Full Length Research Paper

Complement receptor 1 gene polymorphisms in Tunisian patients with systemic lupus erythematosus

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Complement receptor 1 (CR1) is a membrane protein mediating the transport of immune complexes (ICs) to phagocytes, and at least two polymorphisms on the CR1 gene are related to erythrocyte surface density of CR1 molecules, in turn related to the rate of IC clearance from circulation. The aim of the study was to explore whether the polymorphic sites of CR1 gene in exon 22 (His 1208 Arg), and exon 33 (Pro 1827 Arg), leading to amino acid change in the protein sequence are associated with systemic lupus erythematosus (SLE) susceptibility. To investigate this association, genomic DNA of 62 SLE patients and 76 healthy blood donors were genotyped by PCR-RFLP and direct sequencing. The CR1 analysis showed no significant association of the CR1 functional polymorphisms with SLE. However, the C/G genotype in Pro 1827 Arg polymorphism was significantly associated to nephritis and to the presence of cryoglobulins/ ICs compared to C/C and G/G genotypes (OR: 3.68, 95% confidence interval [CI], 1.028 - 13.2; p = 0.038 and OR: 16.6, 95% confidence interval [CI], 3.92 - 31.1; p=0.0002, respectively). This study indicates that the analysed polymorphisms of the CR1 gene do not appear to be primarily involved in the susceptibility of SLE. Nevertheless the Pro 1827 Arg polymorphism could constitute a risk factor of gravity of the disease.

Key words: Complement receptor 1, polymorphism, systemic lupus erythematosus, Nephritis, cryoglobulins, immune complexes.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic auto-immune complex disorder, characterized by auto-antibodies production and immune complexes (ICs) for-

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Abbreviations: CR1, Complement receptor 1; **ICs,** Immune complexes; **SLE,** Systemic lupus erythematosus; **PCR-RFLP,** Restriction Fragment Length Polymorphism; **CR2,** Complement receptor 2; **C4BP,** C4-binding protein; **CR1/E ratio,** number of CR1 molecules expressed on erythrocyte surface; **SCRs,** short consensus repeats; **LHR,** long homologous repeat; **ACR,** American College of Rheumatology; **ECLAM,** European Consensus Lupus Activity Measurement (ECLAM); **SLICC,** Systemic Lupus international Collaborating ClinICs/ACR damage index for systemic lupus erythematosus.

mation that often involves multiple organ inflammation (Arbuckle et al., 2003; Nath et al., 2005). The etiology of SLE is unknown, but the condition seems to be the result of a combination of environmental, genetic and immunologic factors in which an uncontrolled immune response leads to chronic inflammation in genetically predisposed individuals (Hartung et al., 1989; Christiansen et al., 1991; Duits et al., 1995; Manger et al., 1998; Kelly et al., 2002; Sullivan et al., 2003; Nath et al., 2004). Significant linkage in chromosome 1q32, which contains complement components, has been reported. This region encodes several proteins like complement receptor 1 (CR1), complement receptor 2 (CR2) and C4-binding protein (C4BP) thought to play a significant role in the pathogenesis of SLE (Lobatto et al., 1988; Schifferli et al., 1989; Davies et al., 1993; Tsao et al., 1999; Johanneson

et al., 2002; Nath et al., 2005; Birmingham et al., 2007).

The C3b/C4b complement receptor (CR1, CD35) is a polymorphic transmembrane single chain glycoprotein found in different cells, including erythrocytes, phagocytes, B lymphocytes and dendritic cells, and is involved in different activities of the complement system (Fearson and Wrong, 1983; Ross, 1992). Indeed, CR1 plays a critical role in the handling of ICs (Dykman et al., 1983; Walport, 2001): under normal circumstances, ICs are tagged with complement products (C1, C4 and C3). CR1 receptors on the surface of erythrocytes bind the C3b-tagged ICs and transport them to the liver and the spleen where fixed cells of the reticulo-endothelial system take them up (Cornacoff et al., 1983), suggesting that the rate of ICs clearance from the circulation can be directly correlated to the number of CR1 molecules expressed on erythrocytes (CR1/E ratio) (Ripoche and Sim, 1986; Herrera et al., 1998). Thus, the reduced level of CR1/E is speculated as a key mechanism contributing to ICs overload and exaggerated complement activation in SLE (Moldenhauer et al., 1988; Cohen et al., 1989; Kiss et al., 1996; Kavai, 2008; Singh et al., 2008).

