FUNCTIONAL POLYMORPHISMS OF PTPN22 AND FCGR GENES IN TUNISIAN PATIENTS WITH RHEUMATOID ARTHRITIS

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RESUME

Dans le but de rechercher une éventuelle association entre la polyarthrite rhumatoïde (PR) et les polymorphismes fonctionnels de la protéine tyrosine phosphatase non receptor (PTPN22-R620W) et les récepteurs de la fraction Fc des IgG (FcgRIIa-H131R, FcgRIIIa-F158V, FcgRIIIb-NA1/NA2), 133 malades Tunisiens atteints de PR et 100 sujets témoins ont été étudiés par des techniques de biologie moléculaire. L'analyse du polymorphisme R620W de la PTPN22 a montré que l'allèle 620W est statistiquement plus prévalent chez les patients par rapport aux témoins alors que les polymorphismes des FcgRIIa, FcgRIIIb et FcgRIIIa ne semblent pas intervenir dans la susceptibilité à la PR dans notre population. L'étude analytique de ces polymorphismes en fonction des différentes caractéristiques clinico-biologiques, a révélé l'absence d'association entre le SNP R620W de la PTPN22 et la production des auto-anticorps, la présence de manifestations extra-articulaires et la survenue de formes sévères de la PR. En revanche, les génotypes FcgRIIIb-NA2/NA2 et FcgRIIIa-V/V158, semblent influencer le profil clinique et évolutif de la maladie, en favorisant respectivement, la survenue de formes précoces et destructrices de cette pathologie. Une étude portant sur un nombre plus élevé de patients serait nécessaire pour confirmer ces constatations.

Mots clés: *Polyarthrite rhumatoïde, polymorphisme génétique, PTPN22, FcgR.*

ABSTRACT

To investigate a possible association between functional polymorphisms of the protein tyrosine phosphatase nonreceptor type 22 (PTPN22-R620W) and receptors for the Fc fragment of IgG (FcgRIIa-H131R, FcgRIIIa-F158V, FcgRIIIb-NA1/NA2), and rheumatoid arthritis (RA), 133 Tunisian patients with RA and 100 controls were genotyped. We found strong evidence of an association of PTPN22 620W allele and RA. However, analysis does not detect an association between auto-antibodies seropositivity, presence of nodules or erosions and this allele. No significant skewing of any of the three FcgR polymorphisms was seen in this RA group. Nevertheless, we identified FcgRIIIa-V/V158 as the most important FcgR genotype for severe disease subset with joint erosions and observed that patients with FcgRIIIb-NA2/NA2 genotype had an earlier incidence of clinical symptoms.

In conclusion, we have confirmed that PTPN22 620W allele is associated with Tunisian RA but does not constitute a factor influencing clinical manifestations. Conversely, this study supports that the FcgRIIa/IIIa and IIIb polymorphisms could influence the course and the severity of this disease. A large number of samples are required to provide independent confirmation of these findings.

Key words: *Rheumatoid arthritis, gene polymorphism, PTPN22, FcgR.*

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic persistent synovial joint inflammation resulting in bony erosion, cartilage loss and often systemic disorders, such as subcutaneous rheumatoid nodules, secondary Sjögren's syndrome, interstitial lung disease and systemic vasculitis[1]. Autoantibodies are detected in approximately 2/3 of patients with RA and predict more-severe disease [2]. In addition to rheumatoid factor (RF) which is an immunoglobulin specific to the Fc region of IgG [3], many other autoantibodies are found in RA, most notably anti-cyclic citrullinated peptide (anti-CCP) [4]. These autoantibodies recognize peptides in which arginine has been posttranslationally modified to became citrulline [5].

The etiology of RA remains elusive, but it is thought to have both a genetic and an environmental basis [6]. The contribution of human leukocyte antigen (HLA) genes to RA susceptibility was the first described [7] and remains as the best characterized single genetic risk factor contributing to RA [8]. There has been some debate as to whether the RA-associated DRB1 alleles collectively known as "shared epitope (SE)" are susceptibility or severity factors for RA [9]. In addition, HLA has been estimated to account for only one-third of the genetic component in the disease, indicating that genes outside the HLA region also contribute to the disease [10]. Several genome scans have suggested multiple RA loci [11, 12]. Nevertheless, to date, no other candidate genes have been consistently proven to be associated with RA [13]. Some support for linkage was provided for several previously identified regions with linkage evidence in chromosome 18 (18q21), chromosome 20 (20p13) and chromosome 1 (1q41-42).

