Study the Association of *Mycoplasma pneumoniae* and (rs35445101) HLA-DRB1gene Polymorphism with the Immune Susceptibility to Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is an inflammatory immune disease that primarily affects the joints and has its root cause in immune system malfunction. It is unclear what causes RA, but research points to a combination of genetic predisposition, environmental exposures, and microbial infections. **Objectives:** This study aimed to illustrate the relationship between RA and *Mycoplasma pneumoniae*. **Materials and Methods:** The work was performed on 50 RA subjects of various ages, ranging from 25 to 75 years of age, who were treated at the rheumatology clinic in the city of Medical Marjan between February 2022 and October 2022. Blood samples were used for DNA extraction. VEGF-A.HLA-DRB1 and mycoplasma were detected by enzyme-linked immunosorbent assay. **Results:** The results show a significant increase in the serum concentration level of VEGF-A in RA patients infected with *M. pneumoniae* as compared to healthy individuals, but the results of RA patients with *M. pneumoniae* infections and RA patients without *M. pneumoniae* infections were nonsignificant ($P > 0.05$). The results showed a significant increase in the serum concentration level of HLA-DRB1 in the RA patients infected with *M. pneumoniae* as compared to the RA patients noninfected with *M. pneumoniae* and healthy individuals. **Conclusions:** In HLA-DRB1, the SNP rs35445101 shows that A allele behavior as recessive pathogenic allele in which the individual that carries AA genotype has a susceptibility to the disease 9.75 fold compared to an individual that carries GG and AG genotype (odds ratio 9.75 confidence interval 95% 1.19–79.78).

Keywords: HLA-DRB1, mycoplasma, rheumatoid arthritis, VEGF-A

Introduction

One of the most common systemic autoimmune disorders, rheumatoid arthritis (RA), causes synovial inflammation and joint degeneration, which can lead to serious disability and even death. She is one of the 0.5%–1.0% of the adult population affected by RA.[1] The pathogenicity of RA is often complex and has a heterogeneous presentation. Autoantibodies are considered to be characteristic of seropositive RA, and they are biomarkers of erosive disease. Some changes may occur before the clinical onset of disease, a phase that may last for years and is characterized by the development of autoantibodies without evidence of joint inflammation.[2] Genetics and environmental factors play an important role in the development of RA, and some microorganisms have become selective factors for triggering 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

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genetic variation in human leukocyte antigens (HLA) genes is analyzed and interpreted.[4] The smallest self-replicating biological system, *Mycoplasma pneumoniae*, is the causative agent of a wide range of pulmonary and extrapulmonary symptoms and a frequent cause of upper and lower respiratory tract infections.[5] *M. pneumoniae* is a fastidious bacterium. Most of them require cholesterol, a unique trait among prokaryotes.[6] The effectiveness of RA may be more common in women than men, possibly as a result of female hormones, but the role of these hormones in RA progression remains undetermined. RA is more prevalent in women than in men, up to 4 by age 50 but less than 2 after age 60. Furthermore, the peak prevalence of RA occurs at the age of 50 years.[7,8] Many inflammatory mediators involve TNF-α, VEGF-A, 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, the possibility of treatment for RA patients involves monoclonal antibodies and the incorporation of proteins against these molecules.[9] The most potent mechanism involves the regulation of major histocompatibility complex class II DR beta 1 (HLA-DRB1-rs35445101), affecting their regulatory roles in all candidate pathways except the hematopoietic lineage pathway. Previous studies have shown that HLA-DPB1 polymorphisms are significantly associated with the risk of susceptibility to HBV infection.[10] This work aimed to study the relation between immune makers (HLA-DRB1, antimycoplasma IgG, and VEGF-A), genetic polymorphism rs35445101 HLA-DRB1 gene with the susceptibility to RA.

Materials and Methods

Study design

Fifty RA patients (12 males and 38 females) aged 25–75 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 town of Marjan. The sample 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 25–75 years were included, age and sex approximately matched to the RA group.

Subject samples

Whole blood and serum were collected from each subject involved in this study. Serum samples were used for the detection of immunological markers, including Anti-*M. pneumoniae*-IgG antibody, HLA-DRB1, and VEGF-A, were detected according to instructions of enzyme-linked immunosorbent assay kit (Demeditec company/Germany).

