DRB1 allele frequencies in rheumatoid arthritis patients and their correlation with anti-cyclic citrullinated peptide antibodies severity marker

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Abstract

Genetic influence, mainly the HLA-DRB1 on the predisposition of rheumatoid arthritis (RA) disease course is highly contradictive. This study aimed to shed spot light on the DRB1 genetic constitutions and their influence on the anti-citrullinated peptide autoantibodies (ACCP) modulation as a disease activity marker in Iraqi RA patients. A total of 110 RA patients and 50 healthy controls were enrolled in the study. Their HLA-DRB1 allelic constitution and ACCP serum level were evaluated highly sensitive ELIZA technique. The results reveals that the highest DRB1 allele frequencies were in *03, followed by *13, *11, *04, and *07. The majority of Iraqi patients were with positive ACCP and the frequency of this positivity were highest in *10, *16, followed by *04, *03, *11, *08, and *07, allele groups. This study also found that the highest ACCP mean titers were in the allele *13 followed by *11, *04 and *07. In addition, DRB1 *13, 13 homozygotic allelic combination was the highest in frequency (9.1%) and mean titer (3271.7 U/ml) among positive ACCP combinations, followed by *11, 7, *11, 4 and 11, 15. Finally, 44% of the RA-patients DRB1 allelic combination were RA-restricted, whereas 57.1% of the healthy control DRB1 allelic combinations were control-restricted. In conclusion, the distribution of the DRB1 alleles among Iraqi RA patients as well as their ACCP positivity was not subjected to the SE allele roles, and in addition, the presence of RA-restricted and control-restricted allelic combinations is a novel result and of value in differentiating the at-risk from the protecting allelic combination.

Key words: rheumatoid arthritis, HLA DRB1* alleles, anti-CCP, shared epitope, Iraq.

الخلاصة

يتميز التأثير الوراثي المتعلق بأنسجة الخلايا اللمفاوية للانسان خصوصا (HLA-DRB1) على نزعة الإصابة بالتهاب المفاصل الرثواني بكونه متباين جدا. هذه الدراسة سعت لتسليط الضوء على التركيب الوراثي لل(DRB1) ودوره في تعديل الأضداد الذاتية للببتيدات السترولينية (ACCP) كعلامة للنشاط المرضي في مرضى التهاب المفاصل الرثواني العراقيين. شملت الدراسة 110 مريض و 50 من الاصحاءوتم تقييم التركيب الاليلي لل (DRB1) والمستوى المصلي لل (ACCP). أظهرت النتائج أن أعلى تكرارية لل(DRB1) كانت للاليل 03* ثم 13*, 11*, 04* و 07*. غالبية المرضى كانوا ايجابيين لل (ACCP) وكان أعلى تكرارية لهذه الايجابية في الاليلات 10*و16* أعقبها الاليلات 04*, 03*, 11*, 08*و 07*. الدراسة وجدت أيضا أن أعلى معدل معياري لل(ACCP) كان في الاليل 13* اعقبه الاليلات 11*, 04*و 07*. إضافة إلى ذلك كان الزيجوت المتماثل الالائل 13,13* الأعلى من حيث التكرارية والمعدل المعياري لأضداد ال(ACCP) في المرضى الموجبين لهذا المعلم أعقبه 11,7* , 11,4*و 11,15*. أخيرا, كان 44% من الاليلات المركبة بين مرضى التهاب المفاصل الرثواني العراقيين هو غير مرتبط بقواعد التوزيع المستندة على المحددات المستضدية المشتركة (shared epitopes) كما ان وجود اليلات مركبة خاصة بالمرض فقط واليلات مركبة خاصة بالأصحاء فقط هو نتيجة جديدة وغير معلومة سابقا وستكون ذات فائدة كبيرة في تمييز من لديه حماية أو استعداد للإصابة بمرض التهاب المفاصل الرثواني.

الكلمات المفتاحية : التهاب المفاصل الرثوي , التركيب الوراثي (DRB1), مضادات الببتيدات السترولينية ,المحددات المستضدية المشتركة, العراق.

