PTPN22, CTLA4, FcγRIIa, FcγRIIa and FcγRIIIb polymorphisms in Tunisian patients with systemic lupus erythematosus

Background: Pathogenesis of systemic lupus erythematosus (SLE) involves both T cell tolerance breakdown and pathogenic autoantibodies. Polymorphisms in T cell regulatory proteins (PTPN22 and CTLA-4) and IgG receptors (FcγR) genes could impact their functions. Consequently, we aimed to study the role of PTPN22, CTLA-4, FcγRii and FcγRii polymorphisms on either SLE susceptibility or its severity.

Methods: Consequently, PTPN22 rs2476601 (R620W), CTLA-4 rs231775 (+49 A/G), FcγRIIa rs1801274 (R131H), FcγRii rs396991 (F158V) and FcγRiiNa1/Na2 polymorphisms were examined in 137 SLE patients and 100 healthy blood donors matched in age, gender and ethnic origin.

Results: The PTPN22-620*W mutant allele was significantly more prevalent in SLE patients comparatively to controls; p=0.001, OR [95% CI] = 7.8 [1.73-48.85]. Inversely, the frequency of the CTLA-4*G/G homozygous genotype was significantly lower in patients (35%) than in controls; p=0.02, OR [95% CI] = 0.54 [0.31-0.94]. Regarding to FcγR polymorphisms, while the FcγRii*V allele was more prevalent in case of SLE (0.562 vs 0.35); p=0.001, OR [95% CI] = 2.77 [1.38-5.68], FcγRii and FcγRiiib did not show any association with SLE.

Analytic results showed that the prevalence of anti-dsDNA autoantibody was significantly higher in patients carrying PTPN22*W allele p=0.038. Otherwise, no correlation was found between the five studied polymorphisms and either clinical or biological patients characteristics.

Conclusion: PTPN22 R620W, CTLA-A +49 A/G and FcγRIIa F158V polymorphisms seem to be related to SLE susceptibility in Tunisian.

Keywords: Systemic lupus erythematosus • Polymorphism • PTPN22 • CTLA-4 • FcgR

Abbreviations: dsDNA: double stranded Deoxy Ribonucleic Acid; Csk: C-terminal Src (sarcoma) Kinase; CTLA-4: Cytotoxic T-Lymphocyte-Associated Antigen 4; Lyp: Lymphoid Protein Tyrosine Phosphatase; PCR: Polymerase Chain Reaction; PCR-RFLP: PCR- Restricted Fragment-Length Polymorphism; PTPN22: Protein Tyrosine Phosphatase Non-Receptor Type 22; RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematosus; SLEDAI: SLE Disease Activity Index

Introduction

Systemic Lupus Erythematosus (SLE) is a multisystemic autoimmune disease in which the presence of autoantibodies targeting nucleic antigens is the result of loss of both central and peripheral tolerance. SLE is the consequence of multiple defects including reduced ability to clear Immune Complexes (IC) and apoptotic cells and hyperactivation of autoreactive T- and B-cells [1].

PTPN22, known as Lyp protein, is an intracytoplasmic enzymatic glycoprotein mainly expressed by T cells [2]. PTPN22 plays the role of a negative regulator of TCR through intracellular molecules called SHP (src homology region 2 -phosphatase domain) [3]. While previous genetic studies indicate that PTPN22 is one of most implicated genes in autoimmunity [4], PTPN22 mRNA decrease in SLE suggests that its absence is involved in self-tolerance breakdown [5]. Among the polymorphisms in the PTPN22 gene, the rs2476601 SNP, also called R620W, results in an amino acid variation from arginine to tryptophan which disrupts its interaction with Csk (C-terminal Src Kinase) and leads to deregulation of T cells [6]. The rs2466601 SNP have been associated with diverse autoimmune diseases including rheumatoid arthritis (RA), autoimmune diabetes, Graves’ disease, SLE, etc. [7].

