

HLA-DRB1 Gene is Highly Mutated in Omani Patients Affected with Rheumatoid Arthritis

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Abstract

Objectives: Rheumatoid Arthritis (RA) is a multifactorial autoimmune disease affecting synovial joints, surrounding tissues, and many other organs of the human body. *HLA-DRB1* has a major role in the immune system by presenting peptides derived from extracellular proteins on antigen presenting cells and has been associated with RA. In this study, we screened for *HLA-DRB1* mutations in Omani patients affected with RA.

Methods: Thirty blood samples from affected patients were examined in parallel with fourteen healthy control samples. *HLA-DRB1* mutational status was examined using polymerase chain reaction (PCR) and sequencing.

Results: A total of 75 aberrations were identified in *HLA-DRB1*, of which 20% were polymorphisms. From the reported aberrations, 25.5% were silent mutations. Within exon-2, three non-synonymous mutations were predicted to have deleterious effects on protein function. Moreover, six deletions and three insertions were found in 12% of the cases, resulting in significant loss of amino acid information.

Conclusion: The *HLA-DRB1* gene is highly damaged in Omani rheumatoid arthritis patients. Novel mutations have been identified and further analysis is required to test the significance of such mutations.

Keywords: *HLA-DRB1*; Rheumatoid arthritis; Oman; Polymorphisms; Silent mutations

Introduction

Rheumatoid arthritis (RA) is a polygenic inflammatory autoimmune disease that results from the interactions between genetic and environmental factors [1]. RA causes damage to tissues lining the joints that subsequently become swollen, stiff and painful; eventually being completely destroyed. Extra-articular signs of RA include vasculitis causing leg ulcers, pleural or pericardial effusions, pulmonary nodules, pericarditis, myocarditis and lymphadenopathy [1]. The autoimmune process related to RA starts with lymphocyte activation and immune complex deposition in the sites of inflammation. Both CD4⁺ TH-1 and CD4⁺ TH-17 cells play a major role in promoting inflammatory cell and cytokine production, which are directly responsible for bone destruction, loss of cartilage integrity, and accumulation of synovial fluid, fibrosis and joints deformity [2]. Moreover, the immune complexes formed by antibodies such as IgM targeting the Fc portion of IgG (auto-antibodies called rheumatoid factors (RF) are detectable in 30% of rheumatoid arthritis patients [3].

According to the World Health Organization (WHO), RA affected round 1% of the population worldwide in the year 2000, affecting women 2-3 times more as compared to men [4]. In 2005, approximately 1.5 million American adults were affected by RA, a substantial increase from the previously reported data (1.3 million adults in US). In general, Caucasian women as well as individuals with

subcutaneous nodules, cigarette smokers and people with high levels of anti-cyclic citrullinated peptide (CCP) or rheumatoid factor (RF) are considered to be at a higher risk and have a poor prognosis [5,6]. Till date, there is no complete cure for rheumatoid arthritis disease and although prescribed medications including gels, ointments and creams consisting of capsaicin, salicylates, menthol, camphor or turpentine oil can only reduce inflammation, relieve pain and prevent ongoing joint damage, they can put RA on remission. However, above these medications, Complementary alternative medicines (CAM) can aid in reducing pain and inflammation. Fish supplements (Omega-3 fatty acids and DHA) and plant oils are associated with reduced pain and stiffness caused due to RA [7]. Ginger [8] is known to reduce inflammation in RA. The active component of turmeric, curcumin, significantly reduces joint pain and swelling by blocking inflammatory cytokines and enzymes as compared to diclofenac sodium [9,10]. Another CAM known to have anti-inflammatory property includes *Boswellia serrata*, a potential inhibitor of the inflammatory enzyme, 5-lipoxygenase and also slows cartilage damage [11].