Introduction

Rheumatoid arthritis (RA) is a chronic arthritis condition that can lead to deformities and disabilities. The exact pathogenesis is unknown, but both genetic and environmental factors play key roles in this disease process [Sivalingam et al., 2007]. The prevalence of RA is about 1% of the world population, and genetic factors have been estimated to account for 60% of the disease risk [Turesson C *et al.*, 2006]. Human leukocyte antigens (HLA) account for about 50% of the genetic predisposition in most autoimmune diseases [Thorsby E *et al.*, 1997] which play a central role in the immune response by presenting processed antigenic peptides to T cells. The frequency of different HLA-DRB1 allelic groups, alleles and suballeles among RA patients of different racials and ethnicities as well as the role of these alleles in the protection or predisposition for RA is still highly contradictive [Kochi Y et al .,2009]. It is widely accepted that the shared epitope (SE), a common amino acid sequence located from the 70th to the 74th amino acids of the HLA-DR β chain, explains the associations of specific HLA-DRB1 alleles (mainly DRB1*0101, DRB1*0102, DRB1*0401, DRB1*0404,*DRB1**0405, *DRB1**0408, *DRB1**1001, and *DRB1**1402) with RA [Korendowych E *et al* .,2003].

Despite significant improvement in molecular biology techniques, association mechanisms between *HLA-DRB1**SE^+ alleles and RA remain debated and authors have demonstrated that each SE allele does not confer the same risk [Tézenas S et al., 2000]. Rheumatoid factor (RF) has been the only serologic marker in the American College of Rheumatology (ACR) classification criteria for several decades [Arnett F et al., 1988]. RF is a valid prognostic indicator, but the antibody's usefulness for early detection of RA is limited by its moderate sensitivity and relatively low specificity [De Rycke L et al., 2004]. In recent years, the antibody to cyclic citrullinated peptide (anti-CCP) has been shown to be more specific than and as sensitive as RF for diagnosing RA [Forslind K et al., 2004]. Studies indicating that anti-CCP is a better diagnostic tool than RF have been reported for several years and an excellent positive and negative predictive value for RA diagnosis [Ateş A et al.,2007]. The presence of anti-CCP is associated with the severity of the disease, and it predicts radiological joint damage in early RA [Bongi S *et al.*, 2004].

The aims of this study were to determine the frequency of HLA DRB1 alleles in Iraqi RA patients and then their modulation of the immune response as reflected on the positivity and/or the level of positivity of anti-CCP as disease activity markers.

Patients and methods:

This study included 110 patients (102 females and 8 males) with an age range of 24-71 years (average 45.6 ± 12.245) fulfilling the American College of Rheumatology (ACR) criteria for RA. All the subjects were patients of the rheumatology clinic at medical city of Baghdad. Unrelated, apparently healthy Iraqi individuals (50) were randomly chosen as controls. They were refereed to immunology and tissue typing center in Al-Karamah teaching hospital for tissue typing. Blood sample were collected in EDTA tubes from patients and controls. DNA was extracted from the blood using the QIAamp DNA blood mini kit (Innogenetics, Belgium) and the HLA DRB1 alleles were determined in patients and controls by applying polymerase chain reaction with sequence specific primers (PCR-SSP) using the above kit, HLA typing tests are based on the reverse hybridization principle. At first DNA was extracted using QIAamp DNA mini kit. Using an amplification kit(INNO-LiPA HLA DRB1Amp plus) which

contained HLA DRB1 primer solution that contains primers for HLA DRB1 alleles from 1 to 16, an amplified biotinylated DNA material was prepared ,the amplification kit was based on the polymerase chain reaction (PCR). Amplified biotinylated DNA material was chemically denaturated, and the separated strands are hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips already provided. This is followed by a stringent wash step to remove any mismatched amplified material. After the stringent wash, streptavidin conjugated with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with a substrate solution containing a chromogen results in a purple/brown precipitate on specific INNO LIPA strips, the reaction is stopped by a wash step, and the reactivity pattern of the probes is recorded. the corresponding lines on the strips are analysed using specific LIPA interpretation software (LIRAS^{TM}), where each line on the strip corresponds to a particular HLA DRB1 allele group. Anti cyclic citrullinated peptide (ACCP) antibodies was measured using IMTEC-CCP ELISA kit (human, Germany), and rheumatoid factor (RF) antibodies was measured using RF-Screen ELISA kit (Immuchem , Belgium). The chi square test was used for categorical variables, Man Whitney test for variable that are not normally skewed. P value < 0.05 were considered statistically significant