CTLA-4 is a receptor expressed specifically on T cells upon extended activation and shares some homology as well as ligands with the CD28 [8]. Nevertheless, if CD28 delivers activation signal, CTLA-4 ligation provides a regulatory effect on T-cells. In fact, an increase of T-cell proliferation has been obtained in vitro using a soluble anti-CTLA-4 monoclonal antibody that block CTLA-4 to CD80/CD86 ligation.
This study involved 137 SLE patients and 100 controls. Healthy blood donors were used as controls. The identification of the PTPN22 rs2476601 polymorphism was performed using the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method. A standard salting-out procedure [22] has been performed to extract genomic DNA from EDTA blood. The identification of the PTPN22 rs2476601 polymorphism was performed using the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method. To a 10 µL PCR volume containing 50 ng DNA, 100 pmol of each primer, 1 U of Taq polymerase (Promega, USA), and 0.2 mmol/L of each deoxynucleoside triphosphate (dNTP) were added. Taq polymerase reaction was performed according to the manufacturer's instructions. The PCR products were digested with the restriction enzyme HhaI and subjected to electrophoresis in agarose gel.

**Autoantibodies and autoimmunity**

Autoantibodies produced in SLE and most other autoimmune diseases are mainly of the IgG class [16]. IgG-autoantibody/autoantigen complexes are involved in SLE pathogenesis through complement system activation and Fcγ receptors (FcγR) ligation. FcγR are mainly expressed on leucocytes and can bind IgG antibodies, which results in activation of effector actions such as phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) [16]. In humans, there are 3 main classes of FcγR: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) whose cell distribution and affinity vary according to IgG subclasses and regulate immune-inflammatory processes [17]. The binding affinity of the three FcγR, FcγRIIA and FcγRIIB, to IgG displays inter-individual disparity caused, in part, by their genes polymorphisms [18]. The FcγRIIA rs1801274 (R131H) SNP, is due to a guanine-to-adenine (G>A) switch at nucleotide 519. This mutation results in a change of alanine (A) to threonine (T) in the IgG-binding domain which results in an increase in the avidity of IgG2 binding [19]. The FcγRIIB rs936991 (F158V) SNP is a threonine-to-arginine (T->A) switch at nucleotide 499, which results in an increase in the avidity of IgG1, IgG2 and IgG4 binding [20]. Even if the FcγRIIB NA1/NA2 polymorphism has not been reported to influence IgG binding, the wild NA1 variant displays improved efficiency in binding to immune complexes and has been related to autoimmunity [20].

In this peculiar context, we aimed to assess the influence of PTPN22, CTLA4, FcγRIIA and FcγRIIB genetic polymorphisms on SLE predisposition as well as disease severity.

**Material and methods**

**Subjects**

This study involved 137 SLE patients and 100 blood donors exempt from any autoimmune disease. Patients have been collected from internal medicine and nephrology department A and internal medicine department B of the Charles Nicolle university Hospital in Tunis and were diagnosed as SLE patients according to the Criteria Committee of the American College of Rheumatology [21]. Clinical and biological characteristics of the 137 SLE patients are documented in Table 1.

Controls were healthy blood donors matched with patients for age, sex and ethnicity. No evidence of family or personal history of SLE or autoimmunity has been noted. A written informed consent to be included in the present study has been obtained from all patients and controls. The Charles Nicolle Hospital’s local Ethics committee approved the present study.

**Blood sampling and genotyping**

A standard salting-out procedure [22] has been performed to extract genomic DNA from EDTA blood. Taq polymerase reaction was performed according to the manufacturer’s instructions. The PCR products were digested with the restriction enzyme HhaI and subjected to electrophoresis in agarose gel.
was added following primers: forward (5’-TG-CCCATCCACATTTTAT-3’) and reverse (5’-ACCTCCTGGTTTTGTACCTTA-3’). Thermal cycling was performed with an initial activation step at 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing temperature 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR product was incubated with 1 U enzyme Rsal (Promega, USA) in a 15-μL volume at 37°C for 1.5 hours. The PCR generated a 326 bp fragment containing a restriction site for Rsal, which permitted differentiation of the R620- allele (228 bp) and the 620W-allele (272 bp).