Using a candidate gene approach, a genetic association, involving a functional polymorphism of the protein tyrosine phosphatase nonreceptor type 22 (PTPN22) gene was reported to be associated with rheumatoid factor-positive (RF+) RA [14]. The PTPN22 gene, located on chromosome 1p13, encodes for the intracellular tyrosine phosphatase LYP, which acts as a negative regulator of early T-cell activation through binding to the Csk kinase [15].

The PTPN22 single nucleotide polymorphism (SNP) (1858C/T) (rs 2476601) that occurs as a result of an amino acid substitution of arginine to tryptophan at position 620 (R620W), affects a prolin-rich motif of LYP involved in the binding to the SH3 domain of Csk and therefore, the suppression of T-cell activation [16]. Although this loss of function explains an increased susceptibility to autoimmunity for the variant 620W allele [17], a mutually exclusive functional gain has been suggested by other authors, opening up news avenues for exploring disease mechanisms [18, 19, 20, 21]. Several independently works, combining a broad screen of functional single-nucleotide polymorphisms guided by previously published linkage studies, confirm the association between RA susceptibility and PTPN22 polymorphism [22, 23]. In contrast, attempts to replicate associations with this disease in Asiatic and Japanese population have been uniformly negative [24].

The relative importance of different leukocyte subsets and their products in the inflammatory component of RA in humans remains a subject of debate. In a recently described model, in which mice spontaneously develop a disease that has most of the characteristics of RA in humans, B cells were found to be critical for the development of arthritis by secreting IgG-isotype arthritogenic immunoglobulins of particular, although as-yet unidentified, specificity [25]. Therefore immunoglobulins may play a very important role in the initiation and/or progression of RA. However, it remains unclear whether these immunoglobulins are highly pathogenic via an altered function of the receptors for the Fc region of the IgG (FcgR) [26] or merely an epiphenomenon.

Fc receptors constitute a large family of genes belonging to the family of multichain immune recognition receptor. In humans, there are 3 main classes of FcgR: FcgRI (CD64), FcgRII (CD32), and FcgRIII (CD16) which vary in their cellular distribution and affinity for different IgG isotypes and have been shown to play important roles in the initiation and regulation of many immunological and inflammatory processes [27]. The efficacy of IgG-induced FcgR function displays inter-individual heterogeneity due to genetic polymorphisms of three subclasses: FcgRIIa, FcgRIIIa and FcgRIIIb [28]. FcgRIIa is a low-affinity receptor present on phagocytic cells, B cells and dendritic cells. It exhibits significant population polymorphism consisting of a guanine-to-adenine (G A) substitution at nucleotide 519 which results in a switch from arginine (R) to histidine (H) at amino acid position 131 in the immunoglobulin-binding domain. The 131-Arginine (R131) allele binds IgG2 much less avidly than the 131-Histidine (H131) allele [29].

FcgRIIIa is the only intermediate-affinity FcgR expressed on macrophages and NK cells and is belie-

ved to play a pivotal role in the clearance of immune complexes [28]. A thymine-to-guanine point mutation at nucleotide 559 within the FcgRIIIa gene results in a substitution from phenylalanine to valine in the membrane proximal domain of the molecule at amino acid position 158 and affects binding of IgG1, IgG3 and IgG4 subclasses [30]. The presence of phenylalanine (FcgRIIIa-158F isoform) results in receptors with low affinity for IgG1, IgG3 antibodies and immune complexes, while the presence of valine (FcgRIIIa-158V isoform) results in higher-affinity receptors [31]. This last allele could potentially contribute to increase the level of macrophage activation in response to IgG-containing immune complexes and thereby contributing to persistence of inflammation [30].

The two allelic forms of neutrophil-specific FcgRIIIb NA1/ NA2 differ by five nucleotides which results in four amino acid differences in the first extracellular Ig-like domain. Although binding of IgG does not seem to be affected, these two allelic forms have different levels of quantitative function, and the more efficient in binding to immune complexes, the NA1 allele has been reported to be a susceptibility factor for several auto-immune diseases [31].