Despite the tremendous efforts deployed in research, underlying mechanisms related to the onset of RA have not been completely elucidated. Amongst the genes identified in association with RA [2,12-14], a gene found within the Human Leukocyte Antigen class II (HLA) encoding for the beta chain of the DR molecule accounts for a large portion of RA heritability. The class II molecule is a hetero-dimer consisting of an alpha and a beta chain. Its major role in the immune system is to present peptides derived from extracellular proteins on

antigen presenting cells (APC) [14]. The beta chain is approximately 26-28 kDa, encoded by 6 exons located on chromosome 6. Exons-1, 2 and 3 encode the leader peptide and the two extracellular domains respectively. The trans-membrane domain and the cytoplasmic tail are encoded by exons 4 and 5 respectively.

Multiple *HLA-DRB1* alleles are associated with RA including the *DRB1**0401, *0404, *0405, *0101, *0102, *1010, *1001, and *1405 loci [15]. On the other hand *DRB1* alleles such as *0103, *0402, *1102, *1103, *1301 and *1302 are protective against RA [16]. It has been shown that the RA-associated *HLA-DRB1* alleles share a region of sequence similarity or "shared epitope" (SE) at specific amino acid positions in the third hyper-variable region at positions 70-74 of the *HLA-DRB1* molecule [17,18], which represent the most significant genetic risk factor for RA [19]. Apart from the SE region, additional variants in the *DRB1* gene have been associated with increased RA risk [20,21]. In a previous study, MHC knock-out mice consisting of 80-kb deletion within the *DRB* gene (*DRB1**0401 mice) developed arthritis, mimicking the human RA situation [22]. Moreover, various alleles of the *HLA-DRB1* gene are known to be associated with other diseases including Graves' hyperthyroidism, Hashimoto's thyroiditis and Multiple sclerosis [23,24].

According to a previous study conducted in Oman in 1991, the prevalence of RA was estimated at 0.84%, revealing the prevalence of RA in Oman to be lower than that in Caucasian populations [25]. However, these results are outdated and no subsequent studies have been carried out to confirm and update these findings. Rheumatologists in the Sultan Qaboos University Hospital (SQUH) have currently estimated the prevalence of RA among Omanis to nearly 1% with less aggressive phenotype as compared to Europeans [Dr. Hassan B. personnel communication]. Since the Omani population represents heterogeneous ethnicities from Middle East, Africa and Asia, it is worthwhile to determine the status of the *HLA-DRB1* gene and compare it to other populations. The aim of this study is to establish the pattern of *HLA-DRB1* mutations in Omani patients affected with rheumatoid arthritis and to correlate the mutational background with clinical data.

Materials and Methods

Patients and controls

This research was approved by the local Research Ethics Committee at the Sultan Qaboos University (SQU) and the consent was obtained from patients for blood sample collection. The samples were obtained from 30 patients (age ≥ 15 years old, 27 females and 3 males) seen in the outpatient rheumatology clinic in Sultan Qaboos University Hospital (SQUH). Patients having active disease and those in remission were included in this study. Among these, 16 individuals were sero-positive for rheumatoid factor (RF+) and 14 individuals were sero-negative for rheumatoid factor (RF-). Fourteen healthy volunteers from the same ethnic background were enrolled in the study as controls. The diagnosis was based according to the American College of Rheumatology criteria for RA (Arnett et al. (1988)).

Laboratory and radiographic studies

Patient's history, physical results and laboratory analysis recorded in the Hospital Information System (HIS) were retrieved. Patients with additional inflammatory connective tissue diseases, HIV infection, as well as patients undergoing chemo or radiotherapies were excluded

from the study. Patient age, age at diagnosis, previous treatments, history of systemic disease, family history and morning stiffness were recorded. Swollen and tender joints were examined and number of such joints was recorded. DAS28 scores were calculated using C-reactive protein (CRP) values, number of swollen and tender joints. Anti-CCP and RF values were also determined for each patient.