The CR1 structure is characterized by short consensus repeats (SCRs) of 65-70 amino acids with a conserved core of 11-14 amino acids. In the extracellular domain, 7 SCRs constitute a long homologous repeat (LHR). There have been reported functional and structural polymorphisms of CR1 (Herrera et al., 1998; Zorzetto et al., 2002). The structural polymorphism exists in its molecular size: the most common structural allele of CR1 has four LHRs, but alleles known to have three, five or six LHRs also exist (Nelson, 1953; Birmingham and Herbert, 2001; Krych-Goldberg and Akinson, 2001). Three functional polymorphisms have been found on the CR1 gene: intron 27 *Hind*III RFLP, His 1208 Arg and Pro 1827 Arg. The intron 27 *Hind*III RFLP gives rise to two alleles: H allele, associated with allotypes containing three, four, or five LHRs, and L allele, reported only in association with the allotype containing four LHRs. This functional polymorphism determines the quantitative expression of CR1/E, HH, HL and LL (H = allele correlated with high expression and L= for low CR1/E ratio) (Satoh et al., 1991). Erythrocytes from individuals homozygous for the H allele may show as much as a 10 fold higher CR1/E ratio than those from individuals homozygous for the L allele; heterozygous individuals have intermediate CR1 expression (Waxman et al., 1984; Waxman et al., 1986; Wilson et al., 1987; Schifferli et al., 1989, Davies et al., 1992; Moulds et al., 1998; Xiang et al., 1999; Oudin et al., 2000).

In this data, we analysed two polymorphisms in SLE patients and healthy volunteers: His 1208 Arg results from an A→G substitution occurring in exon 22 at nt 3650 and Pro 1827 Arg results from a C→G substitution occurring in exon 33 at nt 5507. The Pro 1827 Arg C allele and the His 1208 Arg A allele have been reported to be associated with H allele, whereas, the Pro 1827 Arg G allele and the His 1208 Arg G allele have been reported to be associated with L allele (Herrera et al., 1998; Xiang et al., 1999; Zorzetto et al., 2002).

MATERIALS AND METHODS

Patients and controls

A total of 62 convenient samples from Tunisian SLE subjects (55 women and 7 men) followed up at the department of Medicine, Charles Nicolle Hospital, were investigated. At the time of the diagnosis, all the patients answered at least 4 revised criteria of the American College of Rheumatology (ACR) (Hochberg, 1997) were included in our study. Demographic data for each patient were obtained retrospectively from the official medical record at the time of the first visit and included sex, age and age at the time of diagnosis. Common clinical manifestations, haematological and immunological parameters, including antinuclear antibodies, ds DNA, cryoglobulins and low C3 and C4, as well as an 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 >500mg/24h (or > 3+) or cellular casts) (Hochberg, 1997). Because the distribution of the CR1 genotypic forms is known to be different between ethnic groups (Sullivan et al., 2003), we limited our study to Tunisian subjects.

As control group, we studied 76 ethnically and geographically matched healthy subjects (60 women and 16 men, mean age was 35 ± 12 years), recruited from the blood donors of the same area than patients. Medical examination and a questionnaire were performed for all controls. Exclusion criteria were autoimmune diseases in the donor or SLE in a first-degree relative.

