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Full Length Research Paper

Genotyping CTLA-4 exon 1 (+49 A/G) and HLA-DRB1 polymorphisms and susceptibility to systemic lupus and rheumatoid arthritis in Tunisian population

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Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a negative regulator of T-cell function, which has been suggested to be involved in a wide-range susceptibility to autoimmune diseases. We sought a probable implication of the CTLA-4 polymorphism (A/G +49) in two autoimmune diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and its possible interaction with HLA-DRB1 polymorphism. CTLA-4 gene polymorphism A/G in exon 1 (+49) was analyzed by PCR-RFLP method in 78 patients with SLE, 132 with RA and 110 normal controls. HLA-DRB1 typing was performed by PCR-SSP (one lambda®) in only 46 SLE patients, the 132 RA patients and 100 normal controls. The results showed that there were no significant differences in exon 1 gene of CTLA-4 polymorphism between SLE, RA and controls. HLA-DRB1*03 and DRB1*15 were significantly more frequent in SLE patients than in controls (p = 0.0001, OR (95% CI) = 4.35 (1.94 - 9.86) and p = 0.01, OR (95% CI) = 3.24 (1.28 - 8.24) respectively). In addition, the frequencies of DRB1*04 and its subtype DRB1*0405 were statistically higher in RA patients than in controls (p = 0.00043, OR (95% CI) = 2.79 (1.54 - 5.07) and p = 0.0001, OR (95% CI) = 5.65 (2.14 - 15.74) respectively). No association between CTLA-4 and HLA-DRB1 polymorphisms was observed in either SLE or RA patients. In conclusion, the CTLA-4 exon 1 polymorphism does not appear to interfere with susceptibility to SLE and RA in Tunisian patients. Corresponding with data in other populations, HLA-DRB1 seems to play major role in conferring susceptibility to SLE and RA.

Key words: Autoimmune diseases, autoimmunity, genetic polymorphism, systemic lupus erythematosus, rheumatoid arthritis.

INTRODUCTION

Autoimmune diseases are clinically heterogeneous condition with complex aetiologies in which environmental and genetic factors are implied. Genetic factors involvedness was suspected by discrepancy in prevalence of those diseases in diverse populations, familial clustering and twins' concordance (Dieude and Cornelis, 2005). According to several studies, different autoimmune diseases are considered as clinically and ethnically heterogeneous conditions with a great variability in diseases progression, therefore their severity and susceptibility may have a genetic component (Huizinga, 2003). Many genetic linkage studies have revealed linkages of those diseases with the chromo-some 6p21 region in the HLA genes map. HLA studies have shown the importance of HLA-DR2 and 3, as well as their linked HLA-DQ specificities DQ1 and DQ2 in systemic lupus

Abbreviations: CTLA-4; Cytotoxic T lymphocyte antigen 4, **SLE**; systemic lupus erythematosus, **RA**; rheumatoid arthritis, **ACR**; American College of Rheumatology, **ECLAM**; European Consensus Lupus Activity Measurement, **SLICC**; Systemic Lupus international Collaborating Clinics/ ACR damage index for systemic lupus erythematosus, **DAS28**; disease activity score, **RF**; rheumatoid factor, **Anti-CCP**; anti-cyclic citrullinated peptide autoantibodies, **SE**; shared epitope, **ICs**; immune complexes, **SNP**; single nucleotide polymorphism.

erythematosus (SLE) (Vargas-Alarcon et al., 2001). The association of rheumatoid Arthritis (RA) with DRB1*0401 was initially discovered by Stastny (1978). Subsequent data have demonstrated that HLA-DRB1 alleles are associated with RA in wide range of populations (Michou et al., 2006). These alleles encode a similar amino acid sequence (QRRAA, QKRAA, RRRAA) defined as shared epitope (SE) and located in position 70 - 74 of the third hypervariable region of the DR-beta chain (Gregersen et al., 1987). Although the contribution of HLA-DRB1 alleles to RA predisposition is significant, the shared epitope hypothesis fails to explain the role of HLA region in disease susceptibility (Van Der Helm-Van Mil et al., 2007).

Different other regions outside the HLA locus have shown suggestive linkage (LOD > 2.2, $p < 0.001$), nevertheless, only a few of them showed significant linkage (LOD > 3.6, $p < 2 \times 10^{-5}$) (Orozco et al., 2006). Interestingly, these linked "outside HLA" regions are shared by many autoimmune disorders, suggesting that there may exists a common genetic background predisposing to autoimmunity, which comprises cytokines, adhesion molecules and immune regulatory molecules, such as CTLA-4 (Orozco et al., 2006).