PCR amplification and sequencing

Blood samples (2-5 ml) were collected from patients and genomic DNA was isolated using QIAamp Blood Maxi kit DNA extraction according to the manufacturer's instructions (QIAGEN, USA). The full coding sequence of the *HLA-DRB1* gene was amplified using primers specific for each of the 6 exons. Amplification included a 10-min denaturation set up at 94°C, followed by 35 cycles each consisting of 50s denaturation at 94°C, 50s of annealing at temperatures ranging from 51 to 62°C depending on each exon's (melting temperature), and 50s extension at 72°C. After the last cycle, the samples were incubated for 10 min at 72°C for final extension. The PCR product from each exon was visualized using 1.5% agarose gel electrophoresis.

PCR products of *HLA-DRB1* gene were sequenced using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit. The conditions of the sequencing reaction included 25 cycles at 95°C (70s), 55°C (5s), 60°C (60s), 4°C (holding temperature). The products were cleaned with 95% and 70% ethanol, solubilized in deionized formamide and analyzed using the ABI Prism Genetic Analyzer.

Test sequences were aligned with the reference sequence obtained from the Ensembl database, and analyzed using the Sequencher 5.0 Demo software. In addition, protein 3-dimensional structures were visualized with the 3D program Ras Win Molecular Graphics windows version 2.7.5.2. The PolyPhen-2 and PROVEAN (PROtein Variation Effect Analyzer v1.1) online tools were utilized to determine the functional alterations caused by the identified variations in the RA patients. The insertions, deletions and premature stop codon mutations were further analyzed to predict the resulting loss of information using the Indelz online tool (<http://www.moseslab.csb.utoronto.ca/amin/indelz.html>).

Results

Clinical data results

Thirty blood samples from Omani patients affected with RA and fourteen samples from healthy subjects were examined for *HLA-DRB1* gene mutations. Females constituted 90% of the patient cohort and the mean age was 40 ± 13.4 with the age range between 19 and 69 years. 63.3% of patients presented with positive anti-cyclic citrullinated peptide and 56.7% were positive for rheumatoid factor. According to the calculated DAS score, 30% of patients presented with high active disease, 47% presented with moderate active disease and 23% were in remission.

Sequence analysis of *HLA-DRB1* gene

The exon sequences of the *HLA-DRB1* gene from the study cohort were examined using the publicly available Sequencher Demo version 5 software. Numerous mutations were identified in exons 1 and 2 (Tables 1 and 2). Analysis of exons 3, 4 and 5 revealed no aberration when compared to sequence references. However, in exon 6, although patients 1-4, 9 and 11-30 displayed a specific product for sequencing,

five patients numbered 5, 6, 7, 8 and 10 failed to display any product consistently after three consecutive assays suggesting that exon 6 may be deleted in people affected with this disease.

Amino Acid Change	Nucleotide Change	Novel/Reported	Frequency in Patients	Frequency Controls	in	Polymorphism/Mutation
S29→A	T85→G (TCT→GCT)	Reported	23/30 (76.66%)	3/14 (21.4%)		Polymorphism
T13→A	A37→G (ACA→GCA)	Reported	15/30 (50%)	0/14 (0%)		Mutation
K5→N	G15→T (AAG→AAT)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
L4→L	C10→G (CTG→GTG)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
L4→L	G12→T (CTG→CTT)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
L4→P	T11→C G12→T (CTG→CCT)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
K5→W	A13→T A14→G (AAG→TGG)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
K5→E	A13→G (AAG→GAG)	Novel	2/30 (6.66%)	0/14 (0%)		Mutation
G8→G	A24→T (GGA→GGT)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
G9→G	C 27→A (GGC→GGA)	Reported	1/30 (3.33%)	0/14 (0%)		Mutation
C11→R	T31→C (TGC→CGC)	Novel	1/30 (3.33%)	0/14 (0%)		Mutation
A14→A	G42→T (GCG→GCT)	Reported	3/30 (10%)	0/14 (0%)		Mutation
R33→R	C97→A (CGA→AGA)	Reported	1/30 (3.33%)	1/14 (7.14%)		Polymorphism

Table 1: A summary of aberrations found in exon 1 of the *HLA-DRB1* gene. Nucleotide numbering starts from the first codon (ATG) encoding for Methionine.