The CTLA-4 rs231775 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’ CAAGGCT- CACGCTGAACCTGGGT 3’ and CTLA-4 (2) reverse: 5’ TACCCTTTACCTCCTGCGTTTG 3’. The PCR was performed using 0.1 μg of genomic DNA, 10 pmol of each primer, 200 μM of dNTPs and 0.5 U 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 incubated at 37°C for 4 h using 5 U 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.

FcyRIIa rs1801274 genotyping was performed by means of a nested-PCR technique. For the first step, a 1000 bp PCR product containing the polymorphic FcyRIIa site was generated using specific primers sense (P63): 5’CAAGGCTCTAGCCTTGGTTATCCTTAGCTGCTG3’ and antisense (P52) primers: 5’GAAGAGCTGCCCATGCTG3’. Briefly, this initial PCR was performed in a Perkin-Elmer thermal cycler using 50 ng of DNA, 100 pmol of each primer, 1.5 mM MgCl2 and 1 U of Taq polymerase (Promega, USA) in a volume of 20 μL of reaction buffer. The PCR conditions were 95°C for 5 minutes, followed by 35 cycles consisting of 95°C for 30 s, 58°C for 45 s and 72°C for 30 s, and then 72°C for 7 minutes. The products of the H131- and R131-specific PCRs were evaluated in ethidium bromide-stained 2% agarose gels for the presence of a band at 290 bp.

A direct sequencing procedure was performed for FcyRIIIa rs396991 polymorphism genotyping. A 199-bp PCR product that contained the polymorphic site was amplified using a specific primer sequences: sense: 5’ TGTAAAACGACGCCTTCCGAGCTTG 3’ and antisense: 5’ CAGGAAACAGCTATGAGCTGAGTCTGCTCTTCA 3’ A 20 μL PCR was performed using 150 ng DNA, 100 pmol of each primer, 200 mM dNTPs, 1.5 mM MgCl2 and 0.5 U of Taq DNA polymerase (Promega, USA). The PCR conditions were 95°C for 5 minutes, followed by 36 cycles at 95°C for 30 seconds, 52°C for 40 seconds, and 72°C for 40 seconds, with a final extension step at 72°C for 10 minutes. Fluorescence automated cycle sequencing of the PCR products was performed using a “big Dye Terminator” reaction kit (Perkin Elmer, APPLIED BIOSYSTEM, Foster City, CA). Electrophoresis was performed on polyacrylamide gels using the ABI Prism 310 DNA Sequencer (Perkin Elmer, APPLIED BIOSYSTEM), and the sequence was analyzed using ABI Prism 310 sequencing software (PE Biosystems). All available sequence data was identified by performing BLAST (basic local alignment search tool) sequence homology searches at the National Centre for Bio-informatics (NCBI).

Genotype analysis of the NA1 and NA2 alleles of the FcyRIIib gene was performed by PCR employing allele-specific sense and antisense oligonucleotide primers. The NA1 sense primer (5’ CAGTGGTTT CACAACTGAA 3’) contained a mismatch at position 4 from the 3’ end in order to prevent mispriming. The sequence of the NA1 antisense primer was as follows: 5’ CATGGACTTCTAGCTGACCCG 3’. The NA1-specific amplification protocol, which
amplifies a 142-bp product, included 1 cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and then 72°C for 7 min to facilitate primer extension. In the NA2-specific PCR, the following sense and antisense primers were employed: 5’ CTCAATGGTACAAGCGTGTT 3’ (sense) and 5’ CTGTACTCTCCACTGTCGTT 3’ (antisense) and the PCR included, like the NA1-specific PCR assays, 50 ng DNA, 100 pmol of each primer, 200 mM dNTPs, 1.5 mM MgCl2 and 0.5 U of Taq DNA polymerase (Promega, USA) in a final volume of 20 μl. The same protocol that NA1 was performed (exception for annealing temperature which is at 60°) generating a 169-bp product.