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a receptor expressed on activated T cells and shares some homology with another T cell surface receptor, CD28 (Cinek et al., 2000; Baroja and Mandrenas, 2003; Friedline et al., 2009). A strong signal via TCR and/or CD28 is required for expression of this molecule (Wells et al., 2001). After incubation with anti-CD3 plus IL-2, expression of CTLA-4 on activated T cells is increased both on the surface and intracellularly (Djukanovic et al., 2000). In contrast to the co-stimulatory activity of CD28, CTLA-4 ligation appears to induce negative regulation of T cell activation both in animals and humans. Indeed, soluble anti-CTLA-4 monoclonal antibodies that block CTLA-4/B7 interaction increased the proliferation of T cells stimulated with anti-CD3 plus anti-CD28 monoclonal antibodies in vitro (Salomon and Bluestone, 2001). In contrast, under conditions of Fc receptor cross-linking, anti-CTLA-4 monoclonal antibodies inhibited T cell proliferation by inducing CTLA-4 signaling (Kosmaczewska et al., 2001). Furthermore, current evidence suggests that CTLA-4 mediates Fas-independent apoptosis of activated T lymphocytes (Davidson et al., 2002).

Several reports indicate that the function of CTLA-4 relates to autoimmunity. Lymphocytes of non obese diabetic mice, an animal model of autoimmune diabetes, have reduced expression of CTLA-4 (Bergman et al., 2001). CTLA-4-deficient mice showed high incidence of a severe lymphoproliferative disorder and autoimmune disease, as well as early lethality (Chambers et al., 2002). In theory, reduced expression or function of CTLA-4 contribute to the pathogenesis of autoimmune diseases (Kosmaczewska et al., 2001). Many studies showed that specific CTLA-4 gene polymorphisms confer susceptibility to several autoimmune diseases, such as Graves' disease (Kouki et al., 2000), primary biliary cirrhosis (Argawal et al., 2000), autoimmune hematological disorders (Pavkovic et al., 2003), insulin-dependent diabetes mellitus (Agarwal et al., 2000), SLE (Ahmed et al., 2001) and RA (Lei et al., 2005). However, it is still unclear how CTLA-4 gene polymorphism contributes to the pathogenesis of these diseases.

Many polymorphisms among the CTLA-4 gene cluster were described, the most frequently studied being an adenine to guanine (A/G) polymorphism at position +49 of the CTLA-4 first exon, that results in an amino acid substitution threonine → alanine (Thr → Ala) at codon 17 of the CTLA-4 leader peptide (Pavkovic et al., 2003). The allele G is associated with an altered spatial conformation which leads to a defect in intracellular circulation of the CTLA-4, thus resulting in a distorted regulation of immune response. High prevalence of the CTLA-4 G allele apparently confers susceptibility to autoimmune disease (Pavkovic et al., 2003).

The present study was undertaken to investigate the possibility that CTLA-4 (A/G +49) and HLA-DRB1 polymorphisms act as a genetic risk factor for susceptibility to and the outcome of, SLE and RA in Tunisian patients.

SUBJECTS AND METHODS

Patients and controls

SLE patients

A total of 78 Tunisian SLE subjects (71 women and 7 men, mean age: 31 ± 14 years) followed up at the department of Medicine, Charles Nicolle Hospital, were investigated. All the patients answered at least 4 revised criteria of the American College of Rheumatology (ACR) (Hochberg, 1997). Demographic data, clinical manifestations and immunological parameters, including antinuclear antibodies (ANA), ds DNA, cryoglobulins and C3 and C4 were collected retrospectively. Activity score (European Consensus Lupus Activity Measurement (ECLAM) (Vitali et al., 1992) and a damage score (Systemic Lupus international Collaborating Clinics/ ACR damage index for systemic lupus erythematosus (SLICC) (Gladman et al., 1996) were recorded at each visit, with a maximum of three visits/year. SLE patients were classified as having nephritis if they fulfilled ACR criteria for renal involvement (persistent proteinuria > 500 mg/ 24 h (or > 3+) or cellular casts) (Hochberg, 1997).