The present study was undertaken to investigate the possibility that PTPN22 and FcgR polymorphisms act as a genetic risk factors for susceptibility to RA and for the outcome of RA in Tunisian patients.

PATIENTS AND METHODS

PATIENTS

One hundred thirty-three RA patients were recruited from the rheumatology outpatient clinics of Charles Nicolle hospital in Tunis. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA [32] and were assessed clinically and serologically. 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).

Early onset was designed when patient developed RA before 50 years. Additional data regarding radiographic evidence of erosions and the presence of nodules founded on examination or previously documented in the patient's medical records were reported. RF and anti-CCP were assayed in RA patients' sera at the hospital attended and HLA-DRB1 typing was performed by sequence-specific PCR primers. The shared epitope was defined as HLA-DRB1*01, *0401, *0404, *0405, *0408, *0409, *0410, *0413, *0416, *0419, *0421, *10 [33].

CONTROLS

As control group, we studied 100 ethnically and geographically matched healthy subjects 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 RA in a first-degree relative.

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

METHODS

• *PTPN22 C1858T (R620W) polymorphism*

Genomic DNA isolated from EDTA-anticoagulated peripheral blood samples of unrelated healthy blood donors and RA patients was extracted by a salting-out process. Genotyping was performed using the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method. To a 10-uL 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 desoxynucleoside triphosphate was added following primers: forward (5'-TGCCCATCCCACACTTTAT-3') and reverse (5'-ACCTCCTGGGTTTGTACCTTA-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 *RsaI* (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 *RsaI*, which permitted differentiation of the R620-allele (228 bp) and the 620W-allele (272 bp).

• *FcgR polymorphisms*

Determination of FcgRIIa genotype

FcgRIIa genotyping was performed by means of a nested-PCR technique. For the first step, a 1000 bp PCR product containing the polymorphic FcgRIIa site was generated using specific primers sense (P63): 5'CAAGCCTCTGGTCAAGGTC3' 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 $MgCl_2$ and 1 U of Taq polymerase (Promega, USA) in a volume of 20 μl of buffer. The first cycle consisted of 5 min of denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 1 min. In the final cycle, extension time was increased to 7 min at 72°C. The first PCR product was employed in a second-step PCR utilizing primers specific for the H131 or R131 allele. Sense primers used in two parallel reactions were as follows: P4A (H131 specific): 5'GAAAATCCCAGAAATTTTTC CA3', P5G (R131 specific): 5'GAAAATCCCAGAAA TTTTTC CG3'.

The antisense primer (P13) used in both reactions was as follows: 5'CTAGCAGCTCACCACTCCTC3'.

Each of the allele-specific PCR assays included 0.6 μl of the first PCR product, 0.5 nmol sense (P5G or P4A) and antisense (P13) primer, 0.2 mM deoxynucleosite triphosphates (dNTPs) and 0.5 U of Taq polymerase (Promega, USA) in a volume of 20 μl of reaction buffer. The amplification protocol consisted of one cycle at 95°C for 5 min, 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 min. 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.

Determination of FcgRIIIa genotype

The FcgRIIIA-158V/F polymorphism was genotyped by direct sequencing. As described previously [34], a 199-bp PCR product that contained the polymorphic site was amplified using a specific primer sequences: sense: 5' TGTAAAACGACGGCCAGTTCATCATAATT CTGACTTCT 3' and antisense: 5' CAGGAAACAG CTATGACCCTTGAGTGATGGTGATGTTCA 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).

Determination of FcgRIIIb genotype

Genotype analysis of the NA1 and NA2 alleles of the FcgRIIIb gene was performed by PCR employing allele-specific sense and antisense oligonucleotide primers. The NA1 sense primer (5' CAGTGGTTT CACAATGTGAA 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' CATGGACTTCTAGCTGCACCG 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' CTCAATGGTACAAGCGTGCTT 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 $MgCl_2$ 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 analyses were performed using the SPSS 13.0 for Windows statistical package (SPSS, Chicago, IL). 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-square test using 2 x 2 and 2 x 3 contingency tables. When numbers in expected cell value was less than 5, Fisher's exact test was used. Two-sided P values less than 0.05 were considered significant throughout. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated to quantify the magnitude of the association between the variants alleles and RA, as an approxi-

Tableau I: Demographic characteristics of patients and disease features.

