

Association of the HLA-DRB1*04 allele and auto-antibodies with disease severity in smokers and non-smokers with rheumatoid arthritis

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Abstract. The present study aimed to investigate the association between the HLA-DRB1*04 allele and the severity of rheumatoid arthritis (RA), as well as the presence of autoantibodies, in patients who were smokers and non-smokers. For this purpose, a case-control study was conducted involving 100 patients with RA (50 smokers and 50 non-smokers) and 50 healthy controls (25 smokers and 25 non-smokers). HLA-DRB1 gene expression was quantified using reverse transcription-quantitative PCR and HLA-DRB1*04 allele genotyping was performed with sequence-specific primers. The levels of serum rheumatoid factor (RF) and anti-cyclic citrullinated peptide (ACCP) antibodies were measured using ELISA. The Disease Activity Score-28 (DAS-28) was assessed by a specialist physician. Positive HLA-DRB1 gene expression was detected in 64% of the patients and 16% of the controls. The HLA-DRB1*04 allele was identified in 32% of the patients and 6% of the controls. Smokers carrying the HLA-DRB1*04-positive allele (n=19) exhibited higher HLA-DRB1 gene expression levels (4.46 ± 4.2 -fold), ACCP antibody levels (77.54 ± 24.6 U/ml) and RF levels (138.48 ± 140.1 U/ml) compared with HLA-DRB1*04-positive patients who were non-smokers (n=13). DAS-28 values were significantly higher in HLA-DRB1*04-positive smokers (4.52 ± 0.9) than in HLA-DRB1*04-negative non-smokers (3.8 ± 1.02 ; $P \leq 0.05$). No significant difference was observed between HLA-DRB1*04-positive non-smokers and HLA-DRB1*04-negative smokers. On the whole, the present study demonstrates that cigarette smoking has an adverse effect on the severity of RA, comparable to that of the HLA-DRB1*04 allele alone. The interaction between smoking and HLA-DRB1*04 positivity links to higher autoantibody

levels and disease activity, highlighting the importance of both genetic and environmental risk factors in the pathogenesis of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by persistent synovial inflammation and progressive joint destruction. The disease results from a complex interaction between genetic susceptibility and environmental triggers that collectively contribute to immune dysregulation and autoantibody production. Accumulating evidence indicates that both genetic predisposition and environmental exposures play critical roles in initiating and perpetuating the autoimmune response in RA (1).

Among the genetic factors involved in RA, the strongest association with susceptibility to RA involves specific alleles of the HLA-DRB1 gene, particularly those carrying the so-called shared epitope (SE) motif. These alleles encode a conserved amino acid sequence within the peptide-binding groove of the major histocompatibility complex class II molecule, enhancing the presentation of modified self-peptides to autoreactive T-lymphocytes (2). Structural and molecular studies have demonstrated that SE-positive HLA molecules preferentially bind citrullinated peptides, thereby promoting autoreactive immune responses and facilitating the development of autoantibodies characteristic of RA. This mechanism represents a key immunogenetic pathway linking genetic susceptibility with adaptive immune activation in the pathogenesis of RA (3,4). Although the condition can appear at any age, the peak age of onset is in the sixth decade of life, and its prevalence is ~3-fold higher in women than in men (5).

Exposure to environmental pollutants, particularly cigarette smoke, markedly amplifies the risk conferred by the SE. Smoking increases the degree of protein citrullination in pulmonary tissues by upregulating peptidyl arginine deiminase 2 enzyme expression. The aryl hydrocarbon receptor (AHR) has been detected in the peripheral blood mononuclear cells of patients with RA who are smokers. The activation of AHR by smoking exacerbates the severity of arthritis and promotes T-cell differentiation, thereby affecting both humoral and cellular immune responses. Notably, smoking is strongly associated with the development

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of RA in individuals carrying HLA-SE susceptibility alleles (6). Additionally, smoking may influence immune responses to citrullinated enolase in genetically predisposed individuals (7). Autoantibodies represent another hallmark of RA (8). Although not entirely disease-specific, the most recognized autoantibodies include rheumatoid factor (RF), which targets the Fc portion of immunoglobulin G (IgG); RA is commonly diagnosed through the detection of RF, particularly the IgM isotype (9). By contrast, anti-cyclic citrullinated peptide (ACCP) antibodies are highly specific serological and can be detected prior to the clinical onset of the disease (10). These antibodies are also considered reliable predictors of the progression of RA (11,12). Recent clinical and molecular evidence suggests that the combined presence of SE alleles, anti-citrullinated protein antibodies and exposure to smoke is associated with more severe disease manifestations, including a higher inflammatory activity and greater structural joint damage. This interaction indicates that environmental triggers may potentiate genetic susceptibility by enhancing autoantigen presentation and promoting autoreactive immune responses, thereby accelerating disease progression in susceptible individuals (13).

However, despite the well-established role of HLA-DRB1*04 and smoking in the pathogenesis of RA, data regarding this interaction within the Iraqi population remain limited. Therefore, the present study aimed to investigate the association between RA and HLA-DRB1*04 (SE) in smokers and non-smokers, as well as to evaluate the association between disease severity and the combined effects of these risk factors.

