibody, HLA-DRB1, IL-6, were detected according to instructions of ELISA kit (Demeditec company/ Germany).

Determination of DNA concentration and purity

The Promega firm of the United States was used to extraction genomic DNA from peripheral blood white blood cells (WBCs) using a high purity PCR template preparation kit. Nano-spectrophotometer (nano-drop) used the scanning ability of diode array from 200 to 320 Nm wave length to detect DNA quantity and quality; the absorbance profile was then processed and evaluated to calculate the 260/280 and 260/230 ratios. Re-extraction would occur if a sample's 260/280 ratio was less than 1.8 and/or 260/230 ratio was less than 2.[12]

rs9271366 HLA-DRB1 gene polymorphism

Restriction Digestion for PCR-RFLP (MnlI) was used for the study of rs9271366 HLA-DRB1 gene polymorphism among RA patients and control.[13] by using the primers, Forward primer TTGTGTGGCCAAGACTAG CA and reverse primer TGCCCAAAGTCAGACATC CA.

Ethical approval

The study was conducted according to ethical principles derived from the Declaration of Helsinki. It was performed after obtaining verbal consent from the patient prior to sample collection. The study protocol and patient informed consent were obtained from the Publication Ethics Committee of the Babylonian Health Authority and the Faculty of Medicine, Babylon University, Iraq, reference number 21 (dated January 15, 2022).

Results

Anti-mycoplasma pneumonia IgG antibody

The concentration of Anti-*Mycoplasma pneumonia* IgG antibody were high significant ($P < 0.05$) increase in the serum concentration level of anti-*Mycoplasma pneumoniae* IgG antibodies in the RA patients infected with *Mycoplasma pneumoniae* as compared to the RA patients non infected with *Mycoplasma pneumoniae* and healthy individuals [Table 1].

The results show significant increase in the serum concentration level of IL-6 in the RA patients infected with *Mycoplasma pneumoniae* (**102.3433 ± 16.91405** pg/L) as compared to the RA patients non infected with *Mycoplasma pneumoniae (***85.9953 ± 13.75934** pg/L) and healthy individuals (**44.2393 ± 8.16846** pg/L) [Table 2].

The results show significant increase in the serum concentration level of HLADRB1 in the RA

Table 1: Concentration of anti-*Mycoplasma pneumoniae* IgG antibody in RA patients and control group

Study group	Antimycoplasma IgG (ng/L)		
	Mean	Std. deviation	*P* Value
RA patients infected with mycoplasma (18)	0.45384	0.0718	< 0.000
RA patients non infected with mycoplasma (32)	0.14162	0.0115	
Control (40)	0.12205	0.0121	

Interleukin-6 level in serum of RA patients and control group.

Table 2: IL6 concentration between RA patients infected with mycoplasma and RA patients non infected with mycoplasma and control group

Study group	IL6 (ng/L)				
	No	Mean	Std. deviation	Std. error mean	*P* Value
RA patients infected with mycoplasma	18	102.3433	16.91405	3.98668	< 0.000
RA patients non infected with mycoplasma	32	85.9953	13.75934	2.43233	
Control	40	44.2393	8.16846	1.29155	

HLA-DRB1 level in serum of RA patients and control group.

Table 3: HLADRB1 concentration between RA patients infected with mycoplasma and RA patients non infected with mycoplasma and control group

Study group	HLADRB1 (ng/l)				
	No	Mean	Std. deviation	Std. error mean	*P* Value
RA patients infected with mycoplasma	18	748.1100	62.47271	14.72496	<0.000
RA patients non infected with mycoplasma	32	502.2916	48.55055	8.58261	
Control	40	398.4232	26.03628	1.29155	

Table 4: Allelic frequencies and Association of rs9271366 with RA

Allele	Control (No. 40)		Patient (No.50)		OR (95% CI)	*P* Value
	Count	Proportion	Count	Proportion		
A	59	0.74	62	0.62	1.72 (0.91–3.27)	*0.0966
G	21	0.26	38	0.38	0.58 (0.31–1.103)	

*$P > 0.05$ is considered no significant

Table 5: rs9271366 exact test for Hardy-Weinberg equilibrium

Allele Frequency	AA	AG	GG	A	G	*P* Value
All subjects	34	53	3	121	59	0.0016
Control	20	19	1	59	21	0.24
Case	14	34	2	62	38	0.0025

patients infected with *Mycoplasma pneumoniae* (748.1100 ± 62.47271 pg/L) as compared to the RA patients non infected with *Mycoplasma pneumoniae (*502.2916 ± 48.55055 pg/L) and healthy individuals (398.4232 ± 4.11670 pg/L) [Table 3].