Statistical analysis
Statistical evaluation was carried out using the SPSS version 11 software (IBM®, Armonk, USA). p-values <0.05 were considered as significant.

Chi-square or Fisher exact tests were used to test the association between categorical variables. Odds ratio (OR) together with 95% confidence intervals [95%CI] were calculated to estimate the strength of the association.

ANOVA, Mann-Whitney U and Kruskal-Wallis tests were used to analyze quantitative and semi-quantitative variables, as appropriate.

The Hardy-Weinberg equilibrium was confirmed for the five studied SNPs via internet (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl).

Results
In total, the present study involved 137 SLE patients and 100 healthy controls. Mean age of the patients group was at 32.51 ± 13.6 years and the sex-ratio (Men/Women) was 0.16 (19/118). Mean onset-age was at 27.49 ± 11.9 years with a median evolution period of 4 [2-10] years. Mean Systemic lupus erythematosus disease activity index (SLEDAI) was at 11.91 ± 6.617 with 99 (72.3%) patients with an active disease (SLEDAI ≥ 8). Nephritis was found in 82 (59.9%) patients while neurolupus was noted in only 19 (13.9%) patients. Serologically, antinuclear antibody (ANA) was detected with high titer (>320) in 100 (73%) patients; anti-dsDNA antibody was found in 93 (67.9%) cases while complement activity was decreased in 111 (81%) patients (Table 1).

Analysis of the PTPN22 rs2476601 polymorphism
The frequency of PTPN22*R/R homozygous wild genotype was significantly lower in patients (88.3%) than in controls (98%); p=0.005, OR [95% CI] = 0.15 [0.02-0.72]. Accordingly, the PTPN22*W mutant allele was significantly more frequent in patients (0.145) than in controls (Table 2).
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**Alleles**

| Genotypes | FcγRIIb NA1/NA2 | Genotypes | Alleles |
|-----------|-----------------|-----------|---------|
| NA1/NA1   | 15 (15%)        | NA1/NA2   | 42 (42%)|
| NA1/NA2   | 43 (43%)        | NA2/NA2   | 50 (36.5%)|

**Alleles**

| FcγRIIb NA1/NA2 | NA1 | NA2 | 0.36 | 0.64 |
|-----------------|-----|-----|------|------|
| Genotypes      |     |     |      |      |
| NA1/NA1         | 0.36| 0.64| 0.437| 0.562|
| NA1/NA2         | 0.38| 0.66|      |      |

(0.01); p=0.001, 7.8 [1.73-48.85] (Table 2).

Analytic results showed that the PTPN22*W mutant allele was significantly associated with the presence of anti-dsDNA antibody, p=0.038. Inversely, the PTPN22 SNP was associated neither to nephritis, nor to neurolupus, nor to SLEDAI. Moreover, no correlation was found between the PTPN22 rs2476601 polymorphism and either elevated titer of ANA or decreased complement activity (DCA).

**Analysis of the CTLA-4 rs231775 polymorphism**

The CTLA-4*G/G homozygous mutant genotype was significantly less frequent in patients (35%) than in controls (50%); p=0.02, OR [95% CI] = 0.54 [0.31-0.94] (Table 2). Moreover, and even lacking significance, the frequency of the CTLA-4*G allele was lower in SLE patients (0.631) comparatively to controls, p=0.09.

Analytic results did not show any association between the CTLA-4 studied polymorphism and the clinical pattern of SLE in patients. Furthermore, there was no correlation of the CTLA-4 SNP with either serological markers or DCA.