Patients and methods

Study participants. The present study employed a case-control design and included 100 patients with RA, of whom 50% were smokers, diagnosed according to the criteria of the American College of Rheumatology (14). The patient cohort comprised 25 males and 75 females, with an age range of 20-70 years. Cases were recruited from the Rheumatology Consultation Clinic at Baghdad Teaching Hospital/Medical City, Baghdad, Iraq.

The control group consisted of 50 healthy individuals confirmed by a clinical examination and laboratory investigations, with smokers representing 50% of the group. Patients with comorbidities, other connective tissue diseases, spondylitis, malignancies or pregnancy were excluded from the study.

A total of 75 patients were receiving therapy with methotrexate and enbrel. The study protocol was approved by the Scientific Ethics Committee of the College of Science, University of Baghdad, as part of a PhD research plan. Informed consent was obtained from all participants, both verbally and in writing, prior to enrollment in the study.

Blood sample collection was conducted from early November, 2023 to the end of March, 2024. Laboratory diagnostic investigations were performed using enzyme-linked immunosorbent assay (ELISA) to measure serum RF levels (Rheumatoid Factor Screen, quant. 96; cat. no. R97422, Dialab GmbH; positive result >25 U/ml) and ACCP antibodies (cat. no. 0323/VER-03, Qualisa; positive result, >25 U/ml). Disease Activity Score-28 (DAS-28) values were assessed by

a specialist physician (Rheumatology and medical rehabilitation) and were used to evaluate the clinical severity of the disease (15).

Molecular analysis. Total RNA was extracted from the peripheral blood samples obtained from the patients with RA and the healthy controls using the TRIzol™ reagent extraction kit (cat. no. ER501-01-01, TransGen Biotech) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total mRNA using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (cat. no. AE311-04, TransGen Biotech). The reverse transcription reaction was performed in a final volume of 20 μ l under the following thermocycler conditions: One cycle of primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min, and reverse transcriptase inactivation at 85°C for 5 sec. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using gene-specific primers for HLA-DRB1 gene (forward, ACAACTACGGGGTTGTGGAG; and reverse, CGTTCAGGAACCACCTGACT). GAPDH was used as the housekeeping gene (forward, GAAATCCCA TCACCATCTTCCAGG; reverse, GAGCCCCAGCCTTCTCCATG). Each reaction was carried out in a final volume of 20 μ l containing a cDNA template, primer, SYBR-Green Master Mix and nuclease-free water. Thermal cycling conditions included one cycle of initial denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 20 sec and annealing at 60°C for 30 sec. A final melting-curve stage was conducted at 55°C for 20 min. Melting-curve analysis was performed to confirm amplification specificity. Relative gene expression levels were calculated using the folding method ($2^{-\Delta\Delta C_q}$) with normalization to the housekeeping gene (16).

For HLA-DRB1*04 typing, a specific PCR technique was performed using allele-specific primers for HLA-DRB1*04, following the protocol described in the study by Parasannanavar *et al* (17), including a reverse primer (TCTCGGTAAGTCTGTGCCTT) and two forward primers (forward 1, GCTACGTGGACGACA CGCT; forward 2, CTCGGTCAGTCTGTGCCTT); HGH was used as the control primer (forward, TGCCTTCCC AACCATTCCCTTA; reverse, CCACTCACGGATTTC TGTGTGTTTC). This method was selected due to its high sensitivity and specificity (100%) in identifying the HLA-DRB1*04 allele, as validated against commercial PCR-SSOP kits in the source (14). To ensure this high level of accuracy and specificity in the clinical samples, the present study strictly adhered to the optimized concentrations and thermal cycling parameters, particularly the annealing temperature of 65°C, as described in the original protocol. The specificity of the results was further confirmed by electrophoresis, demonstrating distinct target bands without non-specific amplification, as presented in Fig. 1. Genomic DNA was extracted from peripheral blood leukocytes (EDTA-anticoagulated samples) using the AddPrep Genomic DNA Extraction kit (cat. no. 100-002, Biotech Ltd.). PCR amplification was performed in a total reaction volume of 25 μ l containing extracted DNA, primers and PCR premix. Amplification was carried out using a G-Storm thermal cycler (Gene Technologies Ltd.) under the following conditions: An initial denaturation at 94°C for