RA patients

One hundred thirty-two RA patients (25 men and 107 women) recruited from the rheumatology outpatient clinics of Charles Nicolle hospitals in Tunis. All patients fulfilled the ACR revised criteria for RA (Arnett et al., 1988). The demographic data included age, sex, disease duration, use of disease-modifying anti-rheumatic drugs and disease activity calculated by using the disease activity score (DAS28). Additional data concerning radiographic evidence of erosions and the presence of nodules founded on examination or

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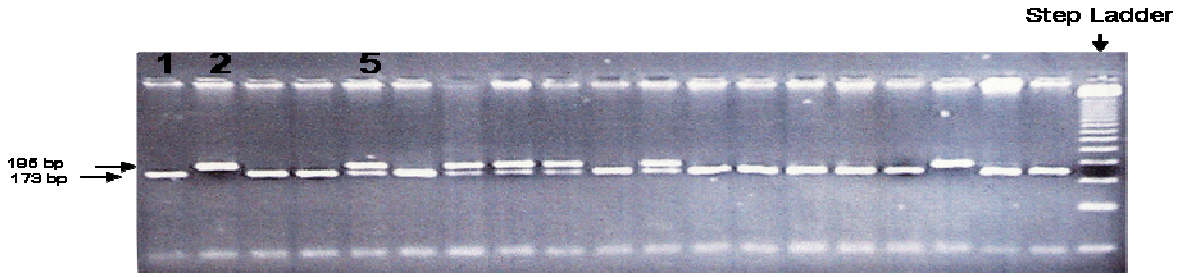

Figure 1. Electrophoretic patterns of CTLA-4 (A/G+49) exon 1 polymorphic site examined on a 4% agarose gel after digestion with *Kpn*I. Sample 1 is homozygous for the G allele (173 bp), Sample 2 is homozygous for the A allele (195 bp), Sample 5 is AG heterozygous. The step ladder is 50pb molecular weight marker (Promega. USA).

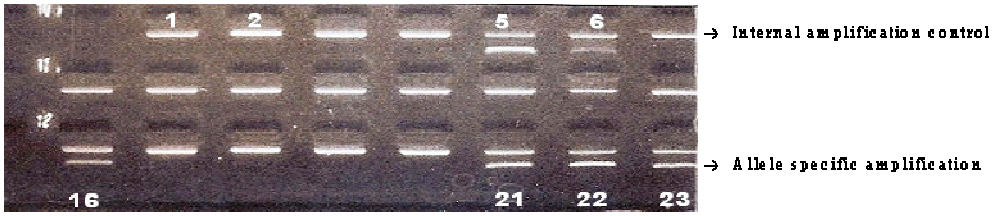

Figure 2. HLA-DRB1 genotyping using PCR-SSP method. 20 primer mixes were used for identifying HLA-DRB1 alleles. Also, in each PCR reaction, a pair of primers specific for non-allelic sequences was included. These primers amplified the third intron of HLA-DRB1 genes and functioned as an internal amplification control. At wells 1, 2 (containing specific primers for HLA-DRB1*0101, *0202/04 and *10 alleles), only internal control primers were amplified with the absence of specific amplification primers. At wells 5 and 6 (containing specific primers for HLA-DRB1*0301, *0302/04 alleles respectively), a 125 bp allele specific amplification was shown. It is the same at wells 16, 21, 22, 23 (containing specific primers for HLA-DRB1*07 alleles) with a 200 bp specific amplification. In conclusion, the HLA-DRB1 genotype of this sample is HLA-DRB1*03 *07.

previously documented in the patient's medical records were reported. Rheumatoid factor (RF) and anti-cyclic citrullinated peptide auto-antibodies (anti-CCP) were assayed in RA patients' sera at the hospital attended.

Controls

As control group, we studied 110 ethnically age and sex-matched healthy subjects. They were recruited from the blood donors of the same area with patients. All theses controls were screened for the presence of autoimmune manifestations using medical examination and a questionnaire. Exclusion criteria were the presence of autoimmune diseases in the donor or SLE/RA in a first-degree relative. This study was approved by the local ethics committee and informed consent was obtained from all subjects.

Methods

Genomic DNA was extracted from peripheral blood by "salting out" procedure after lyse of red and white blood cells.

CTLA-4 exon 1 (+49) polymorphism

This SNP was typed using PCR-RFLP method. The appropriate segment of the CTLA-4 gene was amplified using specific primers: CTLA-4 (1) forward: 5' CAAggCTCAgCTgAACCTgggT 3'CTLA-4 (2) reverse: 5' TACCTTTAACTTCTggCTTTg 3'. The PCR was performed using 0.1 ug of genomic DNA, 10 pmol of each primer, 200 uM of dNTPs and 0.5U of Taq DNA polymerase (Promega. USA). Samples were subjected to initial denaturation for 5 min at 94°C, 35 cycles of 94°C for 40 s, 55°C for 30 s and 72°C for 1 min, with final extension at 72°C for 7 min. A 195 bp fragment was amplified. The substitution created a *KpnI* (promega. USA) restriction site in allele G. Amplified products were incubate at 37°C for 4 h using 5U of *KpnI* per reaction. Digested products were electrophoresed on a 4% agarose gel. Digested G allele yielded fragments of 173 and 22 bp and A allele yielded 195 bp (Figure 1).