Patients	n = 133
Gender	108 females and 25 males
Age in years, median (interquartile range)	52 (39-65)
Age at diagnosis in years, median (interquartile range)	47.5 (32-62)
Disease duration in months, median (interquartile range)	142 (56-240)
Early onset * (%)	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)
Shared Epitope (%)	61 (46)

**: < 50 years.*

mation of the relative risk. The Mann-Whitney U test was used to determine whether there was any significant difference between several genotype groups for continuous data. Finally, multiple logistic regression models were performed to define the possible independently role of FcgRIIa, FcgRIIIa, FcgRIIIb and PTPN22 genotypes as risk factors for outcome criteria like, the presence of nodules or radiographic evidence of erosions.

RESULTS

Characteristics of the RA patients

Data obtained from each patient included gender, age, clinical and biological manifestations of the disease. They were used to define the epidemiological characteristics of the patients and were shown in table I. The RA and control groups had shared epitope (SE) frequencies of 46 % and 18 % respectively. The SE was associated with RA in this cohort of patients (p = 0.00001, OR: 3.86, 95% CIs, [2.01-7.48]).

Genotype distributions of PTPN22 C1858T (R620W) and association with RA

There was no departure from Hardy-Weinberg equilibrium in both controls and patients groups. In RA patients, the 1858T (620W) allele occurred at a significantly greater frequency than in the control group (p: 0.001, OR: 1.83, 95% CIs: [1.56-2.15]) (Table II). However, the demographic data of

Tableau II: Genotype and allele frequencies of PTPN22, FcgR polymorphisms studied in controls and patients.

Polymorphism		Genotype Frequency (%)			Allele frequency		p
PTPN22 R620W	**n**	**R/R**	**W/R****	**W/W**	**R**	**W***	
Controls	100	98 (98%)	2 (2%)	0	0.990	0.010	
Patients	133	100 (75.2%)	30 (22.5%)	3 (2.3%)	0.870	0.130	0.001
FcgRIIa-R131H	**n**	**H/H**	**H/R**	**R/R**	**H**	**R***	
Controls	100	24 (24%)	40 (40%)	36 (36%)	0.440	0.560	
Patients	133	32 (24%)	57 (43%)	44 (33%)	0.455	0.545	0.76
FcgRIIIa-F158V	**n**	**F/F**	**F/V**	**V/V**	**F**	**V***	
Controls	100	43 (43%)	42 (42%)	15 (15%)	0.640	0.360	
Patients	133	44 (33%)	65 (49%)	24 (18%)	0.575	0.425	0.16
FcgRIIIb-NA1/NA2	**n**	**NA1/NA1**	**NA1/NA2**	**NA2/NA2**	**NA1**	**NA2***	
Controls	100	15 (15%)	42 (42%)	43 (43%)	0.360	0.640	
Patients	133	15 (11.3%)	66 (49.6%)	52 (39.1%)	0.361	0.639	0.72

** Chosen allele compared with the other allele; ** Chosen genotype compared with the two other genotypes: OR: 1.83, 95% CI, 1.56 to 2.15; p=0.0001.*

Tableau III: Demographic comparisons between genotypes in PTPN22 and FcgR.

Genotypes	n	Patient age* (years)	Age at diagnosis* (years)
PTPN22			
R/R 620	100	52.5 (40-61)	41 (33.4-50)
W/W 620	3	51 (42-60)	42 (32-52)
FcgRIIa			
H/H 131	32	51.6 (41.3-61)	42.4 (32-52)
R/R 131	44	49.8 (40-58)	40.2 (33-48)
FcgRIIIa			
F/F 158	44	51.2 (40.8-61.2)	42.7 (32.4-52.2)
V/V 158	24	50.4 (42.1-58.3)	39.7 (32-48)
FcgRIIIb			
NA1/NA1	15	50.7 (41.5-60.3)	43.4 (32.4-52.2)
NA2/NA2	52	48.3 (40.2-54.3)	**39.5 (32-48)**
FcgRIIa, FcgRIIIa, FcgRIIIb			
H/H 131, F/F 158, NA1/NA1	9	51.2 (41.2-61.1)	42.8 (32.3-52.1)
R/R 131, V/V 158, NA2/NA2	15	49.5 (40.8-56.8)	**39.1 (31.5-47)**