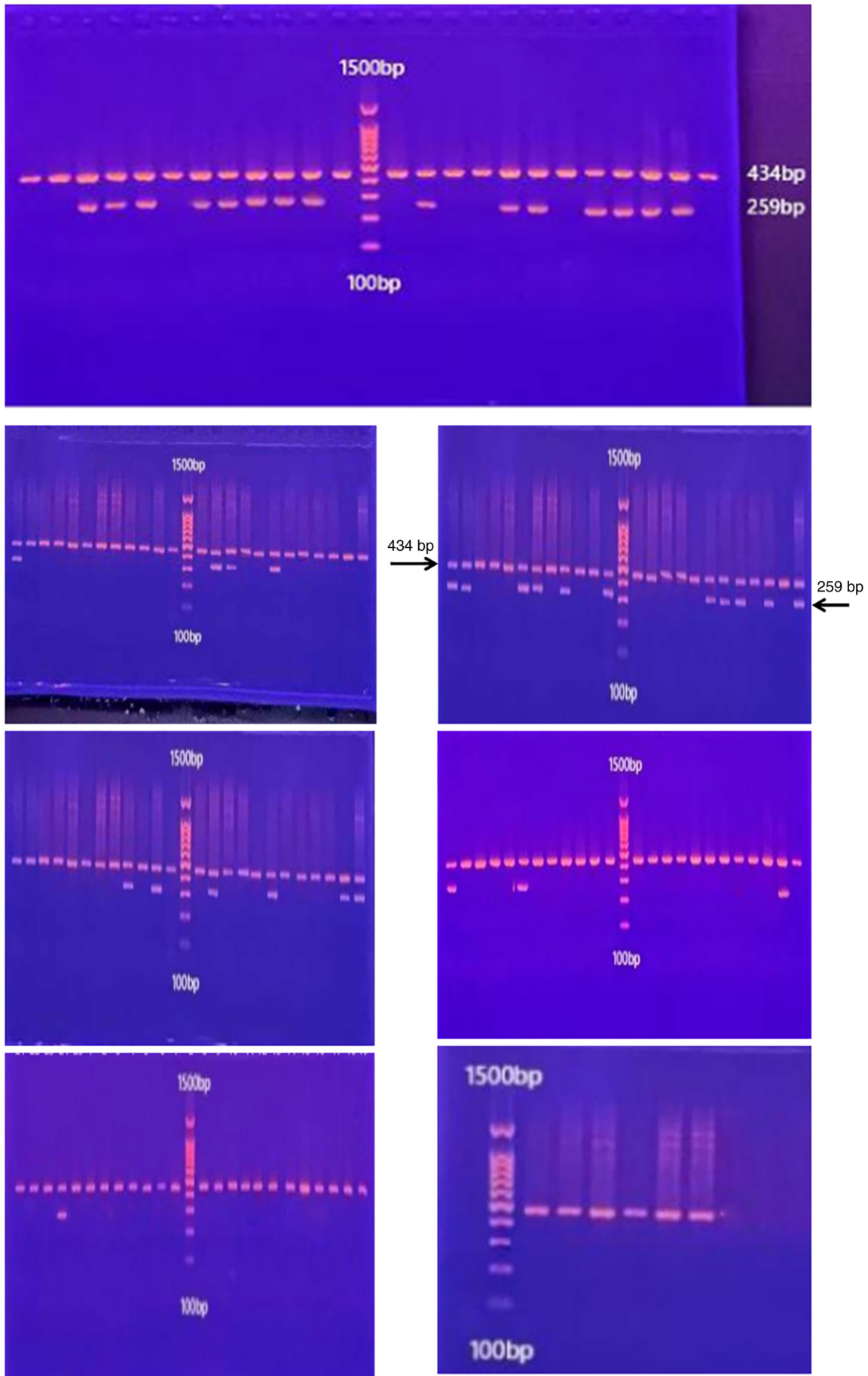


Figure 1. Gel electrophoresis of PCR products on a 2% agarose gel. The 434-bp band represents the control PCR product, while the 259-bp band corresponds to the allele-specific primer product. A DNA 100-bp ladder (lane L) was used for size estimation.

Table I. Baseline characteristics of patients with RA and the controls.

Parameters, mean \pm SD, or %	Patients (n=100)	Control (n=50)
Age, years	53.34 \pm 2.23	43.06 \pm 10.78
Sex (male/female)	25/75	20/30
Duration of RA (years)	4.3 \pm 0.42	-
Family history (yes/no)	19/81	-
DAS-28	4.1 \pm 0.8	-
DAS-28 (mild/moderate/severe)	(10/66/24)	-
Smoking (yes/no)	50/50	25/25
Laboratory parameters		
RF positivity, %	79	0
ACCP positivity, %	87	0

RA, rheumatoid arthritis; DAS, disease activity score; RF, rheumatoid factor; ACCP, anti-cyclic citrullinated peptide.

5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The amplified PCR products (5 μ l) and a 100 bp DNA ladder (molecular weight marker) were separated by electrophoresis on a 2% agarose gel (Promega Corporation) stained with ethidium bromide (MilliporeSigma) for 20 min at room temperature.

Statistical analysis. Statistical analysis was performed using SPSS software (version 20; IBM Corp.). Continuous variables with normal distribution are expressed as the mean \pm standard deviation (SD). A one-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to compare the mean levels of gene expression, autoantibodies and disease activity score across multiple patient groups and controls. A P-value <0.05 was considered to indicate a statistically significant difference, while a P-value <0.01 was considered to indicate a highly significant difference (18).

Results

Baseline characteristic of patients and controls. The general and clinical findings of 100 patients with RA estimated according to the American College of Rheumatology (ACR) criteria are presented in Table I. The mean age of the patients with RA was 53.34 \pm 12.23 years, and the mean age of the healthy controls was 43.06 \pm 10.78 years; the mean disease duration was 4.3 \pm 0.42 years and the mean disease activity (DAS-28) was 4.1 \pm 0.8.

HLA-DRB1 gene expression percentages in the study groups. Gene expression analyses revealed different levels of the HLA-DRB1 gene, ranging from 0.72-18.26 in patients and 0.21-1.98 in the controls. Accordingly, the results revealed the positivity percentage of the HLA-DRB1 gene as 64% in the patients and 16% in the controls (Table II).

HLA-DRB1*04 allele percentages in the study groups. Positive HLA-DRB1*04 allele was recorded in 19 patients who were smokers, and 13 patients who were non-smokers

(total, 32% of 100 patients with RA), while the healthy controls recorded 3 patients positive for the HLA-DRB1*04 allele in smokers only (total, 6%) (Tables III and IV).