Determination of DNA concentration and purity

Genomic DNA was extracted from peripheral blood leukocytes (WBC) using the High Purity PCR Template Preparation Kit provided by Promega Corporation/USA. Using the diode array's scanning capability from 200 to 320-nm wavelength, the DNA quantity and quality were measured using a nanospectrophotometer (nanodrop), then the absorbance curve was processed and analyzed to determine the DNA quantity and quality by calculating the ratio 260/280 and 260/230 to determine. Re-extract if the sample has a 260/280 ratio less than 1.8 and/or a 260/230 ratio less than 2.[11]

rs35445101 HLA-DRB1 gene polymorphism

Restriction digestion for PCR-RFLP (Ppsi) was used for the study of rs35445101 HLA-DRB1 gene polymorphism among RA patients and control (new design). By using the primers, Forward primer GCAGGAAAGCTTTTCATCCTGCAA and reverse primer CTCCTCTCCACTTAGAAAAGGTGT.

Ethical approval

The study was conducted according to ethical principles derived from the Declaration of Helsinki. It was performed after the patient's verbal consent was obtained prior to sample collection. The study protocol and patient informed consent were obtained from the Babylonian Health Authority and the Publication Ethics Committee of the Faculty of Medicine, Babylon University, Iraq, reference number 21 (dated January 15, 2023).

Results

Anti-*Mycoplasma pneumoniae* IgG antibody

The concentration of Anti-*M. pneumoniae* IgG antibodies were highly significant (P-value < 0.05) increase in the serum concentration level of anti-*M. pneumoniae* IgG antibodies in the RA patients infected with *M. pneumoniae* as compared to the RA patients noninfected with *M. pneumoniae* and healthy individuals [Table 1].

VEGF-A level in serum of rheumatoid arthritis patients and control group

The results show a significant increase in the serum concentration level of VEGF-A in RA patients infected

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	

with *M. pneumoniae* (81.3939 ± 14.79369 pg/L) as compared to healthy individuals (48.1068 ± 5.10710 pg/L), but the results of RA patients with *M. pneumoniae* infections and RA patients without *M. pneumoniae* infections were non-significant ($P > 0.05$) [Table 2].

HLA-DRB1 Level in serum of RA patients and control group

The results show a significant increase in the serum concentration level of HLA-DRB1 in the RA patients infected with *M. pneumoniae* (748.1100 ± 62.47271 pg/L) as compared to the RA patients noninfected with *M. pneumoniae* (502.2916 ± 48.55055 pg/L) and healthy individuals (398.4232 ± 4.11670 pg/L) [Table 3].

Association of rs35445101 gene polymorphism with RA

The allelic association analysis, as listed in Table 4, shows that there are no significant differences in allele frequencies between case and control.

On the other hand, cases group showed a significant deviation from Hardy Weinberg compared to control group, which showed no deviation from Hardy Weinberg ($P < 0.05$) as in Table 5. This indicates that G allele is a protective factor in patients with (odds ratio [OR] 1.03 confidence interval [CI] 95% 0.51–2.09) and that allele A considered a risk factor in healthy subjects with (OR 0.97 CI 95% 0.48–1.97).

The results show that A allele behavior as recessive pathogenic allele in which the individual that carries AA genotype have the susceptibility to the disease 9.75 fold compared to individual that carries GG and AG genotype (OR 9.75 CI 95% 1.19–79.78). Furthermore, the genetic association of rs35445101 genotype with RA was analyzed under different model inherit as listed in Table 6 and Figure 1.

Discussion

Anti-*M. pneumoniae* IgG antibody was studied as a marker for chronic and previous infection with *M. pneumonia*.[12] Chu *et al.*[13] elucidate the estimated risk of developing RA related to infections of *M. pneumoniae*. Abbas *et al.*[14] showed *M. pneumoniae* had a role in inflammatory

Table 2: VEGF-A concentration between RA patients infected with mycoplasma and RA patients noninfected with mycoplasma and control group

Study group	VEGFA (ng/l)				
	No	Mean	Std. Deviation	Std. Error Mean	P value
RA patients infected with mycoplasma	18	81.3939	14.79369	3.48691	<0.000
RA patients non infected with mycoplasma	32	80.9641	11.85173	2.09511	
Control	40	48.1068	5.10710	0.80750	

Table 3: HLA-DRB1 concentration between RA patients infected with mycoplasma and RA patients noninfected 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 rs35445101 with RA

Allele	Control		Case		OR (95% CI)	P-value
	Count	Proportion	Count	Proportion		
G	62	0.78	78	0.78	1.03 (0.51-2.09)	0.9361
A	18	0.22	22	0.22	0.97 (0.48-1.97)	