Association of rs9271366 gene polymorphism with RA

The allelic association analysis as listed in Table 4 show that there is no significant differences in allele frequencies between patients and control.

On the other hand cases group showed a significant deviation from Hardy –Weinberg comparing to control group which showed no deviation from Hardy –Weinberg ($p < 0.05$) as in Table 5. This indicates that A allele a protective factor in patients with (OR 0.58 CI 95% 0.31-1.103) and that allele G considered a risk factor in healthy subjects with (0R 1.72 CI 95% 0.91-3.27).

The results shows that G allele behavior as dominant pathogenic allele in which the individual that carry GG and AG genotype have the susceptibility to the disease 2.57 fold comparing to individual that carry AA genotype (OR 2.57 CI 95% 1.07-6.17).furthermore, the genetic association of rs9271366 genotype with RA was analysis under different model inherit as listed in Table 6 and Figure 1.

Table 6: Genotype frequency and association under different model of inheritance for rs9271366 with RA

Model	Genotype	Control	Case	OR (95% CI)	*P* Value
Codominant	A/A	20 (50%)	14 (28%)	1.00	0.1
	A/G	19 (47.5%)	34 (68%)	2.56 (1.06-6.19)	
	G/G	1 (2.5%)	2 (4%)	2.86 (0.24–4.66)	
Dominant	A/A	20 (50%)	14 (28%)	1.00	0.032
	A/G-G/G	20 (50%)	36 (72%)	2.57 (1.07–6.17)	
Recessive	A/A-A/G	39 (97.5%)	48 (96%)	1.00	0.69
	G/G	1 (2.5%)	2 (4%)	1.62 (0.14–8.60)	
Over dominant	A/A-G/G	21 (52.5%)	16 (32%)	1.00	0.049
	A/G	19 (47.5%)	34 (68%)	2.35 (0.99–5.55)	

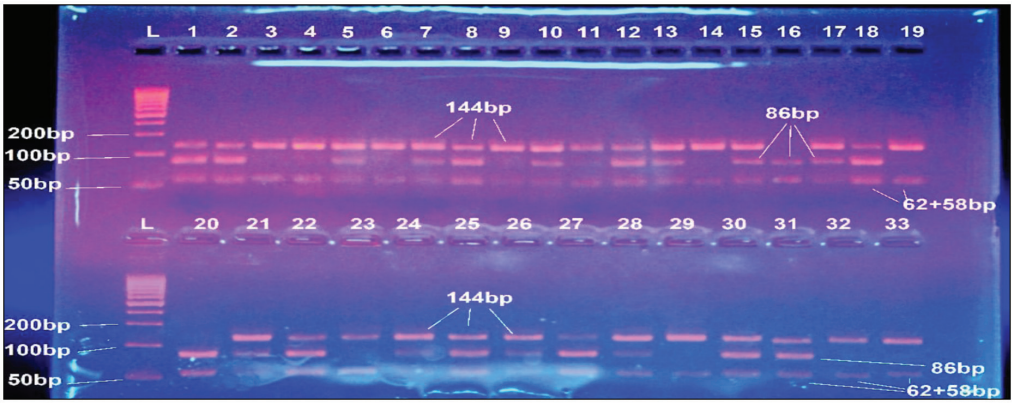

Figure 1: Genotyping of rs9271366 by PCR-RFLP technique, lanes L DNA ladder, lanes 3,4,6,9,11,14,19,23,29,32 and 33 AA genotype; lanes 1,2,5, 7,8,10,12,22,24,25,26,27,30 and 31 AG genotype; lanes 16 and 20 GG genotype

Discussion

Anti-Mycoplasma pneumoniae IgG antibody was studied as a marker for chronic and previous infection with *M. pneumoniae*.[14,15] elucidate the estimated risk of developing RA related to infections of Mycoplasma pneumonia.[16] showed *Mycoplasma pneumoniae* had a role in inflammatory autoimmune diseases.[17] showed *Mycoplasma pneumoniae* antigens have a role to promote production of the IL-6, IL-10 and IL-17.[18] refer that many microorganisms were involved in progression of RA.