HLA-DRB1 typing

HLA-DRB1 types *01-*16 were determined by polymerase chain reaction based method with sequence specific primers (PCR-SSP) (one lambda®) in only 46 SLE patients, the 132 RA patients and 100 normal controls (Figure 2). High resolution typing was also performed by the same method to determine the DRB1*04 subtypes DRB1*0401 - *0422. The shared epitope (SE) was defined as HLA-DRB1*01, *0401, *0404, *0405, *0408, *0409, *0410, *0413, *0416, *0419, *0421, *10 (Gregersen, 1987).

Statistical analyses

Allele frequencies were calculated by direct counting. Correlation with clinical and biological features were analysed by Excel (using

Table 1. Demographic, clinical and biological characteristics of SLE and RA patients.

SLE patients	**n = 78**
Gender	71 females and 7 males
Mean age (years)	31 ± 14
Age at diagnosis (years)	31.2 ± 12.8
Clinical manifestations (%)	
Cutaneous vasculitis	32 (41)
Arthritis	63 (80)
Serositis	28 (36)
Nephritis	44 (56.4)
Neurological disorders	16 (20)
Haematological abnormalities	44 (56.4)
Immunological parameters (%)	
Antinuclear antibodies (ANA)	78 (100)
Ds DNA antibodies	55 (70)
Low C3 and C4	51 (65.4)
Cryoglobulins	16 (20.5)
Immune complexes (ICs)	17 (21.8)
Cardiolipin antibodies (CLA)	32 (41)
ECLAM* (range)	2.9 (0.4-6.2)
SLICC** (range)	2.0 (0-10)
RA Patients	n = 132
Gender	108 females and 24 males
Age in years	
Median (interquartile range)	52 (39-65)
Age at diagnosis in years	
Median (interquartile range)	41.5 (32-62)
Disease duration in months	
median (interquartile range)	125.3 (56-240)
Early onset < 50 years (%)	40 (30)
Nodules present (%)	27 (20)
Joint erosions (%)	63 (47.3)
Disease activity (%)	
High RA activity state (DAS28 > 5.1)	95 (71.4)
Low RA activity state (DAS28 < 5.1)	38 (28.6)
Rheumatoid-factor positive (%)	102 (76.6)
Anti-CCP antibodies	95 (71)

ECLAM*: European Consensus Lupus Activity Measurement: Results are shown as median (range). SLICC**: Systemic Lupus international Collaborating Clinics/ ACR damage index for systemic lupus erythematosus: Results are shown as median (range).

the automatic filter). The chi^2 test with Yates correction and Fisher exact test were used to compare genotypes and alleles frequencies. Relative risks were estimated by calculating the odds ratio (OR) using the Woolf method modified by Haldane: OR = (a × d) / (b × c). P value < 0.05 was considered to be statistically significant. Statistical power was calculated using a web power calculator (http://www.sph.emory.edu/~cdckms/) with two-sided significance level (alpha) at 0.05.

RESULTS

SLE patients

Demographic, clinical and biological characteristics of the patients are recapitulated in Table1. Investigation of CTLA-4 (A/G+49) polymorphism showed no differences in genotype

Table 2. Genotype and alleles frequencies in controls SLE and RA patients.

CTLA-4 (A/G +49)	Controls (n = 110)	SLE patients (n = 78)	p	OR (95% CI)
Genotypes				
AA	10 (9.1%)	7 (8.9%)	0.81 (NS)	0.99 (0.32 - 2.98)
AG	45 (40.9%)	34 (43.5%)	0.82 (NS)	1.12 (0.59 - 2.09)
GG	55 (50%)	37 (47.4%)	0.84 (NS)	0.9 (0.48 - 1.68)
Alleles				
A	0.29	0.31	0.88 (NS)	1.06 (0.66 - 1.7)
G	0.71	0.69		
CTLA-4 (A/G +49)	**Controls (n = 110)**	**RA patients (n = 132)**	**p**	**OR (95% CI)**
AA	10 (9.1%)	17 (12.8%)	0.46 (NS)	1.48 (0.61 - 3.66)
AG	45 (40.9%)	46 (34.8%)	0.4 (NS)	0.77 (0.44 - 1.35)
GG	55 (50%)	69 (52.2%)	0.82 (NS)	1.1 (0.64 - 1.88)
Alleles				
A	0.31	0.3	0.93 (NS)	1.04 (0.69 - 1.56)
G	0.69	0.7		