The study was approved by the local ethICs committee and informed consent was obtained from all subjects

Methods

The two polymorphisms of CR1 gene were studied by restriction analysis for exon 22 and direct sequencing for Pro 1827 Arg (exon 33) polymorphism. Regions encompassing each polymorphism were amplified from genomic DNA, according to Cornillet and Xiang studies (Cornillet et al., 1991; Xiang et al., 1999). Appropriates segments of the CR1 gene was amplified using specific primers:

Exon 22 forward: 5'-TTCACATTGCATAGCCAGAGC-3'
Exon 22 reverse: 5'- CCAGAGGTTAATCTCCCTGGA-3'
Exon 33 forward: 5'-AAGCGCACAGTCACAGGTCAC-3'
Exon 33 reverse: 5'-GGAGGTAGTTCTGTCTCTGAC-3'

Briefly, the amplification was performed using a Gene Amp PCR system 9700 (Perkin-Elmer). 100ng of Genomic DNA were added to 15 ul of amplification buffer containing: 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 5 pmol of each primers and 0.5 U of Taq polymerase (Promega, USA) for a total of 20 ul. Thermal cycling was performed with an initial activation step at 94°C for 10min, then 35 cycles of denaturation at 94°C for 1min, annealing temperature (60°C for exon 22 His 1208 Arg and 56° for exon 33 Pro 1827 Arg) for 15s, extension at 72°C for 2min, and a final extension at 72°C for 10min. The PCR products obtained were 682 bp for exon 22 and 305 bp for exon 33.

These amplification products of exon 22 were digested with 1U *Rsa* I (Promega, USA) in a 15 ul volume at 37°C for 1,5h and two fragments of 520 and 162 bp corresponding to A allele and three

Table 1. Demographic, clinical and biological ccharacteristics of patients.

Patients	n = 62
Gender	55 females and 7 males
Mean age (years)	31 ± 14
Age at diagnosis (years)	31.2 ± 12.8
Clinical manifestations (%)	
Cutaneous vasculitis	42 (68)
Arthritis	50 (80)
Serositis	22 (36)
Nephritis	35 (56)
Neurological disorders	13 (20)
Haematological abnormalities	40 (63)
Immunological parameters (%)	
Antinuclear antibodies	62 (100)
Ds DNA antibodies	44 (70)
Low C3 and C4	44 (70)
Cryoglobulins and/or ICs	26 (42)
ECLAM* (range)	2.9 (0.4-6.2)
SLICC** (range)	2.0 (0-10)

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).

fragments of 458, 162 and 62 bp corresponding to G allele were obtained (Zorzetto et al., 2002). Amplimers of exon 33 was analyzed by sequencing using Big Dye terminator cycle sequencing kit (Applied Biosystems USA), showing the CG, GG or CC genotype electrophoretic pattern.

Statistical analyses

All statistical analyses were performed with the Statistical Package for Social Sciences version 13.0 (SPSS Inc., Chicago, IL, USA). The Hardy-Weinberg equilibrium was assessed by the goodness-of fit test for biallelic markers. Calculation was done using internet programs from (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw-/hwa1.pl). Statistical power was calculated using a web power calculator (http://calculators.stat.ucla.edu/powercalc/). Allelic and genotypic frequencies were evaluated by direct counting. Statistical comparisons were performed, between patients and controls, by Pearson's chi^2 test calculated on 2 x 2 contingency tables. Fisher exact test was used when an expected cell value was less than 5. p value < 0.05 was considered to be statistically significant. The strength of the association of CR1 exon 22 and exon 33 genotypes as genetic factor with the frequency of a certain SLE symptom was estimated by the calculation of the Odds ratios (OR) (OR>1: positive association; OR = 1: no influence; OR < 1: protective) (Bailar and Mosteller, 1986) and 95% confidence intervals (95% CI), using the same software. For groups of equal variance, the correlation between age at the onset of symptoms and CR1 genotypes was followed by two-sample Student's t-test, to compare the means of the individual groups. The Mann-Whitney U test was used to determine whether there was any significant difference between CR1 genotype groups for continuous data.

RESULTS

Data obtained from each patient included gender, age, disease clinical and biological manifestations were used to define the epidemiological characteristICs of the patients and were shown in Table1.