Link between the HLA-DRB1*04 allele and the parameters tested. The results demonstrated a significant association between the presence of the HLA-DRB1*04 allele, and increased levels of HLA-DRB1 gene expression, and ACCP and RF levels in patients with RA compared with the controls. Among the smokers, the HLA-DRB1*04-positive patients exhibited significantly higher gene expression levels (4.46 \pm 4.2 vs. 1.2 \pm 0.0 fold), ACCP levels (77.54 \pm 24.6 vs. 24.4 \pm 0.86 U/ml) and RF levels (138.48 \pm 140.1 vs. 24.83 \pm 0.15 U/ml) compared with the HLA-DRB1*04-positive controls (P=0.001), as shown in Table III and Fig. 2.

Similarly, the HLA-DRB1*04-negative patients who were smokers exhibited significantly higher gene expression levels (1.95 \pm 1.35 vs. 0.75 \pm 0.5 fold), ACCP levels (63.04 \pm 22.9 vs. 7.6 \pm 1.7 U/ml) and RF levels (76.12 \pm 86.6 vs. 17.2 \pm 4.3 U/ml) compared with the controls (P=0.001), as demonstrated in Table III and Fig. 2.

HLA-DRB1*04-positive patients who were non-smokers exhibited significantly higher Gene expression levels (1.75 \pm 0.82 vs. 0.4 \pm 0.0 fold), anti-CCP levels (65.71 \pm 36.22 vs. 8.28 \pm 3.98 U/ml), and RF levels (85.43 \pm 124.1 vs. 10.18 \pm 5.86 U/ml) compared with controls, (P=0.001), as shown in Table IV and Fig. 3.

Likewise, in the HLA-DRB1*04-negative non-smoker patients exhibited significantly higher gene expression levels (1.43 \pm 1.11 vs. 0.4 \pm 0.0 fold), ACCP levels (58.32 \pm 20.2 vs. 8.2 \pm 3.9 U/ml) and RF levels (44.39 \pm 24.4 vs. 10.1 \pm 5.8 U/ml) compared with the controls (P=0.001), as demonstrated in Table IV and Fig. 3.

HLA-DRB1*04 allele-positive allele in patients who were smokers and non-smokers. As presented in Table V and Fig. 4, patients with RA carrying the HLA-DRB1*04 allele who were smokers exhibited significantly higher HLA-DRB1 gene expression levels (4.46 \pm 4.2 vs. 1.75 \pm 0.82-fold, P=0.02), ACCP levels (77.54 \pm 24.6 vs. 65.71 \pm 36.22 U/ml, P=0.04) and RF

Table II. Percentage, range and mean of HLA-DRB1 gene expression between patients and controls.

Groups	No. of participants	Gene expression (%)	Range of the HLA-DRB1 gene (fold)	Mean ± SD	P-value
Patients	100	64	0.72-18.26	1.51±0.59	0.02 ^a
Controls	50	16	0.21-1.98	1.13±0.22	

Data are presented as percent, range and the mean ± SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP≤0.05, significant difference, compared between patients with RA and controls.

Table III. Association of the HLA-DRB1*04 (SE) allele with gene expression and autoantibody in patients with RA who were smokers and the controls.

Smokers group; patients (n=50); controls (n=25)	HLA-DRB1*04 allele	Gene expression (fold)	Mean ± SD		
			ACCP (U/ml)	RF (U/ml)	
Patients	+ve	19	4.46±4.20	77.54±24.6	138.48±140.10
Controls	+ve	3	1.20±0.00	24.40±0.86	24.83±0.15
P-value			0.001 ^a	0.001 ^a	0.001 ^a
Patients	-ve	31	1.95±1.35	63.04±22.90	76.12±86.60
Controls	-ve	22	0.75±0.00	7.60±1.70	17.20±4.30
P-value			0.001 ^a	0.001 ^a	0.001 ^a

RA, rheumatoid arthritis; SE, shared epitope; ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor. Data are presented as the mean ± SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP≤0.01, highly significant difference, compared between the smokers group of patients and controls.

Table IV. Association of the HLA-DRB1*04 (SE) allele with gene expression and autoantibody in patients with RA who were non-smokers and the controls.

Smokers group; patients (n=50); controls (n=25)	HLA-DRB1*04 allele	Gene expression (fold)	Mean ± SD		
			ACCP (U/ml)	RF (U/ml)	
Patients	+ve	13	1.75±0.82	65.71±36.22	85.43±124.1
Controls	-ve	25	0.4±0.0	8.28±3.98	10.18±5.86
P-value			0.001 ^a	0.001 ^a	0.001 ^a
Patients	-ve	37	1.43±1.11	58.32±20.2	44.39±24.4
Control	-ve	25	0.4±0.0	8.28±3.98	10.18±5.86
P-value			0.001 ^a	0.001 ^a	0.001 ^a

RA, rheumatoid arthritis; SE, shared epitope; ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor. Data are presented as mean ± SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP≤0.01, highly significant difference, compared between the non-smokers group of patients and controls.

levels (138.48±140.1 vs. 85.43±124.1 U/ml, P=0.01) compared with non-smokers.