Results:

The results demonstrated the frequencies of HLA-DRB1 allele groups among RA patients compared to their correspondents in healthy controls. Allele groups that showed a significant difference between the two studied groups with a higher frequency among the healthy controls were DRB1*02 (OR= 3.444, CI at 95%= 2.393-4.406, and *P = 0.003*) and DRB1*06 (OR= 3.5, CI at 95%= 2.727-4.493, and P= 0.001). Allele groups that showed a significant difference between the two studied groups with a higher frequency among the RA patients were DRB1*03 (OR= 2.4, CI at 95%= 1.137-4.946, and P= 0.014), DRB1*08 (OR= 3.5, CI at 95%= 0.758-47.483, and P= 0.046) and DRB1*13 (OR= 2.2, CI at 95%= 0.972-5.053, and P= 0.039). HLA-DRB1 allele groups 01, 04, 05, 07, 09, 10, 11, 14, 15, and 16 showed no significant differences between RA patients and healthy controls, whereas DRB1*12 was not exists among any member of both study groups. (Table 1)

Table1. Frequency of DRB1 allele groups among RA patients and healthy controls.

DRB1 alleles	RA patients (n=110)		Control (n=50)		*P-value*	Odd ratio (OR)	CI
	№	%	№	%			
01	6	5.5	6	12	0.13	0.423	0.129-1.384
02	**0**	**0**	**5**	**10**	**0.003***	**3.444**	**2.693-4.406**
03	**50**	**45.5**	**17**	**34**	**0.014***	**2.4**	**1.137-4.946**
04	28	25.5	14	28	0.438	0.878	0.414-1.862
05	0	0	1	2	0.313	3.245	2.571-4.096
06	**0**	**0**	**6**	**12**	**0.001***	**3.5**	**2.727-4.493**
07	14	12.7	10	20	0.169	0.583	0.239-1.422
08	**12**	**10.9**	**1**	**2**	**0.046***	**6**	**0.758-7.483**
09	0	0	1	2	0.313	3.245	2.571-4.096
10	2	1.8	1	2	0.678	0.907	0.080-10.246
11	34	30.9	16	32	0.515	0.951	0.463-1.951
13	**36**	**32.7**	**9**	**18**	**0.039***	**2.2**	**0.972-5.053**
14	14	12.7	4	8	0.278	1.677	0.523-5.379
15	6	5.5	6	12	0.13	0.423	0.129-1.384
16	2	1.8	2	4	0.37	0.444	0.061-3.249

*= Significant, CI= confidence interval.

The relationship between ACCP results and single DRB1 allele groups were demonstrated in table (2). Overlooking of these results would give the impression of a noticeable link between the HLA-DRB1* 03, 04, 08, 11, and 15 allele groups and ACCP positivity, whereas HLA-DRB1* 01 and 13 (and for less extent 07 and 14) allele groups exhibited a more tendency towards ACCP negativity. However, a statistical significant differences for these results reveals a *P value* of less than 0.005 (significant) for the HLA-DRB1* 01 and 13 only as an ACCP negative allele groups. Allele groups HLA-DRB1* 02, 05, 06, 09, and 12 were absent from all members of RA patients. On the other hand, the mean value of the ACCP titer and its association with the allele groups had expressed a higher level among the HLA-DRB1*13 (2857.3 U/ml), *11 (1205.5 U/ml) and *04 (1169.7 U/ml) whereas the allele groups *03, *07, *08, *10, *14, *15 and *16 had expressed a positive ACCP result with a mean titer ranging from low (72.8 U/ml) to intermediate (849.1).

Table 2. Results of ACCP among RA patients according to allele group distribution.