Genotypes of patients and controls were in accordance with Hardy-Weinberg equilibrium. However, there were no significant differences in genotypic and allelic frequencies between SLE patients and controls in His 1208 Arg exon 22 polymorphism (Table 2). Although, we found an increase frequency of the exon 33 Pro 1827 Arg G allele (0.226) in patients compared to healthy controls (0.172), the difference did not reach statistical significance.

The demographic data of patients did not show any significant differences between the exon 22 and exon 33 CR1 genotypes in gender, age and age of onset (Table 3). Notably, the C/G genotype in Pro 1827 Arg exon 33 polymorphism was significantly associated to nephritis (77%) compared to C/C and G/G genotypes (48% and 40%, respectively) (OR: 3.68, 95% CI, 1.028 to 13.2; p = 0.038) (Table 4). Also, this same C/G genotype and G allele of this polymorphism were positively correlated with the presence of cryoglobulins and/or ICs (OR: 16.6, 95% CI, 3.92 to 31.1; p = 0.0002, and, OR: 4.03, 95% CI, 1.64 - 9.89; p = 0.0015, respectively).

In exon 22 polymorphism, the frequency of A/G genotype was increased in patients with nephritis and with cryoglobulins (72% and 54%, respectively) com-pared to A/A (48% and 35%) and G/G genotype (33% and 33%). This same pattern of increase A/G genotype was observed with haematological injuries. But in every case the difference is not statistically significant (Table. 4).

Furthermore, the distribution of the genotypes: AG (exon22) / CG (exon33) was similar in patients with and without nephritis or cryoglobulins (17 vs. 12%) (data not shown).

DISCUSSION

In vivo and *in vitro* abnormalities in both complement and immune complex clearance mediated have been described in SLE (Isaák et al., 2008; Voynova et al., 2008). CR1 plays an important role in ICs clearance (Arora et al., 2004; Boackle et al., 2004) and genetic variation affecting the expression levels or functions of this protein could theoretically lead to variation in the efficiency of proper ICs clearance, and thus to risk for ICs diseases such as SLE (Gergely et al., 2007). In addition, several susceptibility genes for SLE have been located on the long arm of chromosome 1 in the region 1q32 (Botto and Walport, 2002; Manderson et al., 2004; Nath et al., 2005), where complement receptor 1 gene is located, suggesting that CR1 polymorphisms could have a crucial role in determining pathogenesis and course of

Table 2. Genotype and allele frequencies of CR1 polymorphisms studied in controls and patients.

Polymorphism		Genotype Frequency (%)			Allele frequency		p
Exon 22 A3650G	n	A/A	A/G**	G/G	A*	G	
Controls	76	49 (64.4%)	19 (25%)	8 (10.6%)	0.769	0.231	0.93
Patients	62	37 (59.7%)	22 (35.5%)	3 (4.8%)	0.774	0.226	
Exon 33 C5507G	n	C/C	C/G***	G/G	C*	G	
Controls	76	55 (72.4%)	16 (21%)	5 (6.6%)	0.828	0.172	0.30
Patients	62	39 (63%)	18 (29%)	5 (8%)	0.774	0.226	

*Chosen allele compared with the other allele
**Chosen genotype compared with the two other genotypes: OR: 1.53, 95% CI, 0.72 to 3.23; p = 0.26
***Chosen genotype compared with the two other genotypes: OR: 1.58, 95% CI, 0.72 to 3.49; p = 0.24.

Table 3. Demographic comparisons between the CR1 genotypes in Exon 33 and Exon 22.

