Association between severe disease course and nephritis with Q222R polymorphism in DNAse I gene among lupus patients: an Argentine multicenter study

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ACTA REUMATOL PORT. 2016;41:138-144

ABSTRACT

Objectives: Systemic lupus erythematosus is a multifactorial autoimmune disease and the glomerulonephritis is one of the most severe complications, which leads to severe persistent proteinuria, chronic renal failure, and end-stage renal disease. This multicenter study investigated the genetic associations of a non-synonymous single-nucleotide polymorphism in DNase I with the risk of lupus and its influence on development of nephropathy in an Argentinean population.

Methods: Using the polymerase chain reaction restriction fragment length polymorphism method, the Q222R (+2373A G; Gln244Arg) DNase I polymorphism was studied in 156 systemic lupus erythematosus patients and 170 healthy controls.

Results: Although no significant association between Q222R polymorphism and the risk of systemic lupus erythematosus was found, the presence of the A allele was associated with an increased risk for the development of nephropathy (p=0.019, Odds Ratio=2.196, 95 % confidence interval [1.135-4.247]) and a worse disease course [moderate disease course: p=0.006, Odds Ratio=3.250, 95% confidence interval (1.401-7.539); severe disease course: p=0.040, Odds Ratio=2.339, 95% confidence interval (1.040-5.260)].

Conclusions: A better understanding of the genetic basis of systemic lupus erythematosus will help in the development of new and more effectives strategies for the treatment of the disease in the future.

Keywords: DNase I; Susceptibility; Lupus nephritis; Lupus pathology; ISN/RPS classification; Gene polymorphisms; Systemic lupus erythematosus.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease characterized by excessive autoantibody production, immune complex deposition, and immunologically mediated tissue damage. The clinical manifestations and disease severity vary greatly among patients, with periods of illness (flares) alternating with remission. One of the most severe complications of SLE is glomerulonephritis, which leads to severe persistent proteinuria, chronic renal failure, and end-stage renal disease[1,2].

The etiopathogenesis of the disease remains unclear but involves a combination of genetic, environmental, and hormonal factors[3]. Genetic factors are likely to play an important role in susceptibility to the disease, the autoantibody profile of the patient and particularly the disease phenotype[4,5]. Certain major histocompatibility complex allele (HLA A1, B8, DR3) defects in the classical complement pathway and/or defects in apoptotic clearance are strongly associated with SLE. In fact, DNase I, an endonuclease involved in the breakdown of chromatin during apoptosis[6], has been implicated in the pathophysiology of the disease. In 1981, Chitrabamrung et al. found decreased serum DNase I activi-

TABLE I. DEMOGRAPHIC, CLINICAL AND BIOCHEMICAL FEATURES OF THE STUDY SUBJECTS

Demographic features	SLE group n = 156	Healthy controls n = 170
Females; n (%)	143 (92)*	143 (84)*
Age; years, median (IQR)	35 (28-45)*	32 (28-43)*
Age of onset; years, median (IQR)	27 (21-36)	
Disease duration; years, median (IQR)	5 (3-10)	
Numbers of ACR criteria; %		
4-5	44.3	
6-7	35.8	
>7	19.8	
Lupus flares; %		
0	35.8	
1-2	55.0	
>2	9.2	
SLICC score; %		
0	49.0	
1-2	31.8	
>2	19.2	
Disease course; %		
Mild	29.1	
Moderate	34.4	
Severe	36.4	
Nephropathy; %	50.9	
Antiphospholipid antibodies; %	27.6	
Antiphospholipid syndrome; %	6.3	

*The SLE patients and control subjects were statistically comparable with regard to sex and age (p>0.05).
SLE: Systemic Lupus Erythematosus; ACR: American College of Rheumatology; SLICC: Systemic Lupus International Collaborative Clinics; IQR: interquartile range.

ty in patients with SLE[7]. More recently, it was shown that a DNase I knockout mouse develops a lupus-like syndrome[8], and a lower level of DNase I activity was reported along with an extremely high immunoglobulin G titer against nucleosomal antigens in two Japanese SLE patients harbouring a nonsense mutation in the DNase I gene[9]. However, subsequent studies in SLE patients of different ethnic groups have shown that this nonsense mutation is extremely rare.