DRB1 alleles	Patients (n=110)				*P value*	Odd ratios (OR)	CI
	ACCP +ve		ACCP -ve				
	№ (mean titer)	%	№	%			
01 n=6	**0**	**0**	**6**	**100**	**0.003***	**2.7**	**2.124-3.526**
03 n=50	32 (390.3)	68	18	32	0.279	1.359	0.629-2.939
04 n=28	20 (1169.7)	71.4	8	28.6	0.113	1.957	0.773-4.952
07 n=14	6 (849.1)	42.9	8	57.1	0.517	0.874	0.281-2.717
08 n=12	10 (72.8)	50	2	50	0.07	3.750	0.780-18.025
10 n=2	2 (397.2)	100	0	0	0.358	1.688	1.443-1.973
11 n=34	20 (1205.5)	58.8	14	41.2	0.515	0.932	0.409-2.123
13 n=36	**12 (2857.3)**	**33.3**	**24**	**66.7**	**0.000***	**2.5**	**1.589-3.830**
14 n=14	6 (173.3)	42.9	8	57.1	0.134	0.450	0.144-1.402
15 n=6	4 (682)	33.3	2	66.7	0.544	1.355	0.237-7.734
16 n=2	2 (290.2)	100	0	0	0.358	1.688	1.443-1.973

*= Significant, CI= confidence interval.

The highest number of HLA-DRB1 combined allele groups that exhibited an absolute positivity for ACCP serology with a significant difference was HLA-DRB1*13,13 (n=10), followed by HLA-DRB1*11,4 (n=8) then HLA-DRB1* 3,3 and 3,7 (n=6 for each). Other combinations of allele groups with an absolute ACCP positivity were with no significant differences. HLA-DRB1* 13,11 was with the highest frequency (n=6) among the combined allele groups with an absolute negativity for ACCP serology, with a significant difference, followed by allele groups 13,7, 13,1 and 13,4 (n=4 for each) with a significant difference also. All other combinations of alleles groups in Table 4 which exhibited an absolute negativity for ACCP serology were with no significant difference. In the same table, allele combinations of 3,13 3,4 3,11 and 3,14 exhibited a variable results of ACCP. The differences between each of the ACCP categorized groups was significant ($p<0.05$) when a comparison was made between the ACCP positive (50/110 represents 45.5%) X ACCP negative (28/110 represents 25.5%) groups and the ACCP positive X ACCP variable (32/110 represents 29%) groups, whereas this difference was not significant when ACCP negative group was compared with the ACCP variable group.(table 3).

Table 3. Categorization of the HLA-DRB1 combination alleles according to their ACCP results.

Combined HLA-DRB1 allele groups (№ of cases = 110)						
with positive ACCP results (P)			with negative ACCP results (N)		with variable ACCP results (V)	
(№)	FR %	Mean titer	(№)	FR %	(№)	FR %
13,13 (10)	9.1	3271.7	13,7 (4)	3.6	3,13 n=6	5.5
3,3 (6)	5.5	160.4	13,11 (6)	5.5	3,4 n=8	7.3
3,7 (6)	5.5	319	13,1 (4)	3.6	3,11 n=8	7.3
3,8 (4)	3.6	98.2	13,4 (4)	3.6	3,14 n=10	9.1
11,4 (8)	7.3	1963.5	13,8 (2)	1.8		
11,7 (2)	1.8	2439.2	3,15 (2)	1.8		
11,8 (4)	3.6	68.4	11,14 (2)	1.8		
4,10 (2)	1.8	397.1	11,1 (2)	1.8		
4,14 (2)	1.8	70.5	4,7 (2)	1.8		
4,16 (2)	1.8	290.2				
15,8 (2)	1.8	31.1				
15,11 (2)	1.8	1332.8				
Total (50)	**45.5**	**1221.8**	**Total (28)**	**25.5**	**Total (32)**	**29**
Statistics						
P X N groups= <0.05*		**P X V groups= <0.05***		**N X V groups= >0.05**		

FR= Frequency, * = Significant.

A comparison in the frequency of the HLA-DRB1 allelic combinations between the ACCP different category results of the RA patients and the control groups is illustrated in Table 4. For the HLA-DRB1 allele combination with positive ACCP results, there was 12 combinations with a frequency of 45.5% of the total RA patients, whereas only 5 of these 12 allelic combination was found with the control group representing a frequency of 18% of the total number of controls. This difference was significant ($p<0.05$). Patients with negative ACCP results were 9 HLA-DRB1 allele combinations representing 25.5% of the total RA patients. Only 5 of these combinations were found in the control group representing 18% of their total. The difference between the two groups was not significant. For the RA patients group which exhibited a variable ACCP results, the DRB1 allele combinations were 4 representing 29% of the total RA patients. The same allelic combinations were also found in the control group representing a frequency of 16% of the total controls with no significant difference between the two groups. Finally, this study had found 11 DRB1 combinations restricted to the RA patients only (44% of the total) and 20 DRB1 combinations restricted to the control group only (57.1% of the total).