** Results are shown as median (Range). Significant differences are shown in bold.*

patients did not show any significant differences between the PTPN22 genotypes in age. Indeed, patients homozygous or heterozygous for the variant allele 620W developed RA at the same age of onset than patients with wild type allele (Table III). In addition, the analytical study shows also no statistically significant association between the PTPN22 (R620W) polymorphism and the disease activity, presence of nodules or joint erosions (Table IV). Moreover, no correlation was found between the polymorphism studied and the production of RF and anti-CCP (Table IV).

Genotype distributions of FcgR and association with RA

FcgRIIa, FcgRIIIa, and FcgRIIIb were in Hardy-Weinberg equilibrium in both control and RA patient groups. The prevalence of IIa-R/R131, IIIa-V/V158

Tableau IV: Association of FcgR genotypes with clinical and biological parameters of the disease.

Genotypes	Early Onset (%)	Nodules (%)	Erosions (%)	High RA activity state (%)	RF (%)	Anti-CCP (%)
PTPN22						
R/R 620 (n=100)	73 (73)	18 (18)	46 (46)	70 (70)	75 (75)	79 (79)
W/W 620 (n=3)	2 (66)	1 (33)	1 (30)	2 (66)	3 (100)	3 (100)
FcgRIIa						
H/H 131 (n=32)	21 (65.6)	8 (25)	13 (40.6)	27 (84.3)	24 (75)	15 (47)
R/R 131 (n=44)	22 (50)	10 (22)	24 (54.5)	32 (72.7)	35 (79)	22 (50)
FcgRIIIa						
F/F 158 (n=44)	34 (77)	9 (20)	23 (52)	32 (72)	36 (81)	28 (63)
V/V 158 (n=24)	20 (83)	4 (16)	**24 (100)***	19 (79)	18 (75)	12 (50)
FcgRIIIb						
NA1/NA1 (n=15)	6 (40)	5 (33)	6 (40)	12 (80)	13 (86.6)	9 (60)
NA2/NA2 (n=52)	**41 (79)****	10 (19)	27 (52)	34 (65)	38 (73)	22 (54)
FcgRIIa, FcgRIIIa, FcgRIIIb						
H/H 131, F/F 158, NA1/NA1 (n=9)	2 (22)	2 (22)	**6 (66)**	**7 (77.7)**	**7 (77.7)**	**8 (88)**
R/R 131, V/V 158, NA2/NA2 (n=15)	5 (33.3)	4 (26.6)	**10 (66.6)**	**11 (72)**	**12 (80)**	**12 (80)**

** Chosen genotype compared with the other genotype: (p: 0.005, OR: 4.84, 95% CIs: [1.62-18.62]); **: Chosen genotype compared with the other genotype: (p: 0.008, OR: 5.59, 95% CIs: [1.63-19]).*

and IIIb NA2/NA2 among patients with RA was 33% (n=44), 18% (n=24) and 39.1% (n=52) respectively, comparable with that of the control group (36%, 15%, 43%) (Table II). No skewing of the FcgRIIa, FcgRIIIa, FcgRIIIb polymorphisms was seen in this Tunisian RA cohort and there was no significant difference in allele frequencies between patient and control groups. The double negative (IIa-R/R131, IIIa-V/V158) and triple negative (IIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2) homozygous genotypes were detected in 20% (n=27) and 11% (n=15) of patients and 18% and 9% of controls; again no significant skewing, but an overrepresentation of the double negative and triple negative genotypes was found in RA patients.