**Analysis of FcγRIIa rs1801274, FcγRIIIa rs396991 and FcγRIIIb NA1/NA2 polymorphisms**

While genotypes and alleles frequencies were similar in patients and controls for the FcγRIIa rs1801274 and FcγRIIIb NA1/NA2 polymorphisms, the FcγRIIIa*V/V homozygous mutant genotype was significantly more prevalent in patients (32.8%) comparatively to controls (15%); p=0.0018, OR [95% CI] = 2.77 [1.44-5.33] (Table 2). Furthermore, the frequency of the FcyRIIIa*V allele was significantly higher in SLE patients (0.562) than in controls (0.360); p=0.00001, OR [95% CI] = 2.28 [1.56-3.31].

Besides, no association was found of the 3 FcγR studied polymorphisms with either clinical or biological characteristics of patients. Moreover, genetic combinations (haplotypes) of the 3 FcγR polymorphisms did not reveal any correlation with either SLE risk or its outcome.

**Discussion**

In addition to the clinical and evolutionary polymorphism of SLE, significant variations in the expression of the disease are observed between populations from different ethnic origins and seem to involve several factors, not only hormonal and environmental, but also genetic. A part from HLA locus, several non-HLA loci have been linked to SLE susceptibility [23-25]. Some of these chromosomal regions appear to be common to various autoimmune diseases such as RA, type I diabetes, and autoimmune thyroiditis [26]. The analysis of these non-HLA loci by both association studies of multiplex families and case-control studies [27] suggested the implication of candidate genes, coding for molecules, involved in immune response regulation (CTLA-4 and PTPN22) as well as in the clearance of the IC (FcγR) as potential susceptibility factors for SLE [18].

In this study, we noted a significant association between the PTPN22-620*W mutant allele and susceptibility to SLE; p=0.001, 7.8 [1.73-48.85]. This significant finding corroborates the results of 2 meta-analyses [28,29] which stated that the PTPN22 rs2476601 polymorphism confers SLE susceptibility, mainly in Caucasian and Latin populations. Nevertheless, a lack of association between the PTPN22 R620W SNP and SLE predisposition has been reported especially in the Chinese population [29-31]. These discrepancies could be, in part, explained by the great variation of the R620W polymorphism allelic distribution between populations from different ethnic origin. Indeed, the 620W variant is relatively prevalent in European populations, 2% to 3% in Italy and Sardinia, 7 to 8% in Western Europe, and in general >10% in Scandinavian countries, reaching 15% in Finland [32,33]. Inversely, this 620W variant is virtually absent in China and South-Africa [30,31]. This North-
South gradient has also been confirmed by other authors who have raised the possibility of linkage disequilibrium between this 620W allele of PTPN22 and another genetic variation corresponding, in fact, to the real causative allele. However, the association of this 620W allele with various autoimmune diseases supports its direct involvement in the pathogenesis of lupus. In fact, the PTPN22 rs2476601 SNP results in a decrease of the downregulation of T cell activation [6]. This gain in function induced by this SNP could also explain, at the thymic level, the increase of autoreactive T clones that should normally be deleted [34]. In the present study, we found a significant association between anti-dsDNA positivity and the 620W variant, p=0.038. However, this susceptibility-associated allele did not influence the SLE outcome in our patients which agrees with the mainstream of previous studies. Nevertheless, Machado-Contreras, et al. [35] showed, via PTPN22 mRNA quantification, that the R620W variation was predictive of a more active lupus disease. These findings have led some authors to propose this molecule as a potential therapeutic target, not only during SLE, but also, for other autoimmune diseases [36].