Table 4. Matching of HLA-DRB1 combined allele frequency in RA patients according to their ACCP results with healthy controls.

Combined alleles					
With +ve ACCP		**Control № (FR%)**	**With –ve ACCP**		**Control № (FR%)**
Allele	**Patients (P) № (FR%)**		**Allele**	**Patients (N) № (FR%)**	
13,13	10 (9.1)	1 (2)	13,7	4 (3.6)	2 (4)
3,3	6 (5.5)	1 (2)	13,1	4 (3.6)	1 (2)
3,7	6 (5.5)	2 (4)	3,15	2 (1.8)	1 (2)
11,4	8 (7.3)	3 (6)	11,1	2 (1.8)	3 (6)
11,7	2 (1.8)	2 (4)	4,7	2 (1.8)	2 (4)
3,8 11,8 15,8 4,10 4,14 4,16 15,11	18 (16.4)	0 (0)	13,11 13,4 13,8 11,14	14 (12.7)	0 (0)
[12 comb] 50 (45.5)		**[5 comb] 9 (18)**	**[9 comb] 28 (25.5)**		**[5 comb] 9 (18)**

Combined alleles			
With variable ACCP results		**Control № (FR%)**	**Control-restricted alleles № (FR%)**
Allele	**Patients (V) № (FR%)**		
3,13	6 (5.5)	1 (2)	1,4 1,14 2,5 2,6 2,8 2,10 2,11 3,6 4,4 4,6 4,13 4.15 6,6 6,11 7,15 9,13 11,16 13,14 14,15 15,16
3,4	8 (7.3)	1 (2)	
3,11	8 (7.3)	5 (10)	
3,14	10 (9.1)	1 (2)	
[4 comb] 32 (29)		**[4 comb] 8 (16)**	**[20 comb] 24(48)**

Statistics		
P X C1: <0.05*	**N X C2: >0.05****	**V X C3: >0.05****

*: Significant, **: Not significant, [comb]= number of allelic combinations

Discussion

This study had found that HLA-DRB1*02, *05, *06, and *09 allele groups were completely missing in RA patients compared to the healthy controls (with or without significant differences) which points out their protective role against RA among Iraqi population. Similar results were reported in other study, which found HLA DRB1*2, 5, 6 absent in RA patients, while HLA DRB1*9 were present in negligible number in patients and control [Ali A *et al.*, 2006]. On the other hand, HLA-DRB1*03, *08 and *13 allele groups were significantly higher in RA patients than healthy controls indicating the possible predisposition effect of these alleles in RA patients (Table 1). All other HLA-DRB1 allele groups in the patient's group were either with higher frequency (*14) or lower frequency (*01, *04, *07, *10, *11, *15 and *16) than control group but with no significant differences. Before discussing such piece of this study results, we have to remember that the shared epitope (SE) hypothesis described the relationship between HLA-DRB1 and RA [Holoshitz J., 2010]. HLA-DRB1 alleles encoding the SE (DRB1*01, *04, *10, and *14) are

assumed to be associated with severity of RA and have been more recently related with production of anti-citrullinated peptide autoantibodies (ACCP), whereas SE negative genotypes (mainly DRB1*11 and *13) might provide protection against RA susceptibility [Bax M *et al.,* 2011]. Accordingly, the above mentioned results are inconsistent with the SE hypothesis concerning the HLA-DRB1 allelic distribution among Iraqi RA patients. The reason of such contradictory could be in part due to that the major relationship of particular HLA alleles with RA is not constant in all human populations, different geographical areas, or among different ethnic groups [Kochi Y *et al.,* 2009]. Many literatures were investigated the biogeographic distribution of HLA-DRB1 alleles in various ethnicities and races around the world groups [Kochi Y et al., 2009]. For example HLA-DRB1*04 allele was found to be more frequent in RA patients in Morocco [Atouf O *et al*., 2008], and in Iran [Sandoughi M *et al*., 2011] but with no significant difference compared to healthy controls.