There was an increase in the frequency of the FcgRIIIb-NA2/NA2 genotype in RA patients with early onset (< 50 years) compared to FcgRIIIb-NA1/NA2 and FcgRIIIb-NA1/NA1 genotypes (p : 0.008, OR : 5.59, 95% CIs: [1.63-19]) (Table IV). Notably, the median age at diagnosis was 2.2, 3 and 4.2 years earlier in RA patients with IIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2 genotypes than in patients with IIa-H/H131, IIIa-F/F158 and IIIb-NA1/NA1, reaching significance ($p<0.05$) only for IIIb. Interestingly, this difference was even more pronounced in the group of patients with the triple negative (IIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2) homozygous genotypes, who were 4.3 years younger at disease onset than patients with triple positive (IIa-H/H131, IIIa-F/F158 and IIIb-NA1/NA1) homozygous genotypes ($p<0.05$) (Table III).

A possible association of FcgR genotypes with clinical parameters of the disease (nodular disease, activity, bone erosions, rheumatoid factor and anti-CCP) was also investigated. No statistically differences were found between these parameters and FcgRIIa or FcgRIIIb genotypes (Table IV). Nevertheless, there was a trend towards an association between FcgRIIIa and the presence of erosions with each FcgRIIIa-158V allele (p: 0.026, OR : 2.084, 95% CIs: [1.08-4.02]) and FcgRIIIa-V/V158 homozygous genotype (p : 0.005, OR : 4.84, 95% CIs: [1.62-18.62]) appearing to contribute to the overall risk. Indeed, in multiple logistic regression models, including age, sex and FcgR genotypes, we identified FcgRIIIa-V/V158 as the most important FcgR genotype for the occurrence of joint erosions ($p = 0.01$) (Table V).

Interaction of PTPN22, FcgR polymorphisms and the HLA-DRB1 shared epitope in determining the risk of RA

We calculated the risk of developing RA conferred by either the PTPN22 620W allele or the shared epitope allele, and then calculated the risk conferred by both high-risk genes together. The risk associated with the presence of the shared epitope in the absence of the PTPN22 variant allele, determined in the subgroup of individuals PTPN22 620 R/R, was statistically significant (OR 2.3, 95% CI 1.2–5.8, $p = 0.038$). Similarly, there was a risk of RA (OR 1.2, 95% CI 1.1–3.17, $p = 0.04$) when we examined the effect of the PTPN22 620W allele in the subgroup of individuals who were shared-epitope negative. Nearly a 2-fold increase in the OR was apparent when both PTPN22 620W and SE factors were present in the same individual (OR 4.1, 95% CI 1.8–8.3, $p = 0.001$).

The same approach was used to evaluate the risk of developing RA due to interaction of FcgR polymorphisms and HLA-DRB1 SE. Although, no significant differences were found regarding interaction between SE and FcgRIIa-R/R131 genotype or SE and FcgRIIIb-NA2/NA2 genotype, There was evidence of an interaction between SE and FcgRIIIa-V/V158

Tableau V: Homozygous FcgR genotype in RA as prognostic marker for the risk of joint erosions.

	Erosions				
Risk factors	**N (%)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**
FcgRIIa-R/R 131	24 (54.5)	NS	NS	0.23	1.75 (0.69-4.40)
FcgRIIIa-V/V 158	**24 (100)**	**0.01**	**1.83 (1.27-4.52)**	**0.005**	**4.84 (1.62-18.62)**
FcgRIIIb-NA2/NA2	27 (52)	NS	NS	0.41	0.61 (0.19-1.98)
FcgRIIa, FcgRIIIa, FcgRIIIb-R/R 131, V/V 158, NA2/NA2	10 (66.6)	NS	NS	0.37	0.79 (0.48-1.31)
All RA patients	63 (47.7)	Multivariate analysis		Univariate analysis	

Multiple logistic regression analyses. After adjustement for age and sex the genotype FcgRIIIa-V/V 158 is still a risk factor (p=0.01). Significant differences are shown in bold.

genotype (combined group OR= 1.3, 95% CI 1.17–5.7, P = 0.025). Nevertheless, no interaction was found between HLA-DRB1 SE and triple negative (IIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2) homozygous genotypes in RA patients.