Human DNase I is genetically polymorphic and is controlled by six codominant alleles[10]. The most common single-nucleotide polymorphism (SNP), Q222R (rs1053874), is determined by two alleles, A and G[11], and was reported to be associated with SLE susceptibility in Spanish[12] and South Indian populations[13]. However, a study in a Korean population did not find such association[14], though the SNP was linked to the development of anti-RNP and anti-dsDNA antibodies.

As the Q222R polymorphism in the DNase I gene is strictly related to ethnicity[10] and there is insufficient evidence about its association with susceptibility to and/or the course of SLE, we investigated the relationship of this SNP with susceptibility to and clinical features of SLE in an Argentinean cohort. A better understanding of the pathological mechanisms involved in SLE would help in the development of new therapeutic strategies.

MATERIALS AND METHODS

PATIENTS

We studied a total of 326 Argentinean subjects, 156 of whom met the revised classification criteria of the American College of Rheumatology (ACR) for SLE[15].The control group consisted of 170 unrelated

healthy subjects from the general population who had no personal history of chronic inflammatory diseases or autoimmune diseases and were considered to be "metabolically healthy" individuals. All subjects were of Argentine descent and had the same ethno-geographic and social origins. The Argentinean population is the result of genetic admixture processes involving three continental contributors: Native Americans, Western Africans and Europeans (mainly Spaniards and Italians), although the African component detected in different studies was very low (less than 4%). We selected a representative sample of the admixed urban Argentinean population[16,17].

The study was approved by the ethics committees of the medical centers and was performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

GENOTYPE ANALYSIS

Genomic DNA was isolated from 200 µl of whole blood using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The Q222R SNP was analyzed by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism. Primers for the specific amplification of a DNA fragment encompassing the SNP were designed on the basis of the nucleotide sequence of human DNASE I (GenBank accession no. D83195).

PCR amplification was performed in a 50 µl reaction mixture using 10 ng of DNA. The reaction mixture contained 1X buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, 0.08% (v/v), Nonidet P40), 3 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 1.25 U of Taq DNA polymerase (Thermo Scientific, Waltham, Massachusetts, USA). PCR was performed with a protocol consisting of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 10 min.

A 15 µl aliquot of the PCR product was digested with 5 U of *Sty* I at 37°C for 4 h in a final reaction mixture volume of 20 µl. The total digested product was separated in a 2% agarose gel and visualized by staining with SYBR Safe® (Invitrogen, Eugene, OR, USA).

LABORATORY TESTS

We considered the presence of antiphospholipid antibodies in patient samples in which anti-cardiolipin (aCL) antibodies and/or lupus anticoagulant (LA) activity were detected.

aCL antibodies were detected by enzyme immunoassays targeting both the IgG and IgM isotypes. We considered serum aCL titers in the medium or high range (>99th percentile) to be positive results. LA activity was measured following the guidelines of the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Haemostasis[18].

DEMOGRAPHIC, CLINICAL, AND LABORATORY DATA

Data on sex, age, age of onset, disease course (clinical manifestations: mild, moderate, severe), disease duration, number of ACR classification criteria, Systemic Lupus Erythematosus Disease Activity index (SLEDAI), Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) damage index, number of lupus flares, and levels of antibodies (ANA Hep2, anti-dsDNA, ENAs), LA and aCL were collected. A flare was defined according to the international consensus as a measurable increase in disease activity in one or more organ systems involving new or worsening of clinical signs and symptoms and/or laboratory measurements[19]. Generically, the clinical manifestations of SLE can be classified into three levels: mild, moderate and severe. Mild manifestations: those that even affecting the quality of life of patients, are not a threat to life and do not produce irreversible damage or relevant complications. Moderate forms: **t**his includes some inflammation of other parts of the body apart from joints and skin. This may include pleurisy, pericarditis or mild kidney inflammation. Severe forms: major organ involvement, life-threatening and chronic damage production potential with significant organ dysfunction[20].

Anti-phospholipid syndrome (APS) was defined according to the international consensus statement of the classification criteria for definitive APS[21].