Table 5: rs35445101 exact test for Hardy–Weinberg equilibrium

Allele Frequency	GG	AG	AA	G	A	P-value
All subjects	61	18	11	140	40	0.00018
Control	23	16	1	62	18	0.65
Case	38	2	10	78	22	<0.0001

Table 6: Genotype frequency and association under different models of inheritance for rs35445101 with RA

Model	Genotype	Control	Case	OR (95% CI)	P-value
Codominant	G/G	23 (57.5%)	38 (76%)	1.00	<0.0001
	A/G	16 (40%)	2 (4%)	0.08 (0.02-0.36)	
	A/A	1 (2.5%)	10 (20%)	6.05 (0.73-50.40)	
Dominant	G/G	23 (57.5%)	38 (76%)	1.00	0.062
	A/G-A/A	17 (42.5%)	12 (24%)	0.43 (0.17-1.05)	
Recessive	G/G-A/G	39 (97.5%)	40 (80%)	1.00	0.0064
	A/A	1 (2.5%)	10 (20%)	9.75 (1.19-79.78)	
Over dominant	G/G-A/A	24 (60%)	48 (96%)	1.00	<0.0001
	A/G	16 (40%)	2 (4%)	0.06 (0.01-0.29)	

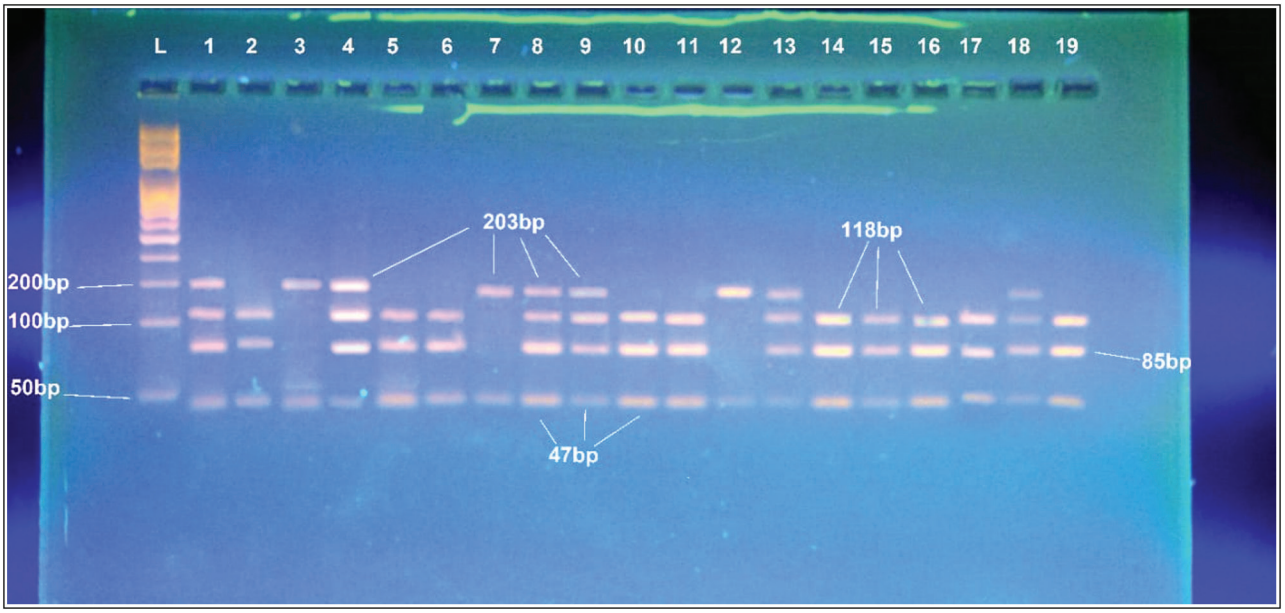

Figure 1: Genotyping of rs35445101 by PCR-RFLP technique, lanes L DNA ladder, lanes 3, 7, and 12 AA genotype; lanes 1, 4, 8, 9, 13, and 18 AG genotype; lanes 2, 5, 6, 10, 11, 14, 15, 16, 17, and 19 GG genotype