*HLA-DRB1*04 allele-negative allele in patients who were smokers and non-smokers.* The effects of cigarette smoke on the levels of HLA-DRB1 gene expression, and ACCP

and RF levels in HLA-DRB1*04 allele-negative patients are demonstrated in Table VI and Fig. 5. The levels of the three parameters were significantly higher in the smokers than in the non-smokers (HLA-DRB1 gene: 1.95±1.2 vs. 1.43±1.1 fold, P=0.05; ACCP: 63.04±22.9 vs. 58.32±20.2 U/ml, P=0.05; and RF, 76.12±86.6 vs. 44.39±24.4 U/ml, P=0.04, respectively).

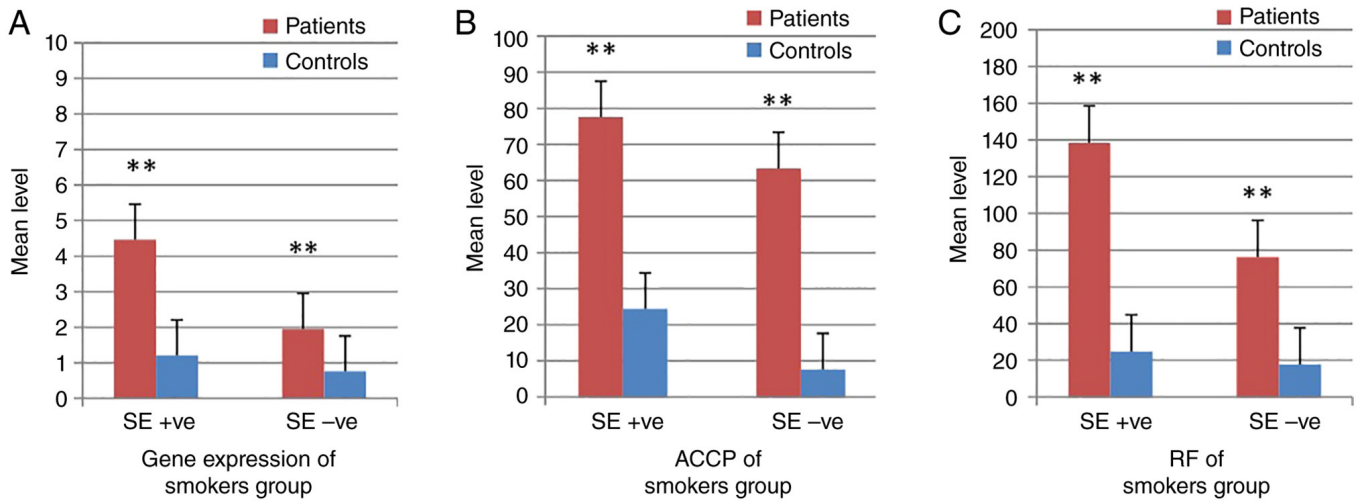


Figure 2. HLA-DRB1*04 allele in patients who were smokers and controls in relation to the mean levels of (A) HLA-DRB1 gene expression, (B) ACCP, and (C) RF. ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; SE, shared epitope. NS, no significant difference. ** $P < 0.001$, highly significant difference (comparison between patients and controls who were smokers).

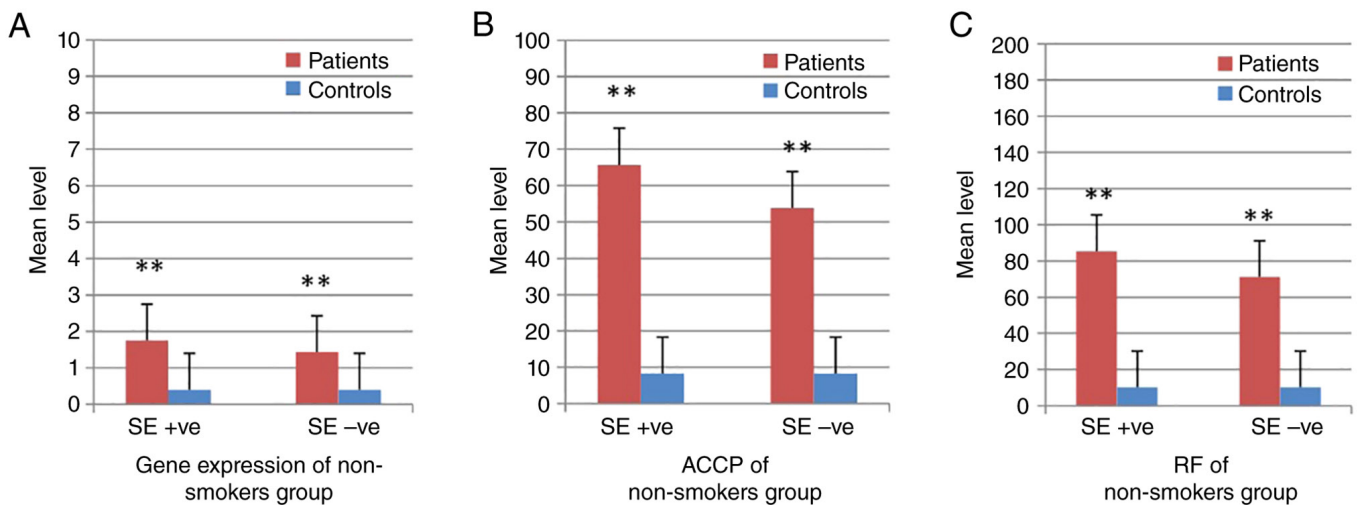


Figure 3. HLA-DRB1*04 allele in patients who were non-smokers and controls in relation to the mean levels of (A) HLA-DRB1 gene expression, (B) ACCP, and (C) RF. ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; SE, shared epitope. ** $P < 0.001$, highly significant difference (comparison between patients and controls who were non-smokers).