Amino Acid Change	Nucleotide Change	Novel/Reported	Frequency Patients	in	Frequency Controls	in	Polymorphism/Mutation
F76→Y	T227→A (TTC→TAC)	Reported	6/30 (20%)		3/11 (27.27%)		Polymorphism
I96→F	A 286→T (ATC→TTC)	Reported	8/30 (26.6%)		1/11 (9.09%)		Polymorphism
E98→E	G294→A (GAG→GAA)	Reported	5/30 (16.66%)		2/11 (18.18%)		Polymorphism
Q99	Insertion of A at position 294	Novel	3/30 (10%)		0/11 (0%)		Insertion
Q99→H	G297→C	Reported	1/30 (3.33%)		0/11 (0%)		Mutation

	(CAG→CAC)				
R101---R	G303→C (CGG→CGC)	Reported	4/30 (13.33%)	2/11 (18.18%)	Polymorphism
V115---G	T344→G G345→T (GTG→GGT)	Reported	9/30 (30%)	6/11 (54.54%)	Polymorphism
K41---T	A122→C (AAG→ACG)	Reported	13/30 (43.33%)	0/11 (0%)	Mutation
R42---S	A124→T G125→C G126→T (AGG→TCT)	Reported	11/30 (36.66%)	0/11 (0%)	Mutation
F55---Y	T179→A (TTC→TAC)	Novel	4/30 (13.33%)	0/11 (0%)	Mutation
Y61---H	T 182→C (TAT→CAT)	Reported	9/30 (30%)	1/11 (9.09%)	Polymorphism
S66---N	T196→A C197→A (TCC→AAC)	Reported	10/30 (33.33%)	2/11 (18.18%)	Polymorphism
S66---Y	C197→A (TCC→TAC)	Reported	3/30 (10%)	0/11 (0%)	Mutation
S66	Insertion of C at position 195	Novel	1/30 (3.33%)	0/11 (0%)	Insertion
D86---D	C258→T (GAC→GAT)	Reported	12/30 (40%)	1/11 (9.09%)	Polymorphism
D86---E	C258→A (GAC→GAA)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
D86---V	A257→T (GAC→GTC)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
A87---A	T261→C (GCT→GCC)	Reported	12/30 (40%)	0/11 (0%)	Mutation
A87---E	C260→A T261→G (GCT→GAG)	Novel	4/30 (13.33%)	0/11 (0%)	Mutation
I96---L	A286→C (ATC→CTC)	Reported	10/30 (33.33%)	4/11 (36.36%)	Polymorphism
L37---L	C109→T (CTG→TTG)	Reported	6/30 (20%)	0/11 (0%)	Mutation
L37	Deletion of G and C at positions 109 and 111	Novel	3/30 (10%)	0/11 (0%)	Deletion
Q39---S	C 115→A A 116→G G117 →T (CAG→AGT)	Novel	3/30 (10%)	0/11 (0%)	Mutation