STATISTICAL ANALYSIS

A statistical analysis was performed using the SPSS statistical package (version 15.0 for window SPSS, Chicago, IL, USA). Because the data were not normally distributed, they are presented as medians and interquartile ranges (IQRs), with categorical variables as frequencies and percentages. Nonparametric tests (U-Mann–Whitney) were used to compare quantitative data, and the Chi square test or Fisher's exact test was used to compare proportions. A binary logistic regression analysis was performed to test the association between the alleles and the studied variables. The odds ratio (OR) was estimated to assess the strength of the association, and

TABLE II. GENOTYPE DISTRIBUTION AND ALLELE FREQUENCY OF Q222R SNP IN THE DNASE I GENE IN SLE AND CONTROL GROUPS

Q222R SNP	SLE group n = 156	Healthy controls n = 170	p
Genotype distribution; n (%)			
GG	66 (42.3)	83 (48.8)	0.248
AG	74 (47.4)	77 (45.3)	
AA	16 (10.3)	10 (5.9)	
Allele frequency			
G Allele	0.66	0.72	0.293
A Allele	0.34	0.28	

SLE: Systemic Lupus Erythematosus; SNP: single-nucleotide polymorphism

inheritance models were established using a likelihood test ratio. A $p<0.05$ was considered statistically significant (bold values).

RESULTS

This multicenter study included 156 SLE patients and 170 metabolically healthy controls belonging to the Argentinean population. The SLE patients and control subjects were statistically comparable with regard to sex and age ($p>0.05$). The demographic, clinical and biochemical features of the SLE patients included in the study are given in Table I. We analyzed the frequency of the Q222R (+2373A G) SNP in all the study participants, and the genotypes of the controls and patients were concordant with Hardy-Weinberg equilibrium ($p>0.05$). Table II shows the distribution of the DNase I Q222R SNP genotype and its allele frequencies in the SLE group and healthy controls. No significant genetic associations were found between DNase I polymorphism and the risk of SLE.

We investigated the influence of the Q222R polymorphism on different clinical and biochemical features among SLE patients; according to statistical tests, the inheritance model was dominant to the presence of the A allele. As shown in Table III, there was a statistically significant association between the presence of the A allele and the development of nephropathy [$p=0.019$, OR=2.196, 95 % CI (1.135-4.247)]. Furthermore, the presence of the A allele correlated with a worse disease course [moderate disease course: $p=0.006$, OR=3.250, 95% CI (1.401-7.539)]; severe disease course: $p=0.040$, OR=2.339, 95% CI (1.040--5.260)] (Table III). A trend towards a higher ACR criteria was also found in the presence of the A allele (Table III); however, a logistic regression analysis showed no statistically significant differences. We did not observe any other significant influence of the Q222R SNP for the remaining clinical and biochemical features recorded, including variables that are known to be associated with an increased risk of major organ involvement, such as age of onset of the disease and disease duration.

DISCUSSION

SLE is a systemic autoimmune disease with a strong genetic predisposition. However, single gene defects related to lupus-like phenotypes have infrequently been reported (approximately 1%), with the majority of the identified genetic SLE risk factor common variants showing a modest degree of risk[4]. In the present multicenter study, we analyzed the possible role of the Q222R SNP in the DNase I gene with susceptibility to SLE and its potential impact on SLE clinical manifestations.

Although our data suggest that the Q222R polymorphism is not associated with SLE susceptibility, the presence of the A allele of the Q222R SNP in DNase I was found to confer a potential risk for the development of nephropathy and a worse disease course. In fact, we found that A allele carriage increases the risk of developing nephropathy by more than twofold and the risk of having a worse disease course by more than threefold.

Our results indicate that the Q222R SNP is common

TABLE III. MAIN CLINICAL FEATURES OF THE SLE PATIENTS WITH OR WITHOUT THE PRESENCE OF THE A ALLELE OF THE Q222R POLYMORPHISM IN THE DNASE I GENE

	A Allele		
Clinical features	**Absent**	**Present**	**p**
Disease course; n (%)			**0.016**
Mild	27 (41.3)	18 (20.5)	
Moderate	17 (25.4)	37 (40.9)	
Severe	22 (33.3)	35 (38.6)	
Nephropathy; n (%)			**0.019**
Absent	40 (60.3)	37 (40.9)	
Present	26 (39.7)	53 (59.1)	
Age of onset; years, median (IQR)	26 (20.5-35.5)	27.5 (23-37)	**0.185**
Disease duration; years, median (IQR)	5 (3-11.5)	5 (2-10)	**0.259**
Number of ACR criteria; n (%)			
4-5	35 (52.4)	35 (38.6)	**0.179**
6-7	22 (33.3)	34 (37.5)	
>7	9 (14.3)	21 (23.9)	