*Disease severity in relation to HLA-DRB1*04 and smoking status.* Regardless of disease severity, the highest DAS-28 values were observed in the HLA-DRB1*04-positive patients who were smokers (4.52 ± 0.9); these values were significantly higher compared to those in the HLA-DRB1*04-negative patients who were non-smokers (3.8 ± 1.02 ; $P = 0.04$). Conversely, no statistically significant difference was found when comparing HLA-DRB1*04-negative patients who were smokers (4.18 ± 0.8) with the HLA-DRB1*04-positive patients who were non-smokers (4.02 ± 0.85 ; $P > 0.05$) (Table VII and Fig. 6).

Discussion

RA is a chronic, autoimmune disease with destructive polyarthritis. It is multifactorial with prominent genetic-environmental interactions as the most key risk factors for the development of the disease. RA association with HLA-DRB1

alleles, including HLA-DRB1*04, which code to SE contain a common amino acids sequence in position of 70-74; this has been traditionally recognized as link the greatest heritable influence in disease susceptibility (19).

The present study demonstrated (Tables III and IV, and Figs. 2 and 3) the effects cigarette smoke on HLA-DRB1 gene expression levels; the quantity of HLA-DRB1 levels was affected by the cigarette smoke factor; the level was recorded as 4.46 ± 0.42 in smokers and 1.75 ± 0.82 in non-smokers. The risk of developing RA is associated to SE-positive HLA-DRB1 alleles (20). In the present study, the HLA-DRB1*04(SE) allele was found in 19 patients who were smokers, and in 13 patients who were non-smokers, with total a percentage of 32% (32/100) of the total RA patient group. Exposure to other environmental contaminants, including cigarette smoke, interacts with the HLA-DRB1*04 allele and markedly increases the risk of developing disease (21). The HLA-DRB1*04 allele percentage

Table V. Positive HLA-DRB1*04 allele in the patient group in relation to gene expression and autoantibodies.

Patients with positive HLA-DRB1*04 allele	n=32	Mean ± SD		
		Gene expression (fold)	ACCP (U/ml)	RF (U/ml)
Smokers	19	4.46±4.2	77.54±24.6	138.48±140.1
Non-smokers	13	1.75±0.82	65.71±36.22	85.43±124.1
P-value		0.02 ^a	0.04 ^a	0.01 ^a

ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor. Data are presented as mean ± SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP≤0.05, significant difference, compared between the smokers and non-smokers who positive HLA-DRB1*04 allele in the patient group.

Table VI. Negative HLA-DRB1*04 allele in the patient group in relation to gene expression and autoantibodies.

Patients with negative HLA-DRB1*04 allele	n=68	Mean ± SD		
		Gene exp. (fold)	ACCP (U/ml)	RF (U/ml)
Smokers	31	1.95±1.2	63.04±22.9	76.12±86.6
Non-smokers	37	1.43±1.1	58.32±20.2	44.39±24.4
P-value		0.05 ^a	0.05 ^a	0.04 ^a

ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor. Data are presented as mean ± SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP≤0.05, significant difference, compared between smokers and non-smokers who negative for the HLA-DRB1*04 allele in the patient group.

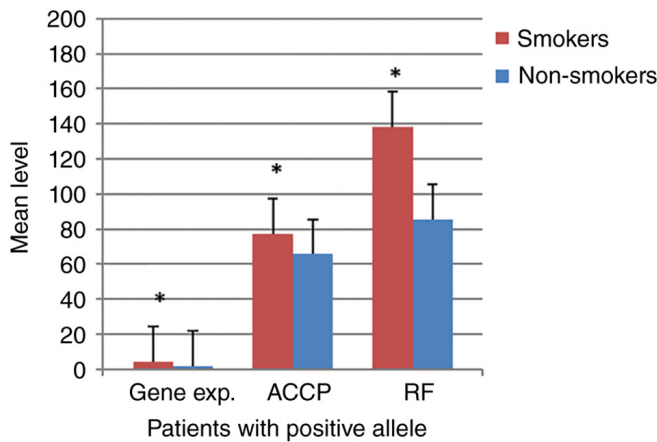


Figure 4. Positive HLA-DRB1*04 allele in patients who were smokers and non-smokers in relation to the mean levels of HLA-DRB1 gene expression, ACCP and RF. ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; SE, shared epitope. *P<0.05, significant difference (comparison between smokers and non-smokers who positive for the HLA-DRB1*04 allele in the patient group).