Q39--V	C 115→G A 116→T G117→C (CAG→GTC)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
Q39	Deletion of C at position 115	Novel	1/30 (3.33%)	0/11 (0%)	Deletion
Q39--H	G 117→C (CAG→CAC)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
Q39--Y	C115→T G117→C (CAG→TAC)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
P40	Insertion of T at position 119	Novel	3/30 (10%)	0/11 (0%)	Insertion
A 100--R	G98→A C99→G (GCG→AGG)	Novel	3/30 (10%)	2/11 (18.18)	Polymorphism
R101	Deletion of C at position 301, STOP codon at TGA (383)	Novel	3/30 (10%)	0/11 (0%)	Deletion
R101	Deletion of G at position 303, STOP codon at TAA (378)	Novel	2/30 (6.66%)	0/11 (0%)	Deletion
A103--P	G107→C (GCG→CCG)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
A103--G	C108→G (GCG→GGG)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
A103àR	G107→C C108→G (GCG→CGG)	Reported	4/30 (13.33%)	0/11 (0%)	Mutation
A103	Deletion of C at position 308	Novel	2/30 (6.66%)	0/11 (0%)	Deletion
T106--N	C117→A (ACC→AAC)	Reported	8/30 (26.66%)	2/11 (18.18%)	Polymorphism
W38-stop codon	G124→A (TGG→TGA)	Novel	2/30 (6.66%)	0/11 (0%)	Mutation
W38--E	T122→G G123→A (TGG→GAG)	Reported	4/30 (13.33%)	0/11 (0%)	Mutation
P40--S	C118→T (CCT→TCT)	Reported	6/30 (20%)	0/11 (0%)	Mutation
R42--G	A124→G G126→T (AGG→GGT)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
H45--Y	C133→T (CAT→TAT)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
F55--L	T179→G (TTC→TGC)	Novel	2/30 (6.66%)	0/11 (0%)	Mutation
D57--E	C172→G	Reported	2/30 (6.66%)	0/11 (0%)	Mutation

	(GAC→GAG)				
Y59--H	T 176→C (TAC→CAC)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
Y89--S	A266→C (TAC→TCC)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
Y107--Y	C121→T (TAC→TAT)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
V114--A	T 141→C (GTT→GCT)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
V67---C	G 199→C (GTG→CTG)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
A100---T	G98→A (GCG→ACG)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
A100--E	C99→A (GCG→GAG)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
A100--K	G98→A C99→A (GCG→AAG)	Reported	5/30 (16.66%)	2/11 (18.18%)	Polymorphism
A102--G	C 305→G (GCC→GGC)	Reported	5/30 (16.66%)	0/11 (0%)	Mutation
A102	Deletion of G at position 304, STOP codon at TGA (383)	Novel	2/30 (6.66%)	0/11 (0%)	Deletion
Q63 --Q	G 189→A (CAG→CAA)	Reported	4/30 (13.33%)	0/11 (0%)	Mutation
Q99---D	C295→G G297→C (CAG→GAC)	Reported	4/30 (13.33%)	2/11 (18.18%)	Polymorphism
F36---F	C108→T (TTC→TTT)	Novel	1/30 (3.33%)	0/11 (0%)	Mutation
T119--T	A297→G (ACA→ACG)	Reported	2/30 (6.66%)	0/11 (0%)	Mutation
H110--H	C330→T (CAC→CAT)	Novel	1/30 (3.33%)	0/11 (0%)	Mutation
N111---D	A331→G (AAC→GAC)	Novel	1/30 (3.33%)	0/11 (0%)	Mutation
R42---M	G125→T (AGG→ATG)	Reported	1/30 (3.33%)	0/11 (0%)	Mutation
E51---K	G151→A (GAG→AAG)	Novel	1/30 (3.33%)	0/11 (0%)	Mutation
N48--- T	A143→C (AAT→ACT)	Novel	1/30 (3.33%)	0/11 (0%)	Mutation

Table 2: A summary of aberrations found in exon 2 of the *HLA-DRB1* gene. Nucleotide numbering starts from the first (ATG) codon.

As summarized in Figure 1, among the total aberrations identified in the *HLA-DRB1* gene in this study, 20% were polymorphisms, 26.6% of which were silent. The remaining 68% were mutations, 25.49% of them being silent mutations; thus showing no change in the amino acid sequence. Additionally, 6 deletions and 3 insertions were identified.