In the present study, the CTLA-4 rs231775 SNP conferred a protective role against SLE occurrence; p=0.02, OR [95% CI] = 0.54 [31-0.94]. This was similarly the case in Indian, Thai and Chinese populations [37-40]. Inversely, this association to SLE susceptibility has not been found in other independent cohorts of the same populations or in different ethnic groups [41]. Indeed, 2 previous meta-analyses confirmed these discrepancies, arguing that this SNP is likely to be a genetic factor of susceptibility to SLE in Asians, but not in Caucasians [40, 41]. The protective role of the CTLA-4*G variant was estimated at 0.85 [0.73-0.99] in another recent meta-analysis [42]. Functionally, the CTLA-4*G variant could alter the spatial conformation and intracellular circulation of the protein as well as its regulatory function [11]. Besides, in our study, analysis of this SNP according to the clinical-biological SLE features did not reveal any association with either the active forms of the disease or the presence of anti-dsDNA antibody. However, the impact of the CTLA-4 rs231775 SNP on SLE outcome and autoantibodies profile was variably estimated [41,42] and could be explained by the heterogeneity of studied groups in term of SLE-disease profile.

In this study, a significant association was noted between the FcγRIIa*V/V mutant genotype and SLE risk; p=0.0018, OR [95% CI] = 2.77 [1.44-5.33]. This result corroborates those found in German [43], Chinese [44] and Afro-American [45] populations. However, in a study performed in a Brazilian population, Grecco et al. [46] did not find any association between the FcγRIIa F158V SNP and the SLE susceptibility. Besides, if the present study did not show any influence of the FcγRIIa rs396991 SNP on SLE profile and outcome, the *G allele has been associated to lupus nephritis [45,47,48]. Indeed, the FcγRIIa*V variant has a higher affinity towards IgG1 and IgG3, compared to the FcγRIIa*F allele [44], which most likely results in a higher and more lasting activation of macrophages, via IC recognition. This stronger macrophage activation could explain its association with the occurrence of lupus nephritis.

In the present study, the FcγRIIa R131H and FcγRIIib NA1/NA2 polymorphisms, which decrease the affinity of IgG binding and alter IC clearance [48], were not associated to either SLE susceptibility or lupus nephritis occurrence. The influence of these polymorphisms on both SLE susceptibility and outcome was diversely estimated. In fact, Vigato-Ferreira et al. [49] noted, in a case control study in a Brazilian population, significant associations of the FcγRIIa*R allele with SLE risk and lupus nephritis; p=0.02, OR = 1.44 and p=0.01 OR = 2.09, respectively. Equally, one study has reported significant association between FcγRIIib-NA1/NA2 polymorphism and SLE in a Japanese population [50]. Inversely, 3 recent meta-analyses of Lee et al. [51], Zhang et al. [52] and Yuan et al. [53] showed that FcγRIIa R131H and FcγRIIib NA1/NA2 polymorphisms were not associated either to SLE susceptibility or to lupus nephritis. The lack of association between these 2 polymorphisms and SLE risk and lupus nephritis reported in the majority of previous available studies could be explained that the decrease of IC clearance ability in spleen and liver would be offset by the lower activation of macrophages and neutrophils in the sites of IC deposit.

In summary, the present study noted that PTPN22, CTLA-4 and FcγRIIa genes polymorphisms could confer an SLE risk in Tunisian corroborating the results of the majority of previous reports. Our findings support that SLE is the result of T cell central and peripheral...
tolerance failure together with a decreased IC clearance ability.

**Conclusion**

PTPN22 rs2476601 R620W, CTLA-A rs231775 +49 A/G and FcyRIIa rs396991 F158V polymorphisms seem to influence SLE susceptibility-risk in Tunisian.

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**Competing and conflicting interests**

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

**Author’s contribution**

Professor Yousr Gorgi proposed the study. Imen Sfar realized all the practical part of the study. Tarak Dhaouadi wrote the draft. Tarak Dhaouadi, Imen Sfar, Taieb Ben Abdallah and Yousr Gorgi analyzed the data. All authors contributed to the design of the study. Yousr Gorgi is the guarantor of the integrity of this study.

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