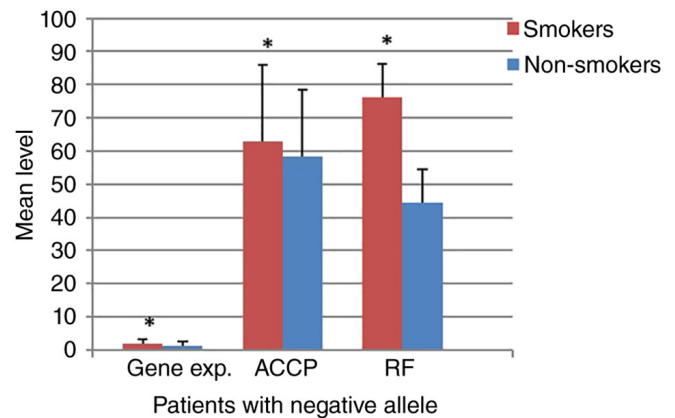


Figure 5. Negative HLA-DRB1*04 allele in patients who were smokers and non-smokers in relation to the mean levels of HLA-DRB1 gene expression, ACCP, and RF. ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; SE, shared epitope. *P<0.05, significant difference (comparison between smokers and non-smokers who were negative for the HLA-DRB1*04 allele in the patient group).

was previously recorded in Egyptian patients with RA as 60 and 25% in normal subjects (22). Another study in Iraq recorded the HLA-DRB1*04 allele in 70% of patients with RA and 23.3% in the controls (23). Another study in the Babylon governorate in Iraq recorded the HLA-DRB1*04 allele in 59% of patients with RA and 24.4% of the controls (24).

Positivity of the HLA-DRB1*04 (SE) allele was recorded in 32% of 100 patients with RA in the present study; this may

be due to the fact that herein, there were 75 female and 25 male patients with RA, and despite the fact that RA is more prevalent among women, it was previously shown that SE alleles were more frequently identified in men (25). In addition, the family history percentage of the participants in the present study was recorded as 19, and 81% had no family history (Table I). Another possible explanation for this variation may be geographic and racial factors; a local study in the

Table VII. Association between disease activity with HLA-DRB1*04 (SE) and smoking status.

Patient group		Mean \pm SD DAS-28	P-value
Smokers/SE-positive	19	4.52 \pm 0.9	0.05 ^a
Non-smokers/SE-negative	37	3.8 \pm 1.02	
Smokers/SE-negative	31	4.18 \pm 0.8	NS
Non-smokers/SE-positive	13	4.02 \pm 0.85	

SE, shared epitope; DAS, disease activity score. Data are presented as mean \pm SD, and analyzed using one-way ANOVA with Tukey's post hoc test; ^aP \leq 0.05, significant difference, compared between SE-positive smokers and SE-negative non-smokers; NS, not significant, compared between SE-negative smokers and SE-positive non-smokers.

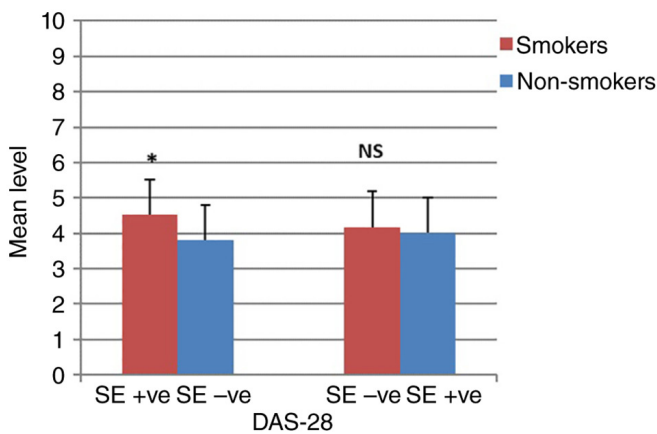


Figure 6. Disease activity (DAS-28) in relation to HLA-DRB1*04 (SE) and smoking. ACCP and RF. ACCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; SE, shared epitope; NS, not significant. *P \leq 0.05, significant difference between smokers who were SE-positive and non-smokers who were SE-negative.

Kurdish region in the north of Iraq reported a strong association between disease susceptibility, and HLA-DRB1*0404 and HLA-DRB1*0405 in patients with RA (26). It has been reported that some geographical areas have recorded no significant association between HLA-DRB1*04 and RA (27). According a previous study, there is no RA-specific HLA-DR; instead, the SE is coded by several HLA-DRB1 alleles (25).

In the present study, 29 out of the 32 HLA-DRB1*04-positive patients were ACCP-positive and 25 were RF-positive; HLA-DRB1 alleles are the sole risk factor for RA among individuals with positive ACCP antibodies, according to two sizable cohort studies conducted in the USA and Europe using various genetic epidemiology techniques (28,29). SE-positive HLA-DRB1 alleles are linked to RA that is ACCP-positive rather than ACCP-negative, that presents them to T-cells that aid in the production of ACCP-producing B-cells; ACCP is linked to the pathophysiology of RA (30).

The genetic findings in the present study (Tables III and IV, and Figs. 2 and 3) indicate a positive association between HLA-DRB1 gene expression and the levels of ACCP and RF, with statistically significant differences observed between patients who were smokers and non-smokers (P \leq 0.01). Evidence suggests that genetically susceptible individuals exposed to various environmental pollutants have a substantially increased risk of developing RA (31). In the present study,

HLA-DRB104-positive patients exhibited higher ACCP levels (77.54 \pm 24.6 U/ml) compared with HLA-DRB104-negative patients (58.32 \pm 20.2 U/ml). ACCP antibodies are strongly associated with the presence of the HLA-DRB1*04 allele (32).