Exon 33	CC (n = 39)	CG (n = 18)	GG (n = 5)	p*
Patient age in years: median (range)	32.5 (18 - 50)	28 (15.4 - 41)	29 (16 - 42)	p = 0.56
Age at diagnosis in years: median (range)	21.7 (15 - 27)	20 (15 - 25)	22.5 (15 - 29)	p = 0.36
Gender (women/men)	34/5	16/2	5/0	p = 0.24
ECLAM score: median (range)	3.1 (0.5 - 4.8)	2.7 (1.4 - 5.1)	3.0 (0.8 - 5.2)	p = 0.52
SSLICC score: median (range)	3.0 (0 - 10)	2.5 (0 - 8)	2.0 (0 - 8)	p = 0.32
Exon 22	AA (n = 37)	AG (n = 22)	GG (n = 3)	p*
Patient age in years: median (range)	33(17.8 - 49)	26.6 (15 - 42)	31 (16 - 46)	p = 0.48
Age at diagnosis in years: median (range)	22 (14 - 30)	18 (12 - 24)	20 (12 - 28)	p = 0.38
Gender (women/men)	33/4	19/3	3/0	p = 0.28
ECLAM score: median (range)	3.4 (0.5 - 4.3)	2.3 (1.4 - 3.4)	3.0 (0.9 - 3.8)	p = 0.50
SSLICC score: median (range)	3.0 (0 - 8)	3.0 (0 - 10)	2.0 (0 - 8)	p = 0.28

*Two-sided *P* values were calculated to determine whether there was any significant difference between CR1 genotype groups using the Mann-Whitney U test for continuous data and the Pearson chi-square test for dichotomous data. For groups of equal variance, the correlation between age at diagnosis and CR1 genotypes was followed by two-sample Student's t-test, to compare the means of the individual groups.

Table 4. Analysis of CR1 SNPs with clinical and biological features of SLE.

Exon 33	CC (n = 39)	CG (n = 18)*	GG (n = 5)	C	G	p	OR (95% CI)
Nephritis	19 (48%)	14 (77%)	2 (40%)	0.742	0.258	p = 0.038	3.68 [1.028 - 13.2]
Cutaneous vasculitis	25 (64%)	13 (72%)	4 (80%)	0.750	0.250	p = 0.38	1.2 [0.7 - 2.9]
Arthritis	29 (74%)	16 (88%)	5 (100%)	0.740	0.260	p = 0.08	2.31 [0.58 - 9.12]
Haematological injuries	25 (64%)	12 (66%)	2 (40%)	0.795	0.205	p = 0.50	1.41 [0.38 - 5.2]
Cryoglobulins/ICs	9 (23%)	15 (83%)	2 (40%)	0.630	0.365**	p = 0.0002	16.66[3.92 - 31.1]
Exon 22	AA (n = 37)	AG (n = 22)*	GG (n = 3)	A	G	p	OR (95% CI)
Nephritis	18 (48%)	16 (72%)	1 (33%)	0.742	0.258	p = 0.33	0.78 [0.2 - 2.9]
Cutaneous vasculitis	25 (67%)	14 (63%)	3 (100%)	0.762	0.238	p = 0.38	0.7 [0.39 - 1.28]
Arthritis	30 (81%)	18 (81%)	2 (66%)	0.780	0.220	p = 0.63	0.79 [0.19 - 2.61]
Haematological injuries	21 (56%)	17 (77%)	1 (33%)	0.756	0.244	p = 0.53	1.12 [0.27 - 4.6]
Cryoglobulins/ICs	13 (35%)	12 (54%)	1 (33%)	0.730	0.270	p = 0.32	1.43 [0.77 - 2.56]

*Chosen genotype compared with the 2 other genotypes
**Chosen allele compared with the other allele: OR: 4.03, 95% CI, 1.64 to 9.89; p = 0.0015.

this disease (Tsao et al., 1999; Johanneson et al., 2002).

The present data document an association between the tested exon 33 CR1 polymorphism, the prevalence of nephritis and occurrence of cryoglobulins and/or ICs in Tunisian patients with SLE. To our knowledge, this is the first report providing information about the clinical impact of this polymorphism on the course of SLE.