In Syrians, the frequency of HLA-DRB1*04 as well as *01 and *14 exhibited a strong association with the disease susceptibility [Jamil Mourad et al.,2013]. However, Peruvians [Castro F *et al.,* 2001], Mexican Americans [del Rincon et al.,1999] and Pakistanis [Ali A *et al*.,2006] showed no significant correlation between HLA-DRB1*04 and RA susceptibility which is similar to the results of the current study (OR= 0.878, 95% CI= 0.414-1.862, P= 0.438). Concerning HLA-DRB1*09 allele group which was completely missing in RA patients of this study, was reported to be associated with RA proneness among Turkish [Uçar F *et al.*,2012], Malaysians [Kong K et al.,2002] and Koreans [Lee H *et al.*,2004], whereas in some other studies, this allele was found in a negligible frequency in both RA patients and healthy controls with no significant difference [Ali A *et al.*,2006].

Unlike the results of our study, it had been reported that DRB1*01 was highly frequent among RA patients in many world populations like Italians [Bongi S et al.,2004], Turkish [Uçar F *et al*.,2012], Brazilians [Louzada-Junior P et al.,2008] and Pakistanis [Ali A *et al*.,2006]. In this study, the frequency of HLA-DRB1*14 was higher among the RA patients but with no significant difference, whereas HLA-DRB1*08 was also higher among RA patients with significant difference (OR= 6, 95% IC= 0.758- 7.483, P= 0.046). Similar results were reported in Peruvians [Castro F et al., 2001], Mexican Americans [del Rincon et al.,1999] and Saudi Arabians [Al-Swailem R et al.,2006]. The protective role of DRB1*02 and *06 alleles which is reported in our study was similar to some other studies [Swailem R et al., 2006 and Larsen B et al., 1989].

For DRB1*13, the role of this allele in protection or predisposition for RA is highly controversial. In some literatures, it had been concluded that this allele is highly protective against RA [Jamil Mourad *et al.,* 2013], or it might play a dual role in the development of RA, by protecting against ACCA-positive RA but, in combination with DRB1*03, increasing the risk of ACCP-negative RA [Lundström E et al., 2009]. In our study, HLA-DRB1*13 seems to be predisposing rather than protecting against RA as it was more frequent among the RA patients than healthy controls with significant value. A study conducted in 2011 stated that only DRB1*1301 is associated with protection, whereas all other DRB1*13 alleles might be associated with risk of developing RA. [Bax M *et al.,* 2011]. This would come in consistency with the results of this study as the non-*1301 HLA-DRB1 alleles frequency (including *1302, *1316, *1328, *1335, *1351, *1361, *1373, and *1374) was found to be much higher than the *1301 HLA-DRB1 frequency (data not shown in this paper). The frequency of DRB1*03 allele in this study was the highest among both patients and control (45.5% and 34% respectively) compared to other DRB1

allele groups. The difference in this frequency was significantly higher among RA patients compared to the healthy controls; a result looks for certain extent an unusual. In certain studies, *03 allele group frequency is almost lower in the RA patients than in the controls [Sandoughi M et al., 2011] or nearly the same with no significant difference from healthy controls [Ali A *et al.*,2006].

These overall differences between our study and other studies can be explained by many additional reasons. First, factors causing genetic variation do so in response to the development, migration, and structure of populations. Due to human history, each population has been exposed to different environments, likely creating different evolutionary pressures to preserve or delete genetic variants in their genomes [Bamshad M et al., 2003]. Second, is related to the MHC which is a dense cluster of over 260 genes on chromosome 6p21.3 containing a high percentage of immune-related genes, in particular the highly polymorphic human leukocyte antigen (HLA) genes involved in antigen presentation and thus any gene in this dense region has the opportunity to be expressed in any geographical area of the world [Kelley J et al., 2005]. Third, the number of RA-associated DRB1 genes present in the genotype configuration of RA patients influences the level of DRB1 gene expression in peripheral B cells, independently of disease activity, severity and drug regimen. This particular regulation of DRB1 gene expression in RA either genetically determined or resulting from unidentified modulating factors may be part of the molecular mechanisms involved in the association of RA with the HLA class II component [Kerlan-Candon et al., 2001]. One more thing about the modern classifications of the protective and predisposing DRB1 alleles. One of these classifications is the Tezenas du Montcel system which divided the DRB1 alleles into susceptible (S) and non-susceptible (X) alleles according to the presence or absence of the amino acid sequence RAA at amino acid positions 72-74 [Tezenas du Montcel S *et al.*, 2005].