NS: not-significant.

in genotypes and alleles frequencies between SLE patients and controls (Table 2). HLA-DRB1 typing for 46 SLE patients showed a significant association with DRB1*03 and DRB1*15 ($p < 0.05$). In contrast DRB1*11 and DRB1*12 were less frequent in patients than in controls (Table 3). Analysis of CTLA-4 SNP with clinical and serological features showed that the frequencies of pericarditis, cutaneous vasculitis, hypocomplementaemia, cryglobulins, immune complexes (ICs) and cardiolipin antibodies (CLA) were higher in patients carrying G allele than those with A allele, but the difference did not reach the level of statistical significance (Table 4). However, no differences observed in the frequencies of nephritis, haematological manifestations and ANA generation between the two alleles. Also, we did not show any correlation between this polymorphism and early age of onset or SLE activity (data not shown).

No associations were observed between HLA-DRB1 alleles and the frequency of CTLA-4 exon 1 genotypes (data not shown). However, CLA and high titer of anti-nuclear antibodies (ANA) (over 1/400) were significantly more frequent in DRB1*03 positive patients ($p < 0.05$) (table 4). No difference in the frequencies of others clinical and/or biochemical parameters was shown between DRB1*03 + and DRB1*03 - patients. It is the same for HLA-DRB1*15 (Table 4).

RA patients

One hundred eight women and twenty four men with RA were studied. The average onset age was 41.5 years with middle length evolution of 125.3 months. Radiological abnormalities including bone lesions and osteoporosis were found in 63 patients. Activity of RA was investigated by calculating DAS28 at the moment of sample harvest (Table 1).No differences were found in genotypes and alleles frequencies of CTLA-4 (A/G +49) between RA patients and controls (Table 2). The results of HLA-DRB1 genotyping in RA showed a significant increase of DRB1*04 allele, SE and 2 copies of SE in patients comparatively to controls (Table 3). However, no increase was observed in the frequencies of DRB1*01, *DRB1*10 or DRB1*14 in RA. Inversely, the DRB1*07 allele, known to be protective against RA, was significantly less frequent in patients than in controls ($p < 0.05$). It the same for DRB1*11 and DRB1*12 (Table 3).

The most frequent subtype in patients was DRB1*0405 (26.5%), while DRB1*0403 allele was significantly less frequent in patients than in controls (Table 3). Analysis of CTLA-4 SNP with clinical and biological features showed that bone lesions frequency was significantly higher in patients carrying AG genotype than in those having AA and GG genotypes ($p = 0.049$ and $p = 0.01$ respectively) (Table 5). However, no significant differences were observed between CTLA-4 SNP and the frequencies of HLA-DRB1*04 allele, shared epitope (SE), rheumatoid factor (RF) and early onset of the disease (age of onset < 50 years old).Again, analysis of HLA-DRB1 alleles and shared epitope (SE) with clinical and biological features showed no associations with either early onset of RA, bone lesions or RF (data not shown).

Table 3. HLA-DRB1 typing in controls SLE and RA patients.