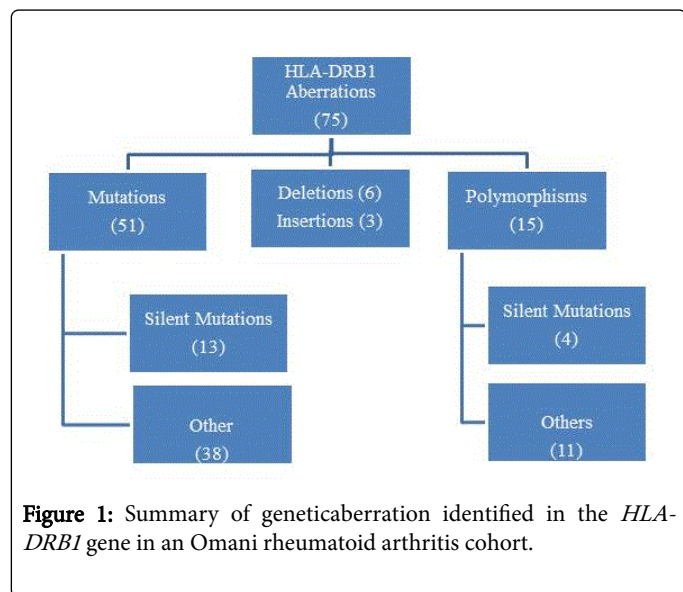


Figure 1: Summary of genetic aberration identified in the *HLA-DRB1* gene in an Omani rheumatoid arthritis cohort.

The main aberrations found in exon 1 are summarized in Table 1 where the status of the *HLA-DRB1* in rheumatoid arthritis patients is compared to healthy controls. Out of 30 cases and 14 controls analyzed

we confirmed 2 polymorphisms, which were previously reported, and eleven mutations, 3 of which have been previously reported. Six of the identified mutations were silent mutations. Amongst the non-synonymous mutations, we identified two novel mutations, L4P and C11R in the RA patient cohort and were absent in the controls. The PolyPhen-2 and PROVEAN (protein variation effect analyzer v1.1) online tools were utilized to determine the functional alterations caused by the identified variations in the RA patients. The PolyPhen-2 variation score ranges from 0 (Benign) to 1.0 (Damaging) whereas the PROVEAN tool has a set threshold score of -2.5, below which variations are classed as deleterious. Prediction of the functional effect of the variation using the PolyPhen-2 (v2.2.2r398) online tool, classed these two mutations as “benign” (score=0.082 and 0.001, respectively). The S29A and T13A variations, which have been previously reported, were present at a high frequency in the patient cohort compared to the other identified polymorphisms/mutations. However, these reported variations have not been associated with RA or any other disease and were also classed as “benign” by the PolyPhen-2 prediction tool. The identified benign variants may be in strong linkage disequilibrium with other causal alleles.

Several mutations were identified in exon 2 of *HLA-DRB1* in rheumatoid arthritis patients compared to healthy controls and are listed in Table 2. Thirteen polymorphisms were recorded, of which 11 have been previously reported, in addition to three insertions and six deletions. Moreover, 40 mutations were identified in total, 10 of which have not been previously reported, 7 of which were synonymous and one caused a stop codon (W38-, G124A). Only 3 mutations displayed overlapping results in both the PolyPhen-2 and PROVEAN tools as functionally deleterious variations: V67C, E51K and N48T (Table 3), all 3 of which occur in the conserved MHC II beta domain.

Mutation	Nucleotide Sequence Change	Polyphen-2 Prediction	Polyphen-2 Score	Provean Prediction	Provean Score
V67C	G 199→C (GTG→CTG)	Probably Damaging	0.974	Deleterious	-3.183
E51K	G151→A (GAG→AAG)	Possibly Damaging	0.596	Deleterious	-3.231
N48T	A143→C (AAT→ACT)	Possibly Damaging	0.936	Deleterious	-5.249

Table 3: Mutations identified by both the PolyPhen2 and PROVEAN online tools as functionally deleterious.