DISCUSSION

In this study, we investigated the significance of the PTPN22 variant allele on the risk of RA in Tunisian population. Our results confirm recently published data indicating an association of the 1858T allele (620W) and this disease [22, 23, 37]. It has been proposed that there is a gene dose effect thus individuals homozygous for the PTPN22 620W would have more severely reduced binding of LYP with Csk than would individuals who are homozygous PTPN22-R/R 620, thereby reducing the ability to down-regulate T-cell activation [21]. So, T cells are likely to be hyperactive and more readily able to develop auto-immune reactivity [21]. Our data may support this hypothesis, since we found that there was an increased risk of RA when both PTPN22 620W and SE factors were present in the same individual. The clear link between PTPN22 and lymphocyte signaling pathways and the association of 1858T (620W) variant with multiple autoimmune diseases such as RA, systemic lupus and type 1 diabetes, suggests that it may influence a pivotal and common pathway leading to the development of autoimmunity. More recently studies using transfected Jurkat cells have shown that the LYP 620W variant has an enhanced inhibitory effect on TCR signaling [21]. The same authors and others [20] have also demonstrated in human T cells that carrying this variant allele produced a reduced signaling in response to anti-CD3. While these data are certainly consistent with the increased enzymatic activity, it is also possible that lack of binding to Csk, or other binding partners such as Zap70 and CD3ε, could also contribute to the cellular phenotype by virtue of changes in the location of PTPN22 within the cell.

Numerous confirmations of the RA association have been reported in ethnically different populations. However, geographic differences in the 1858T allele frequencies have been shown. Indeed, the frequency of this variant allele decreases from northern to southern Europe, from around 12.5% in the English and Finnish populations, to around 6% in the Italian and Spanish populations and it is almost absent in African, American and Asian populations [38]. In our Tunisian RA controls, the allelic frequency of 620W was only 1%. Although these differences, it seems that the association between the SNP and autoimmunity is population-independent, as shown by studies carried out in sufficiently diverse populations [23, 37, 39].

It has been also suggested that the R620W polymorphism plays a role mainly in multiples diseases characterized by a brisk autoantibody production. In fact, a functional activity variation of PTPN22 could lead to quantitative changes in the thresholds for thymic selection with increased numbers of autoreactive T cells escaping negative selection [38]. In line with this hypothesis, seropositivity of both rheumatoid factor and anti-CCP auto-antibodies has been associated, by several data, with 620W allele in RA patients [22, 40, 41]. Plenge et al. [23] have demonstrated that the presence of T1858 allele and of anti-CCP led to a 350 times increased risk of developing RA. Nevertheless, the association with RF or anti-CCP is controversial, since other authors have not confirmed this correlation [42, 43]. In our study, we did not replicate a published association between auto-antibody-related RA, such as RF or anti-CCP and PTPN22 variant allele. Also, we did not found any association between 620W allele of PTPN22 polymorphism and clinical parameters of the disease, especially presence of nodules or joint erosions, reported by numerous studies [22, 41]. However, the wide CIs in many of these analyses reflect the relatively small numbers in some of the subgroups, with a consequent loss of statistical power. A larger patient cohort would therefore be required for us to reduce the width of the CIs and maybe confirm these observations. More studies are needed in order to clarify if determination of the patients' PTPN22 genotypes could be a useful prognostic factor in RA clinics.

Although PTPN22 has emerged as the strongest common genetic risk factor for RA apart from the major histocompatibility complex (MHC), several previous studies have suggested that multiple genes involved in immune complex handling and clearance are implicated in RA [44, 45, 46]. Indeed, significant genome–wide linkage studies have suggested that chromosomal region 1q23, which contains genes that encode FcgR, is one of the strongest candidate regions for human RA. Therefore, we examined FcgR genes which were known to have a key role in activation and modulation of immune response by contributing to immune complex handling [47] and we evaluated polymorphic variants which had been

shown by others to have functional consequences [48]. We, as well as others [46], found no significant differences in the distribution of genotypes or genotype combinations between patients with RA and controls of an ethnically homogenous group, thus excluding the FcgRIIa and IIIb polymorphisms as susceptibility factors for this disease in Tunisian population. Sullivan et al. 48 and other data [49] have also observed no significant relation between these polymorphisms and systemic lupus, with or without renal disease in Caucasians. Moreover, no associations were found between FcgRIIa genotypes and any of a series of clinical parameters. However, the present study documents an association between the tested NA1/ NA2-FcgIIIb polymorphism and an earlier occurrence of RA disease. Human FcgIIIb is expressed mainly in neutrophils and it is necessary for the neutrophil function tethering to immune complexes (IC). About the FcgIIIb-NA2 variant it has been reported a lower binding ability to human IgG1 and IgG3 IC than NA1, and it is therefore plausible that reduced binding ability of FcgRIIIbNA2 may lead to reduced clearance of IC which may then promote the persistence of inflammation and increase the severity of articular disease.