autoimmune diseases. *M. pneumoniae* antigens were shown to play a role in promoting the production of IL-6, IL-10, IL-17, and VEGF-A.[15] Sepúlveda-Delgado *et al*.[16] showed that many microorganisms are involved in the progression of RA. Endothelial dysfunction is an essential characteristic of rheumatoid synovitis. The creation of additional blood vessels permits the delivery of nutrients and oxygen to the increasing number of inflamed cells, hence contributing to the persistence of joint illness. VEGF is a powerful, endothelial cell-specific growth factor that is increased by proinflammatory cytokines and hypoxia. These findings are congruent with,[17] which showed that increased serum VEGF levels correspond with disease activity in RA. VEGF produces proinflammatory alterations in chronic inflammation, such as leukocyte accumulation, collagen deposition, and vascular abnormalities. Active angiogenesis leads to joint invasion, destruction, and pain in the etiology of chronic arthritis, making increased vascularity one of the hallmarks of RA synovitis. VEGF has been found to have a crucial role in the creation and maintenance of pannus and is highly expressed in the synovial fluid and serum of patients with RA.[18,19] Multiple inflammatory cell types cooperate to sustain mutual activation networks in RA joints, sustaining the autoimmune cycle. When stimulated by VEGF, endothelial cells secrete chemokines such as MCP-1 and IL-8. These chemokines can recruit monocytes around synovial endothelium. Furthermore, newly introduced macrophages, which can activate endothelial cells in addition to external activation, can also activate resident synoviocytes.[18,20] As a result, TNF- and IL-6 further enhance macrophage and synoviocyte VEGF secretion. A positive feedback loop is formed when this causes endothelial cells to activate macrophages through cell contact. Therefore, it is possible that VEGF acts as a functional bridge between macrophages and synoviocytes, and endothelial cells.[21] CD4+ T lymphocytes undergo activation when they engage APCs expressing HLA or major histocompatibility complex class II (MHC-II) molecules and costimulatory molecules.[22] The presence

of Mycoplasma spp. leads to the regulation of MHC class II antigen presentation on the cell surface. A role for CD4+ T cells in chronic RA inflammation is also supported by their association with a specific MHC-II, HLA-DRB, in the third hypervariable region, which is amino acid similar to DR4. This interaction then leads to more aggressive RA.[23,24] 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.[25] However, based on the fact that the HLA-DR molecule (encoded by the HLA-DRB1 gene) is required for the activation of CD4+ T cells, we hypothesized that the HLA-DRB1 risk allele is likely to carry a somatically induced ACPA hypermutation leading to pathogenicity.[26] T cell-mediated immune responses are generally considered to be important for protection against mycoplasma that cause local respiratory infections.[27] T cells play a key role in regulating the immune response and critically impact the development of mycoplasma-induced pneumonia.[28] A common epitope encoded in the MHC (a five amino acid motif spanning positions 70–74 of the HLA-DRB1 chain) is present in approximately 70% of ACPA-positive RA patients.[29,30] Citrullinated autoantigen epitopes are thought to bind to SE-containing HLA-DRB1 and presented to CD4+ T cells that contribute to autoimmunity.[31] Furthermore, SE is an important risk factor for severe bone-destructive diseases.[32,33] The major histocompatibility complex is encoded in the HLA gene cluster on chromosome 6. Among MHC class II molecules, distinct HLA-DR alleles are associated with RA susceptibility in several ethnic groups. In addition, some HLA-DRB1 alleles are also associated with RA severity and outcome.[34] Both the MHC class I and class II gene groups are located on chromosome 6 to make up the HLA system.[35] DRB1, expressed in immune cells and a part of the HLA class II genes, plays a crucial role in regulating the immune response to foreign antigens and is a characteristic that separates the self from foreign antigens.[36] More and more genetic association studies have found that changes in the gene HLA-DRB1 are associated with a wide range of immune-mediated illnesses, including RA and head and neck cancer.[37] Previous investigations have demonstrated that the prognosis and clinical course of inflammatory diseases like RA and lupus are influenced by the HLA-DR genotype. Additionally, inflammatory mechanisms like complement activation lead to tissue destruction.[38] The HLA-DRB1*1501 allele plays a highly immunogenic role in autoimmunity.[39]

In conclusion, we found that rs35445101 was significantly associated with RA (OR 9.75 CI 95% 1.19–79.78), where the A allele represented a pathogenic recessive allele, but not the allele or genotype was significantly associated with Mycoplasma An association with infection was shown (P >0.05), Therefore, we argue that rs35445101 in exon 5 of the HLA-DRB1 gene independently contributes to the risk of RA in Babylonians. Patients at risk for RA can be identified through the use of the HLA-DRB1SNP (rs35445101).

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Conflicts of interest
There are no conflicts of interest.

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