HLA-DRB1	Controls (n = 100)	SLE (n = 46)	RA (n = 132)	p; OR (95% CI)*	p; OR (95% CI)**
DRB1*01	17 (17%)	4 (8.7%)	12 (9%)	0.28; 0.46 (0.12 - 1.6)	0.1; 0.49 (0.21 - 1.15)
DRB1*03	23 (23%)	26 (56.5%)	40 (30.3%)	0.0001; 4.35 (1.94 - 9.86)	0.27; 1.46 (0.77 - 2.76)
DRB1*04	27 (27%)	8 (17.4%)	67 (50.75%)	0.29; 0.57 (0.21 - 1.48)	0.00043; 2.79 (1.54 - 5.07)
DRB1*07	36 (36%)	14 (30.4%)	28 (21.2%)	0.63; 0.78 (0.34 - 1.75)	0.018; 0.48 (0.26 - 0.89)
DRB1*08	5 (5%)	1 (2.17%)	3 (2.2%)	0.72; 0.45 (0.02 - 3.91)	0.22; 0.44 (0.08 - 2.19)
DRB1*09	1 (1%)	0 (0%)	3 (2.2%)	-	0.42; 2.3 (0.21 - 58.33)
DRB1*10	8 (8%)	3 (6.5%)	12 (9%)	0.52; 0.8 (0.16 - 3.56)	0.95; 1.15 (0.42 - 3.23)
DRB1*11	28 (28%)	5 (10.8%)	22 (16.6%)	0.03; 0.31 (0.1 - 0.94)	0.0551; 0.51 (0.26 - 1.01)
DRB1*12	7 (7%)	0 (0%)	2 (1.5%)	0.06; 0 (0 - 1.67)	0.035; 0.2 (0.03 - 1.11)
DRB1*13	28 (28%)	7 (15.2%)	25 (18.9%)	0.14; 0.46 (0.17 - 1.24)	0.14; 0.6 (0.31 - 1.16)
DRB1*14	5 (5%)	1 (2.17%)	2 (1.5%)	0.72; 0.45 (0.34 - 1.75)	0.12; 0.29 (0.04 - 1.75)
DRB1*15	13 (13%)	15 (32.6%)	26 (19.7%)	0.01; 3.24 (1.28 - 8.24)	0.24; 1.64 (0.75 - 3.61)
SE +***	18 (18%)	-	61 (46.2%)	-	0.00001; 3.91 (2.03 - 7.59)
2 copies of SE	1 (1%)	-	13 (9.8%)	-	0.01; 10.8 (1.45 - 6,16)
DRB1*04 subtypes					
*0401	0 (0%)	-	3 (2.2%)	-	-
*0402	4 (4%)	-	14 (10.6%)	-	0.10: 2.85 (0.84 - 10.62)
*0403	13 (13%)	-	6 (4.5%)	-	0.03: 0.32 (0.1 - 0.94)
*0404	0 (0%)	-	4 (3%)	-	-
*0405	6 (6%)	-	35 (26.5%)	-	0.0001: 5.65 (2.14 - 15.74)
*0406	3 (3%)	-	1 (0.7%)	-	0.21: 0.25 (0.01 - 2.71)
*0407	1 (1%)	-	6 (4.5%)	-	0.11: 4.71 (0.55 - 105.61)
*0408	0 (0%)	-	1 (0.7%)	-	-

*: Statistical comparisons between controls and SLE patients.
**: Statistical comparisons between controls and RA patients.
***: SE (shared epitope) was not estimated in SLE patients.

Table 4. Analysis of CTLA-4 SNP with clinical and biological features of SLE.

	CTLA-4 (+ 49) SNP**		HLA-DRB1 polymorphisms			
	A (n = 48)	G (n = 108)	DRB1*03 – (n = 20)	DRB1*03 + (n = 26)	DRB1*15 + (n = 15)**	DRB1*15 – (n = 31)
Early Onset < 25 years	16 (0.307)	36 (0.693)	15 (75%)	11 (42.3%)***	10 (66.6%)	16 (51.6%)
Nephritis	26 (0.295)	62 (0.705)	9 (45%)	11 (42.3%)	7 (46.6%)	13 (42%)
Haematological injuries	26 (0.295)	62 (0.705)	10 (50%)	10 (38.4%)	7 (46.6%)	13 (42%)
Cutaneous vasculitis	17 (0.265)	47 (0.735)	15 (75%)	23 (88.4%)	12 (80%)	26 (83.8%)
Pericarditis	9 (0.250)	27 (0.750)	5 (25%)	6 (23%)	5 (33.3%)	6 (19.3%)
Hypocomplementaemia	27 (0.264)	75 (0.735)	11 (55%)	15 (57.7%)	10 (66.6%)	16 (51.6%)
Cryoglobulins	8 (0.250)	24 (0.750)	6 (23%)	7 (26.9%)	5 (33.3%)	8 (25.8%)
ICs	9 (0.264)	25 (0.735)	8 (40%)	10 (38.4%)	6 (40%)	12 (38.7%)
CLA	18 (0.281)	46 (0.718)	7 (35%)	18 (69.2%)****	10 (66.6%)	15 (48.4%)
Titer of ANA > 1/400	18 (0.300)	42 (0.700)	9 (45%)	21 (80.7%)*****	10 (66.6%)	20 (64.5%)

ICs: immune complexes.
CLA: cardiolipin antibodies.
ANA: antinuclear antibodies.
** *p* value and odds ratio (OR) did not reveals any differences between the two alleles.
*** p; OR (95% CI): 0.028; 0.24 (0.06 - 1.03).
**** p; OR (95% CI): 0.04; 4.18 (1.03 - 17.74).
***** p; OR (95% CI): 0.02; 5.13 (1.17 - 23.99).

Table 5. Analysis of CTLA-4 SNP with clinical and biological features of RA.