Other classification is the De Vries system who defined the DRB1 SE susceptibility sequences as those encoding the amino acids LQKAA, LQRAA, and LRRRA at positions 67, 70, 71, 73, and 74 in the third hypervariable region. The SE-negative alleles were further subdivided as protective (P) and neutral (N) alleles according to the amino acid at position 67, with an isoleucine (I) conferring protection [De Vries N et al., 2002]. In addition, many other classification systems are suggested, hence our future plan is to classify the DRB1 alleles in RA Iraqi patients according to the most reliable and credible updated system as soon as all facilities and finance for such project become feasible.

Table (2) illustrates the results of anti-citrullinated peptide (ACCP) among RA patients according to the allele group distribution. Concerning the frequency of DRB1 alleles with positive ACCP compared to negative ACCP results among RA patients, allele groups *03, *04, *08, *10, *11, *15, and *16 were with higher positive ACCP than negative ACCP frequency but with no significant differences. Other DRB1 alleles were with lower positive ACCP than negative ACCP frequency with significant differences (DRB1*01 and *13) or without significant differences (DRB1*07, and *14). On the other hand, categorization of the positive ACCP DRB1 alleles according to their mean titer reveals that DRB1*13 was with the highest mean titer (2857.3 U/ml) followed by *11 (1205.5 U/ml), and *04 (1169 U/ml). The lowest titer was with the alleles *08 (72.8 U/ml) and *14 (173.3 U/ml).

Patients with RA show considerable variability in disease activity, which can be difficult to predict at the onset of disease. Anti-CCP antibodies have proven useful in identifying those patients who are likely to have clinically significant disease activity [Pinheiro GC., 2003]. It also correlated with higher ESR, CRP, swollen joint count,

and worse physician global assessment ratings [Kastbom A., 2004]. Back to the SE hypothesis, some literatures had referred to certain association between DRB1 SE alleles and ACCP-positive RA but not associated or only weakly associated with ACCP-negative RA [Ohmura K et al., 2010, Morgan AW et al., 2009] which suggests that ACCP-negative RA is genetically distinct from ACCP-positive RA.

When we apply such hypothetical aspect on the current study, we find it only partially applicable with RA Iraqi patients as two out of the four main DRB1 SE alleles (*4 and *10) were with high frequency for positive ACCP results, whereas the other two DRB1 SE alleles (*01 and *14) were with low frequency of positive ACCP results.

Apart from the qualitative aspects of ACCP positivity for these SE alleles, only *04 was found to be with a high mean titer of ACCP (1169.7 U/ml) compared with the other three SE alleles (*01, *10, and *14) which were ranging from 0 U/ml – 397.2 U/ml. Very similar results were noticed among Portuguese population, in which HLA-DRB1*04 and HLA-DRB1*10 groups were found highly associated with rheumatoid arthritis [Ligeiro D et al.,2007]. The association between the non-SE DRB1 alleles (*03, *07, *08, *11, *13, *15, and *16) and the ACCP results was variable. Take the allele group *13 as an example; in spite of the low frequency of positive ACCP among the carriers of this allele in RA patients (33.3%) compared to the healthy controls (66.7%), the mean titer of ACCP was the highest among all other allele groups (2857.3 U/ml) within the RA patients group. This difference between the qualitative and quantitative scales in this allele reflects the duel effects of the allelic contents of this allele group on the amelioration of the immune response in RA patients. Similar results were noticed in other study which found that 6 of 7 (85%) patients carrying DRB1*13 were ACCP positive and would produced significantly more ACCP than did any other non-SE HLA-DRB1 subtypes ($P < 0.01$) [Kapitány A et al.,2008]. In summary, the frequency of ACCP positivity as well as the mean titer of ACCP positive in Iraqi RA patients is not associated with the DRB1 SE alleles Such result contradicts with Syrian study which found that the frequency of ACCP antibodies was higher in SE-positive RA patients compared with SE-negative patients (OR = 5.5, 95% CI = 2–15.1, $P = 0.00054$) [Jamil Mourad et al.,2013]. Here, we have not to forget that HLA molecules with specific SE are considered to constitute about 30% to 40% of the genetic risk of RA [Kurkó J et al.,2013] and that other genetic risk factors might be associated with ACCP antibody production [Annette H et al.,2008] and that more than 30 loci involved in RA pathogenesis [Kurkó J et al.,2013].