SLE: Systemic Lupus Erythematosus; ACR: American College of Rheumatology

among Argentines. The allele frequencies in the healthy population were found to be similar to those reported in the Spanish population by Bodaño et al[12]. However, the association between the GG genotype of the Q222R SNP and SLE susceptibility reported by these authors[12] has not been found in Argentinean patients. A recent study in South Indian SLE patients also reported an association between the Q222R SNP and SLE susceptibility[13], with a higher frequency of a heterozygous genotype in SLE patients than in healthy subjects. In contrast, and in accordance with our results, a study in a Korean population did not find the Q222R SNP to be a genetic risk factor for SLE[14]. This discrepancy between different studies could be related to environmental factors, differences in genetic background of the populations or the influence of sample size on the power of the study. Thus, further studies are needed to determine the real role of Q222R SNP in SLE susceptibility.

There are few works focusing on the clinical aspects of SLE patients and the presence of Q222R SNP. Our results suggest that the presence of the A allele of the Q222R SNP confers a potential risk for development of nephropathy and a worse disease course. However, we did not find any significant association between the Q222R SNP and the presence of anti-DNA autoantibodies (data not shown), which play an important role in initiating nephritis in SLE patients. An association of the Q222R SNP with nephritis was also reported in South Indian SLE patients[13], with a higher frequency of the heterozygous genotype found in those patients who developed nephritis.

DNase I is required for chromatin breakdown during apoptosis as well as necrosis. Loss of this enzyme activity may lead to the accumulation of chromatin fragments in glomeruli, a core factor that imposes progressive renal inflammation in SLE. Moreover, recent evidence shows that neutrophil extracellular traps (NETs), web-like structures of DNA decorated with histones and antimicrobial proteins[22] that trap and kill a wide variety of microbes, also participate in the pathogenesis of a variety of inflammatory and autoimmune diseases, including SLE. An imbalance between NET formation and clearance in SLE patients may play an important role in the perpetuation of autoimmunity and the exacerbation of disease, as well as the induction of end-organ manifestation[23]. In fact, NETs were detected in affected kidney and skin of SLE patients[24]. It was also reported that a subset of SLE patients display impaired DNase I-mediated NET degradation in their serum[25,26], mostly during flares; however, DNase I-mediated NET clearance can be partially restore during remission. It has been proposed that inhibitory antibodies against DNase I or anti-NET antibodies might protect NETs from DNase degradation. The defective NET degradation in SLE tends to be correlated with higher

levels of anti-NET and anti-dsDNA autoantibodies as well as with lupus nephritis and lupus flares[25,26]. Yasuda et al. reported that the Q222R substitution (A→G) in the DNase I gene is associated with the production of an enzyme with reduced *in vitro* activity[10]. Thus, it is tempting to speculate that being a carrier of the G allele could lead to the synthesis of an enzyme with reduced enzymatic activity. In accordance with this hypothesis, we expected a higher development of nephropathy in patients harboring the G allele. However, unexpectedly, our results suggest that it is the presence of the A allele in the Q222R SNP that confers a potential risk for the development of nephropathy and a worse disease course. Within this context, we could hypothesize that in our cohort of patients, the presence of the A allele leads to the synthesis of a DNase I isoform that could dismantle NETs faster than the isoform produced when the A allele is absent, leading to a direct exposure to NET components, such as proteases and citrullinated histones. The higher exposure to NET-derived proteases and citrullinated histones could damage endothelial cells[27] and constitute new autoantigens for the immune system[28,29], which in turn could exacerbate renal damage, leading to a worse disease course. In fact, it has been reported that citrullinated histones are recognized in preference over non-modified histones by antibodies from patients with SLE[30]. However, further studies are needed to corroborate this hypothesis.

Finally, to our knowledge, this is the first study from Argentina to deeply explore the association between the Q222R SNP in DNase I gene and lupus susceptibility and its clinical manifestations. A better understanding of the mechanisms involved in lupus will help in developing new strategies to treat and restore quality of life in our patients.

CONTRIBUTORS

GDL and SAM had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. GC and GDL made substantial contribution to the conception and design of the study or data analysis. All authors made contributions to data interpretation and drafting of the article and its final approval.

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