Certain serological indicators for RA, namely in individuals who test positive for HLA-DRB1*04, have been demonstrated to be ACCP positive; these findings suggest that ACCP may be directly linked to RA (20). It has been noted that patients with RA with SE alleles have three times the frequency of positive titers for antibodies against citrullinated peptides compared to those without them (33).

Effects of smoking on SE-positive and -negative patients.

In the present study, SE-positive patients who were smokers had higher ACCP levels than SE-positive patients who were non-smokers; SE-negative patients who were smokers had higher levels ACCP than those who were non-smokers (P \leq 0.05) (Tables V and VI, and Figs. 4 and 5). A large US study indicated that the degree of smoking exposure is crucial in supporting the function of SE-smoking interaction (34). It has been mentioned that tobacco exposure increases the risk factor for ACCP antibodies only in SE-positive patients with RA and is not observed in undifferentiated arthritis (35).

In present study, the larger percentage of patients developed RA with no genetic or familial susceptibility. Although RA susceptibility is genetically defined, it has been proposed that non-genetic (i.e., environmental), epigenetic, or post-translational processes may be responsible for the onset of the illness (36). Cigarette smoking and HLA-DRB1SE alleles have been shown to interact strongly, which aids in the development of ACCP positive RA (4). High levels of both ACCP and RF may be linked to smoking. Notably, herein, RF levels were increased in all patients, although the effect of smoking on ACCP levels was only observed in those with SE alleles. Quitting smoking prior to the onset of RA reduces the chance of future ACCP-positive disease (37).

In the present study, HLA-DRB1*04 positive patients who were smokers had higher RF levels than HLA-DRB1*04-positive non-smokers; HLA-DRB1*04-negative patients who were smokers had higher RF levels than non-smokers (P \leq 0.05). It has been shown that smokers have increased levels of RF and are more prone to developing RA (13). Both current and former smokers have an increased prevalence of developing elevated RF levels compared to non-smokers, and the proportion of smokers increases with increasing RF titers (35).

*Disease severity in relation to HLA-DRB1*04 and smoking status.* The risk of developing RA increases with cigarette smoking, which may also have a dose-dependent negative effect on the severity of RA. Tobacco smoke exposure is associated with RF positivity among subjects without RA, by increasing RF levels and altering immune function both in the lungs and systemically; smoking may have a biological link on the course of RA illness; thus, cigarette smoke has a marked effect on the development and severity of RA (38).

The present study demonstrated (Table VII and Fig. 6) the DAS-28 values in relation to both risk factors cigarette smoke and genetic susceptibility. The analysis of the results revealed that the DAS-28 value of HLA-DRB1*04-positive smokers differed significantly with that of HLA-DRB1*04-negative non-smokers ($P \leq 0.05$); no significant difference was observed between the DAS-28 values of HLA-DRB1*04-negative smokers and HLA-DRB1*04-positive non-smokers. The results of the present study demonstrated that the effect of cigarette smoke as a single risk factor is equipotent to the presence of the SE allele alone, which reflects the importance of the smoking-HLA-DRB1*04 interaction in the adverse progression of RA (35). The highest DAS-28 value (4.52 ± 0.9) was associated with the highest ACCP level (77.54 ± 24.6 U/ml), a strong association of HLA-DRB1*04 with anti-CCP positivity, and disease activity was recorded in patients with RA (30).

The present study had certain limitations which should be mentioned. The sample size was relatively modest, particularly in the HLA-DRB104-positive control group, which may have limited the statistical power and generalizability. In addition, the cross-sectional study design precluded the assessment of causal associations between smoking, HLA-DRB1*04 allele status and RA severity.

Furthermore, the majority of patients were receiving anti-rheumatic treatment at the time of sampling, which may have influenced autoantibody levels, gene expression and DAS-28 scores. Environmental and lifestyle factors other than smoking were not fully controlled. Finally, the genetic analysis was limited to the HLA-DRB1*04 allele and did not include other SE alleles or gene-gene interactions. Further longitudinal studies with larger cohorts and expanded genetic analyses are warranted.

In conclusion, cigarette smoking, as an independent risk factor, exerts an effect comparable to that of the SE-positive allele. However, the interaction between cigarette smoking and SE positivity produces the greatest severity of RA. The highest levels of ACCP antibodies were observed in patients who were smokers carrying SE-positive alleles. These findings highlight the critical importance of integrating genetic screening strategies with robust smoking cessation programs for individuals at elevated risk. Further studies are required to explore the molecular pathways via which smoking interacts with particular common epitope alleles in the Iraqi population.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Both authors (KE and DSS) were involved in the conceptualization of the study and in the study methodology. KE was involved in data investigation and data collection, and in the writing of the original draft of the manuscript. DSS was involved in the formal analysis and data interpretation, and in the writing, reviewing and editing of the manuscript. DSS also supervised the study. Both KE and DSS confirm the authenticity of all the raw data, authors have read and approved the final manuscript.

Ethics approval and consent to participate

Participants were provided with detailed information about the study objectives and signed an institutional informed consent form in their native language, including the purpose of the study, detailed procedures, and any potential benefits of participation. Healthy participants voluntarily participated in the present study. Formal approval was obtained from the Ethics Committee/College of Science/University of Baghdad, reference no. CSEC/1123/0101, November 2, 2023. Official letter/Ministry of Health No. 48321-December 25, 2023.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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