The insertions, deletions and premature stop codon mutations were further analyzed to predict the resulting loss of information by utilizing the Indelz online tool (<http://www.moseslab.csb.utoronto.ca/amin/indelz.html>), which provides a Loss score D ranging from 0 to 1, where 0 signifies no change to the protein and 1 signifies complete loss of amino acid information for proper protein function [26]. Interestingly, all the identified insertions and deletions led to a significant loss of information (>75%) due to protein truncation. The most significant effect was displayed by the deletions at nucleotide position 109, 111, 115, insertion at position 119 and a premature stop codon at amino acid position 38, all of which span the first beta strand domain of the *HLA-DRB1* protein.

Amongst the variations identified in exon 2, amino acid substitution at position 41 occurred at the highest frequency in the studied RA cohort, whereby the native threonine residue was more frequent than the lysine residue in the RA cases (43.33% compared to none in the

control samples). However, this variation was classed as benign/neutral by both the PolyPhen-2 (score=0.003) and PROVEAN tools (score=3.691) indicating the possibility of strong linkage disequilibrium between this variation and another causal mutation. Three-dimensional modeling of the *HLA-DRB1* protein with amino acid variation at position 41 indicated a conformational change between the native (A) and K41 (B) form. The affected beta strand domain harboring the mutation is indicated by an arrow. The function of the affected domain juxtaposed to the folding domain on the *DRB1* is not yet known.

Discussion

The heritability of rheumatoid arthritis has been predicted to be approximately 50%, laying emphasis on the strong role of genetics in RA disease susceptibility [27]. Moreover, only 30% of the disease risk

i.e. half of the heritability, could be explained by common causal variants identified by GWAS studies, thus pointing out to the role of rare causal variants in RA patients that need to be identified by sequencing. Although GWAS studies have identified several disease risk loci [28] in genes such as peptidyl arginine deiminase type 4 (PADI4), protein tyrosine phosphatase nonreceptor 22 (PTPN22), tumour necrosis factor alpha-induced protein 3 (TNFAIP3), signal transducer and activator of transcription 4 (STAT4) and chemokine receptor 6 (CCR6) in numerous RA cohorts, the *HLA-DRB1* gene has been the major focus of RA studies since the identification of variant alleles that confer risk or protection against the disease in RA patients. The purpose of this study was to establish the pattern of known and novel mutations of *HLA-DRB1* in Omani patients affected with rheumatoid arthritis and the association of these genetic variants with serological and clinical data in the cohort.

Amongst the genetic aberrations identified in the Oman RA patients, a majority segregated in exon 2 of the *HLA-DRB1* gene. Three novel non-synonymous mutations were predicted to have a damaging effect on protein function: V67C, E51K and N48T, which occur in the MHC class II beta domain. The V67 amino acid occurs in the beta-chain region of the *HLA-DRB1* protein and the substitution of valine, a non-polar amino acid, with cysteine, a polar amino acid, may potentially affect the antigen-presentation properties of the *HLA-DRB1* protein. On the other hand, E51 and N48 both flank the first beta-turn region of *HLA-DRB1*. Mutations at these amino acid residues resulting in charge alterations could affect the proper folding of the protein. These mutations were identified in only one patient each highlighting their rare occurrence. Although these mutations do not occur in the shared epitope conserved domain (spanning amino acid residues 70-74) that has been associated with RA and the levels of citrullinated protein antibody (ACPA) [29], they may indirectly affect protein function by altering the 3-dimensional structure of *HLA-DRB1*. Moreover, several insertions and deletions in exon 2 of *HLA-DRB1* were identified in the RA patients that resulted in significant loss of genetic information and protein truncation. The inability to amplify exon 6 in 5 RA patients also highlighted the possibility of the presence of deletions in this region of the gene.