Table 3 categorizes the HLA-DRB1 allelic combination according to the ACCP results in RA patients. Among the total 110 RA patients, 50/110 (45.5%) with 12 different HLA-DRB1 allelic combinations were ACCP positive, 28/110 (25.5%) with 9 allelic combinations were ACCP negative, and 32/110 (29%) with 4 allelic combinations were ACCP variable (mixed of positive and negative results). An interesting result is that one of the the homozygotic DRB1*13,13 allelic combination which had exhibited the highest frequency (9.1%) and highest mean titer (3271.7 U/ml) than all other combinations of ACCP positive RA patients. The second highest frequency (7.3%) was noticed with the heterozygotic DRB1*11,7 combination which was also the second highest mean titer (2439.2 U/ml) among all ACCP positive combined alleles. All combinations of this allele including those with SE alleles (*13,1, and *13,4) and those with non-SE alleles (*13,7, *13,11, and *13,8) were negative for ACCP which verifies the neutralizing effect of DRB1*13 on SE and non-SE alleles for their induction of ACCP positivity among RA patients [Lundström E et al.,2009].

The only exception was with the homozygotic combination (DRB1*13,13) which seems to be related to the gene-dose effect on the stimulation or inhibition of the ACCP autoantibodies. Similar effects for the homozygotic combinations was noticed with DRB1*03. All DRB1 *3,3 combinations were ACCP positive (although with a low mean titer of 160.4 U/ml) in RA patients, however, certain heterozygotic combinations of this allele (3,7, and 3,8) were also positive for ACCP. Other *03 heterozygotic combinations exhibited either a negative ACCP (3,15) or variable ACCP results (3,13, 3,4, 3,11 and 3,14). The homozygocity of certain DRB1 alleles (as *0901) but not heterozygocity, is associated with RA in Japanese [Wakitani S *et al.*, 1998]. In the same table, the mean titer for each DRB1 allelic combination is illustrated which is ranging from 31.1 U/ml for 15,8 combination to 3271.7 U/ml for 13,13 combination with an average of 1221.8 U/ml for all combinations. It is worth mentioning that the DRB1 homozygotic phenomenon in the ACCP positive was seen only with non-SE alleles, but not with SE alleles.

Matching of HLA-DRB1 combined allele frequency in RA patients according to their ACCP results with healthy controls is shown in Table 4. These results point out that 20 out of the 34 total DRB1 allelic combinations of the control (57.1%) were control-restricted combinations, and on the other hand, there were 11 out of the 25 total DRB1 allelic combinations of the RA patients (44%) were RA-restricted combinations. These interesting results would be of great value in the classification of DRB1 allelic combination into at-risk or protecting alleles among Iraqi population when a larger scale population sample is available.

In conclusion, the frequency of the DRB1 allele groups among Iraqi RA patients were not subjected to the SE roles. Certain DRB1 alleles were missing in Iraqi RA patients including *02, *05, *06 and *09 indicating their protective role. The frequency of DRB1 alleles with ACCP positive results and the mean titer of the ACCP positive alleles in RA patients were also not associated with DRB1 SE alleles. The majority of RA patients were with allelic combination that exhibited ACCP positivity with a significant difference when compared with allelic combinations that exhibited ACCP negativity. The presence of DRB1 allelic combination that exhibited both ACCP positivity and negativity would deny the hypothesis that ACCP positive RA and ACCP negative RA are genetically separated diseases. Finally, the presence of high percent of RA-restricted DRB1 combinations, and control-restricted DRB1 combinations, is a novel result which if strengthen by a larger cohort study it would be of value in differentiating the at-risk from the protecting allelic combination.

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