CTLA-4 genotype	AA (n = 17)	AG (n = 46)	GG (n = 69)	P	OR (95% CI)
HLA DRB1*04 +	6 (35.2%)*	21 (45.6%)	40 (57.9%)	0.26	0.48 (0.5 - 1.54)
SE +	6 (35.2%)*	24 (52.1%)	31 (44.9%)	0.47	0.6 (0.18 - 1.9)
RF +	12 (70.5%)*	36 (78.2%)	54 (78.2%)	0.33	0.67 (0.19 - 2.41)
CCP +	10 (58.8%)*	36 (78.2%)	46 (66.6%)	0.45	0.8 (0.5 - 3.5)
Bone lesions +	6 (35.2%)	29 (63%)*	27 (39.1%)	0.01	2.74 (1.23 - 6.15)
Early onset < 50 Y.O.	10 (58.8%)*	34 (73.9%)	49 (71%)	0.4	0.55 (0.17 - 1.77)

* Chosen genotype compared with the 2 other genotypes: AG genotype compared to AA genotype (p: 0.049, OR (95% CI): 3.2 (0.9-9.98))AG genotype compared to GG genotype (p: 0.01, OR (95% CI): 2.65 (1.22 - 5.72)).

DISCUSSION

Association of the CTLA-4 (A/G +49) SNP with SLE was controversial. A number of studies observed a significant association of SLE with allele G (Ahmed et al., 2001; Fernandez-Blanco et al., 2004). Other studies showed a lack of association with that allele (Matsushita et al., 1999; Liu et al., 2001). In our study, we found no association of that SNP with SLE. This disparity among diverse populations is probably due to a different genetic background and/ or a possible small degree of involvement of this SNP in SLE predisposition, which might have been missed in underpowered studies. In our study, analysis of CTLA-4 SNP with clinical and serological features showed that, despite not significant, the frequencies of pericarditis, cutaneous vasculitis, hypocomplementaemia, cryglobulins, immune complexes (ICs) and cardiolipin antibodies are higher in patients carrying G allele than those with A allele. Inversely, no differences were observed in the frequencies of nephritis and haematological injuries between the two alleles. Unfortunately, only a few studies have looked for a possible association between this SNP and SLE and none of them have analyzed the severity of various clinical manifestations, such as nephritis (Ahmed et al., 2001; Fernandez-Blanco et al., 2004). Although the CTLA-4 exon 1 SNP may have a small impact in SLE predisposition, it might modify the clinical course of that disease. Nevertheless, we are aware that the major limitation of our report is the failure of statistical power since a small size of patients and controls. A larger patient cohort would therefore be required for us to confirm these observations.

CTLA-4 exon 1 SNP was also disparately quoted in RA, as some studies found a significant association between RA and allele G or homozygous genotype GG (Vaidya et al., 2002; Lee et al., 2003; Cai et al., 2005), while others showed no such association (Barton et al., 2000; Milicic et al., 2001; Lee et al., 2002). Besides, in our study, no association was observed between this SNP and RA. Functional evidence that the G allele leads to reduced CTLA-4 expression and hence, possibly to increased proliferation of autoreactive T cells has been reported by several studies (Kouki et al., 2000; Barreto et al., 2004; Teutsch et al., 2004). In accordance with this hypothesis, Plenge et al. (2005) have shown a stronger association between CTLA-4 exon 1 polymorphism and production of RF or CCP autoantibodies in a larger meta-analysis of 4000 samples from North America and Sweden. These findings corroborate the results of Matsushita et al. (1999), however, in our study, no association of this polymorphism with any of the clinical or biological features of the disease was found. John et al. (2005) have also demonstrated the lack of association between CTLA-4 exon 1 SNP and FR or anti-CCP (+) in RA patients. Our data reveals a correlation between AG genotype and bone lesions. This result would support the view that this SNP may constitute a factor influencing the RA severity, which is hard to assess in various ethnic populations. Several other CTLA-4 polymorphisms, such as, a dinucleotide repeat (AT)n in the 3' untranslated region (Slavcheva et al., 2001, Rodriguez et al., 2002), reported to affect the molecule m-RNA stability and subsequent CTLA-4 expression and a recently described CT60A/G dimorphism (SNP 3087243), which was shown to be associated with lower mRNA levels of soluble CTLA-4 isoform (sCTLA-4) (Van Blezen et al., 2004, Orozco et al., 2006), were considered to be a strong candidate factors also for susceptibility to RA and SLE. Thus, further explorations of these polymorphisms are needed in order to more fully examine RA and SLE associations with CTLA-4 locus in Tunisian population.