Analysis of a large reference population identified the risk associated with amino acid positions 11, 13, 71 and 74 of *HLA-DRB1* in seropositive rheumatoid arthritis patients [20]. Although a rare mutation (Cysteine to Arginine) in the amino acid 11 was identified in the Omani RA cohort, none of the previously highlighted high-risk alleles coding for Val-11 or Leu-11 are reported here. A non-synonymous mutation at position 13 (Threonine to Alanine) was also reported in 50% of the Omani RA patients, the most common variation in the studied cohort. This variant was not also associated with RA in the Raychaudhuri et al study and hence, may indicate a novel rare mutation or strong linkage with alternative causal alleles at position 11.

In exon 1, S29A is a well-established polymorphism associated with RA in a Korean population. This polymorphism occurs in 21.4% of healthy subjects and 76.66% of patients and may affect the folding of the protein structure due to the change from a polar (serine) to a nonpolar amino acid (alanine).

A 67-year old RA female suffering from small tender swelling joints and positive RF with an abnormally high level of mutations in the *HLA-DRB1* gene, when compared to other patients, displayed 2 polymorphisms, 19 mutations, 3 deletions and one insertion. Among these aberrations, two mutations (W38-, R101-) resulted in the

incorporation of premature stop codons in the protein. Thus, these aberrations in combination may have contributed to the formation of an abnormal protein or its complete absence, in line with the critically high RF value displayed by this patient implying the highly aggressive nature of the disease.

The large number of polymorphisms and mutations identified in this study albeit the small size of the population, further confirms the highly polymorphic nature of the *HLA-DRB1* gene, the presence of broad linkage disequilibrium and the contribution of multiple genetic aberrations in determining disease aggressiveness.

Both ACCP and RF levels have shown strong association with rheumatoid arthritis severity in several studies and has hence, been included in the RA classification criteria [29,30]. Autoantibodies specific to cyclic citrullinated peptides appear at early stages of RA onset and their specificity and production have been shown to be regulated by HLA SE alleles [15]. It also has a better predictive value compared to RF, which is non-specific and absent in early stages of RA [31]. It has been suggested that the higher affinity of HLA SE alleles to bind to citrullinated peptides may play a key role in activating the T cell response and B-cell mediated production of autoantibodies [32]. However, the lack of binding of certain citrullinated peptides (e.g. Epstein Barr virus-derived) or the ability of some *HLA-DRB1** alleles to equally bind both the native and citrullinated forms of the peptide indicates the presence of alternative mechanisms than antigen-presentation by which ACCP is involved in RA pathogenesis. In this study no association was identified between the number of *HLA-DRB1* genetic aberrations and ACCP or RF status. This observation is in line with results from a study indicating that the various *HLA-DRB1* genotypes affect the expression level of the protein irrespective of disease activity or severity [33]. Hence, this further strengthens the claim that serum markers such as ACCP and RF and the *HLA-DRB1* gene aberration status independently contribute to the pathogenesis of the RA. Thus, the laboratory analysis criteria used to diagnose RA does not reflect the patients' genomic status and may affect appropriate disease diagnosis. It is of primary interest to dissect the contribution of each of these parameters and their effect on RA prognosis with molecular biology studies to ensure systematic patient and disease management.

Although this study is the first investigation of the association of *HLA-DRB1* genetic aberrations, in rheumatoid arthritis within the Omani population, there are some caveats that need to be addressed in the future. Larger cohort with matched controls and detailed description of diagnostic parameters are necessary to determine the significance of these mutations and their association with the clinical and pathological data. The gender skew in the sample population reflects the higher disease prevalence of RA in females with a worldwide ratio of 3:1 [2], although this ratio was higher in the Oman cohort studied despite the relatively lower prevalence of RA in Oman, further stressing on the need to collect larger sample populations to prevent biased observations. The possibility that the rare variations identified in this study display ethnic specificities needs to be tested by sequencing studies in different ethnic populations.

In conclusion, this study identified several novel polymorphisms and mutations in the *HLA-DRB1* gene in an Omani rheumatoid arthritis population irrespective of disease severity. The functional consequences of mutations predicted to have a deleterious effect on the protein need to be elucidated in detail, in addition to the confirmation of these genetic aberrations in larger populations.

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