Activated macrophages secrete IL-6, IL-1β and IL-23, cytokines that potentiates Th17 cell development, thus enforcing chronic inflammatory response within RA inflammatory joints. Recent data in mice suggest that Th17 may also increase germinal center B cells to produce higher amounts of autoantibodies.[19,20]

IL-6 plays important role in regulating immune functions.[21] It is involved in the infection process of MP and plays an important role in the pathogenesis of MP.[22]During activation of T-cells, CD4+ T-cells interact with human leukocyte antigen (HLA) or major histocompatibility class II (MHC-II) molecules as well as co-stimulating molecules that are expressed on the surface of APC.[23]

The presence of Mycoplasma spp. leads to the regulation of MHC class II antigen presentation on the cell surface. The role of CD4+ T-cells in RA chronic inflammation is also supported by its association with the particular MHC-II, HLA-DRB in the third hyper variable region which is the similar to amino acid with DR4. This interaction then leads to a more aggressive form of RA.[24,25]

In recent studies, the occurrence of HLA-DRB1 risk alleles predisposes the formation of N-glycosylation sites in (anti-citrullinated protein antibodies) ACPA-IgG. Still, this predisposition's precise mechanism is unknown.[26] Nevertheless, due to the fact that HLA-DR (encoded by HLA-DRB1 gene) molecules are necessary for the activation of CD4+ T cell, we assume that it is likely that HLA-DRB1 risk alleles induce somatic hypermutation of ACPA, leading to their pathogenicity.[27] T cell-mediated immune responses are generally considered important for protection against Mycoplasmas causing local respiratory infections.[28]

T cells are key in the regulation of immune responses and have a critical impact on the development of Mycoplasma-induced pneumonia.[29] The shared epitope (a five-amino acid motif encompassing positions 70 to 74 of the HLA-DRB1 chain) encoded in the major histocompatibility complex (MHC) is present in approximately 70% of patients with ACPA-positive RA.[30,31] It is thought that citrullinated autoantigen epitopes bind to HLA-DRB1 that contain the SE and are presented to CD4+ T cells,

which contribute to autoimmunity.[32] Moreover, SE is an important risk factor for severe bone destructive disease.[33,34]

Anti-cyclic citrullinated peptide has been identified as a potential diagnostic and prognostic marker of RA. Citrulline is an unusual amino acid resulting from an enzymatically modified arginine residue present on certain human proteins. The presence of anti-CCP antibodies is highly specific and sensitive for RA.[35]The major histocompatibility complex is encoded in human leukocyte antigen gene clusters on chromosome 6. Among MHC class II molecules, various HLA-DR alleles have been associated with susceptibility to RA in several racial groups. In addition, some HLA-DRB1 alleles have also been related to the severity and outcome of RA.[36]

These disease-associated HLA molecules share a common amino acid sequence in the third hyper variable region of the beta chain of the HLA-DR molecule (HLA-DRB1), hence the term "shared epitope." SE is a sequence of five amino acids. Among the SE variants, the QKRAA, QRRAA and RRRAA motifs have been described within various DRB1 variants.[37,38] who showed the SNP rs9271366 (tag SNP for HLA-DRB1*15:01) confers the highest risk for Systemic lupus erythematous (SLE) among the 13 MHC gene alleles that display association with SLE (P = 8.748E-10; OR = 3.5). Among the 26 non-MHC gene alleles analyzed.

[39] who showed that the SNP rs9271366 (OR = 2.06, 95% CI: 1.71–2.50) the highest risk for Systemic lupus, HLA-DRB1 the main and major association for autoimmune disease in Europeans and in most populations.[40] who found four independent SNPs in the MHC region associated with risk of SLE in African-American women. The most strongly associated SNP with SLE was rs9271366 near the HLA-DRB1 gene. Conditional haplotype analysis revealed three other SNPs (rs204890, rs2071349 and rs2844580) that were associated with SLE independent of the rs9271366 SNP. A genotype score combining the four newly identified SNPs showed an additive risk according to the number of high-risk alleles.

In conclusion, we found that rs9271366 was significantly associated with RA (OR 2.57 CI 95% 1.07-6.17) the G allele represented a pathogenic dominant allele and also found rs9271366 to be the G allele associated with an increased risk of mycoplasma infection The G allele represents the dominant pathogenic allele (OR 4.80 CI 95% 0.94-24.62). The HLA-DRB1 and IL-6 in RA patients infected with *Mycoplasma pneumoniae* was significant ($p<0.05$) as compared to the RA patients non infected with *Mycoplasma pneumoniae* and controls. The levels of HLA-DRB1 and IL6 were significantly correlated between RA patients.

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Conflicts of interest
There are no conflicts of interest.

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