Wong et al. (2005) have reported a positive and significant correlation between plasma sCTLA-4 concentration and SLE activity. The above result should provide new postulates for the immunopathological role of this co-stimulatory molecule in the exacerbation of auto-immune diseases and should facilitate recent advances in the exploration of therapeutic agents targeting T-cell activation in these diseases (Wong et al., 2005). Besides, Kremer et al. (2003) have demonstrated that the therapeutic use of antibodies against the sCTLA-4 form (CTLA-4 Ig) versus placebo induces a significant better clinical outcome of RA.

In our study, HLA-DRB1*03 was associated with SLE, corroborating the results found in most of other populations including Caucasian (Tsuchiya et al., 2001; Marchini et al., 2003; Gladman et al., 2004), Latin American

(Vargas-Alarcon et al., 2001) and Asian (Shankarkumar et al., 2003). Interestingly, DRB1*15 allele was also significantly more frequent in SLE patients than in controls, which was observed in studies of Asian (Azizah et al., 2001; Lee et al., 2003) and Latin American (Cortes et al., 2004) populations. On the contrary, none of the studies in Caucasian populations has found any association with this allele. As in Latin American populations (Castano-Rodriguez et al., 2008), DRB1*11 and DRB1*12 alleles appear to play a protective role against SLE in Tunisians, as well as Taiwanese people (Pan et al., 2009), while in Caucasians, DRB1*07 allele is rather protective (Marchini et al., 2003).

In a meta-analysis of HLA-DRB1 polymorphism in Latin Americans, Castano-Rodriguez et al. (2008) showed that the different alleles of susceptibility to SLE share a degree of homology in the variable region of DRβ chain comprising 3 pockets (1, 4 and 9) of susceptibility. Moreover, Marchini et al. (2003) showed that in Italian SLE patients, DRB1*1501 allele was associated with nephritis. Additionally, McHugh et al. (2006) found a significant correlation between DRB1*03 and the positivity of anti-SSA/SSB antibodies. In our study, HLA-DRB1*03 allele was significantly associated with the presence of CLA and high titer of ANA in SLE patients.

These results were in agreement with the concept of an autoantigen driven process involving T-helper cell/ HLA-DR3 recognition (McHugh et al., 2006). But neither DRB1*03 nor DRB1*15 were associated with nephritis, early age of onset and arthritis activity suggesting that HLA-DRB1 polymorphism may be more strongly linked to autoantibody generation but do not constitute a risk factor for SLE severity in Tunisian patients. In accordance with most of the previous studies (Kinikli et al., 2003; Alsaeid et al., 2006 Hughes et al., 2008), our results revealed the classic association between HLA-DRB1*04 allele and RA. However, no association was observed between HLA-DRB1*01 allele and RA patients, in controversy with results showed in Caucasian populations (Hughes et al., 2008). The most frequent subtype in our patients was HLA-DRB1*0405 corroborating the results found in other Mediterranean and Arabic patients (Kinikli et al., 2003; Alsaeid et al., 2006). Inversely, the most frequent subtypes in Caucasian are HLA-DRB1*0401 and *0404, which may contribute to much more severe forms of RA in these populations. Apart from disease susceptibility, some studies showed that HLA-DRB1 polymorphism is a risk factor for disease severity with an increased prevalence of developing erosions (Kinikli et al., 2003) and may also influence response to treatment (Miceli-Richard et al., 2008). The most severe outcome is seen in rheumatoid patients homozygous for *0401 or *0404 or *0401/*0404/*0408 heterozygotes (Alsaeid et al., 2006). Other authors report that homozygosity of HLA-DRB1*0401 has been shown to be significantly increased in patients with major organ involvement and that the compound heterozygote *0404/*0401 has a significantly greater risk of severe erosive RA (Feitsma et al., 2008).

In our study, no association was found between HLA-DRB1 and any of the clinical and biological features, which may be due to the predominance of DRB1*0405 allele. The contribution of the shared epitope to RA susceptibility is open for debate. Notably not all patients with RA are SE positive and ≈ 30% of healthy subjects are HLA-DRB1*04 positive. An alternative theory suggests that susceptibility to RA is rather encoded by HLA-DQ, modulated by certain protective HLA-DRB1 alleles (Dieude and Cornelis, 2005). This effect, however, is probably a refection of linkage disequilibrium with SE alleles (Kilding and Wilson, 2005).

In conclusion, in Tunisian population, the CTLA-4 exon 1 SNP does not seem to contribute to disease susceptibility and severity of SLE and RA. As shown in most of the previously studied populations, HLA-DRB1 appears to play major role in the susceptibility to these diseases also in Tunisians, but does not seem to influence the course or severity of SLE and RA.

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