Initial studies (Satoh et al., 1991) suggested that the low expression genotype might be responsible for the low CR1 levels that have been observed in SLE patients. Subsequent studies (Wilson et al., 1987), however, indicated that the lower CR1 levels in SLE reflect an acquired defect, because H and L alleles did not show altered frequencies. Our study showed no significant differences in the distribution of exon 22 and 33 CR1 genotypes between patients with SLE and controls of an ethnically homogonous group, thus excluding the CR1 functional polymorphisms as susceptibility factors for SLE. A recent meta-analysis (Nath et al., 2005), also showed no association of the CR1 functional polymor- phisms and SLE in both Asian and Caucasian patients, suggesting no association of lower CR1 levels in SLE and the CR1 functional polymorphism. However, this data revealed the association of the S structural variant of CR1 and this disease. CR1 exhibits four structural polymorphic forms differing in terms of their molecular weights. Although no functional differences have so far been detected between the structural variants of the CR1 (Kumar et al., 1995; Sullivan et al., 2003), other studies (Cornillet et al., 1992; Moulds et al., 1996) suggest the association of the S structural variant of CR1 and SLE. However, it is unclear how the structural polymorphism of the CR1 may play a role in the pathogenesis of this disease.

We paid particular attention to the Pro 1827 Arg polymorphism (exon33) since this was available in the largest series of subjects and because the C$\rightarrow$G substitution leading to the Pro$\rightarrow$Arg amino acid change at position 1827 of the protein sequence in the proximal extramembrane region of the L allotype, according to Herrera et al. (1998), may create a cleavage site allowing its accelerated proteolysis. De Carvalho et al. (2004) showed that reduced erythrocyte complement receptor type 1 in systemic lupus erythematosus is related to a disease activity index and not to the presence or severity of renal disease. Also, Birmingham et al. (2006) have demonstrated that consumption of erythrocyte CR1 is associated with protection against SLE renal flare. These data contrast with study of Verma et al. (2005) and others (Katyal et al., 2004), who found an association of leukocyte CR1 gene transcription with the disease seve- rity and renal involvement in SLE. Our study shows that G allele of CR1 gene exon 33 polymorphism is more frequent in subjects with SLE than ethnically-matched healthy controls, but the difference was not statistically significant. We also had evidence that the CG genotype, which correlated intermediate CR1/E expression (Herrera et al., 1998; Xiang et al., 1999; Zorzetto et al., 2002), was

significantly more frequent in SLE patients with nephritis and with cryoglobulins and/or ICs than others genotypes. But we did not find any statistically significant association with the other clinical or biological variables and with either ECLAM or SLICC activity scores of SLE. These facts could be explained mainly by the reduced number of patients in our study. Although, we may postulate a role for the Pro 1827 Arg polymorphism in the occurrence of nephritis, it constitutes a weak predictor of disease severity and exacerbation activity of SLE. Nevertheless, we are aware that the major limitation of our report is the lack of a relationship between genetic issues and the corresponding phenotype, because the CR1/E ratio was not determined in our study. But, even if we may consider our report as a sort of serendipitous finding, we believe that it might be of some interest from a genetic point of view, because it opens up interesting speculations about many things, from the gene to the role of the protein on pathogenetic processes in SLE and it suggests future areas of therapeutic research.

Both polymorphisms Pro 1827 Arg and His 1208 Arg exon 22 do not appear to be involved in the susceptibility of SLE or in the occurrence of nephritis. In fact, the distribution of the genotypes AG (exon22) / CG (exon33) was similar in patients with and without nephritis or cryoglobulins. Further work in a larger study with more detailed linkage analysis of the CR1 gene, especially the intron 27 *Hind*III RFLP polymorphism, which is in linkage disequilibrium among the two other polymorphisms (Zorzetto et al., 2002), is required to confirm these results.

In conclusion, our study demonstrated no difference in the CR1 functional polymorphisms between SLE cases and controls. So they do not appear to be a genetic predisposing factor to this disease in our population. Although not relevant, a significant association between SLE patients with nephritis in the Pro 1827 Arg polymer- phism of the CR1 gene speculates about a possible role for this polymorphism as a partial predictor gene of severity of SLE in Tunisian patients.

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