Morgan et al. [31, 33] have shown a strikingly relation between FcgRIIIa-158V allele and RA in larger cohort interesting different ethnical patients, while other authors [46] reported similar distribution of FcgRIIIa-158 genotypes in relation to this pathology. Nieto et al. [50] have, in contrast, found that subjects who are homozygous for FcgRIIIa-158F are more susceptible to develop RA in Spanish population. Such discrepancies may certainly be caused by the difference in the genetic background of each population. In our Tunisian cohort, there was no significant difference of FcgRIIIa-158 in allele frequencies between patient and control groups.

Numerous data [31, 44] have suggested that FcgRIIIa-158V allele may play a role in determining disease severity with the development of nodules or erosions. Indeed, this intermediate-affinity for immune complexes receptor is thought to be the major receptor involved in IgG1 and IgG3 immune complex clearance [30] and an increased affinity of this receptor observed with FcgRIIIa-158V allele may have important implications for antibody-mediated immune surveillance and macrophage activation leading to release of proinflammatory mediators, reactive oxygen species and proteolytic enzymes [33]. These molecules could be responsible for much of the synovial inflammation and may mediate tissue damage. This hypothesis supports the fact that FcgRIIIa-158V isoform, through binding IgG-RF, could more likely activate connective tissue macrophages leading to risk of developing rheumatoid nodules. Although we did not found any significant relationship between the FcgRIIIa tested polymorphism and seropositivity of RF or presence of nodules in Tunisian patients, the present data document an association between the FcgRIIIa-158V variant allele and highest risk for occurrence of severe disease subset with bone erosions.

Despite the weaker genetic effect of FcgRIIIa polymorphism in susceptibility to RA, the possibility of an additive effect with the rheumatoid shared epitope was again raised [31]. Thus, previous analysis [33] demonstrated a possible interaction between the FcgRIIIa-158V and the shared epitope in determining susceptibility to RA and have reported some evidence that this variant allele only increases susceptibility to the disease in individuals who are shared-epitope positive. This finding, according to Morgan et al [33] could explain why the chromosomal region containing FcgRIIIa has not been highlighted as an independent genetic susceptibility locus for RA in genome-wide screens. The authors suggested a possible explanation for the observed interaction is that the high-affinity FcgRIIIa-158V isoform increases the uptake of IgG-containing immune complexes or opsonized pathogens into the antigen-presenting cell. This may then result in more efficient presentation of arthritogenic peptides by feeding them directly into the antigen-processing pathway [33, 58]. Accordingly to this hypothesis, we have also found an interaction between SE and FcgRIIIa-V/V158 genotype in Tunisian RA.

In other data, Morgan et al. [44] have demonstrated that the haplotype FcgRIIIa- FcgRIIIb 158V-NA2 was strongly associated to RA susceptibility, especially in the presence of nodules. Although an overrepresentation of the double negative (IIa-R/R131, IIIa-V/V158) and triple negative (IIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2) homozygous genotypes was found in RA patients of our cohort, no significant skewing was observed. However, analysis with clinically relevant subsets of RA reveals that the onset of RA diagnosis was found at younger age in patients with triple negative homozygous genotypes (FcgIIa-R/R131, IIIa-V/V158 and IIIb-NA2/NA2)

compared to those with triple positive homozygous genotypes. This significant association was also observed by Manger et al. [49] in German patients with systemic lupus erythematosus.

In conclusion, despite several limitations in our study, including the limited number of patients, our results provide support for PTPN22 620W as RA susceptibility factor in Tunisians but failed to demonstrate linkage evidence for the involvement of this allele in RF positive RA predisposition. Moreover, this study shows that the FcgRIIa/IIIa and IIIb polymorphisms could represent genetic markers influencing the clinical manifestations as well as the severity of this disease in our population. These findings require independent confirmation with a large number of samples to provide additional insights into the pathogenesis of RA.

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