Association of a non-synonymous single-nucleotide polymorphism of DNASEI with SLE susceptibility

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Objectives. To investigate the association of a non-synonymous single-nucleotide polymorphism (SNP) in DNASEI with susceptibility to systemic lupus erythematosus (SLE) and the production of autoantibodies to nuclear antigens.

Methods. The Gln244Arg (rs1053874) SNP was studied in 276 SLE patients and in 368 healthy controls of Spanish ancestry. Its relationship with SLE susceptibility, serum DNase I activity, anti-ribonucleoprotein (RNP), anti-double-stranded DNA (dsDNA), anti-nucleosome and anti-single-stranded DNA (ssDNA) antibodies was determined.

Results. An association of the Gln244Arg SNP with SLE susceptibility that followed a recessive genetic model (P=0.002) was found. The GG genotype was more common in SLE patients (59.8%) than in controls (47.3%). However, the Gln244Arg genotype did not correlate with DNase I activity in sera from SLE patients or from controls. In addition, the Gln244Arg SNP did not influence autoantibody titres significantly.

Conclusion. The association of the Gln244Arg SNP with SLE susceptibility indicates that common polymorphisms in DNASEI play a role in the genetics of SLE. However, the lack of effect of the Gln244Arg SNP on serum DNase I activity calls into question the direct involvement of this specific SNP.

Key words: Systemic lupus erythematosus, Single-nucleotide polymorphism, DNASEI gene, autoantibodies.

Systemic lupus erythematosus (SLE) is characterized by a wide range of clinical manifestations and the production of autoantibodies directed to ubiquitous intracellular antigens. Its aetiology is incompletely known, although a genetic predisposition has been recognized. Several genome-wide linkage studies have discovered a number of loci with reproducible linkage to SLE [1–3]. Other studies to identify candidate genes have revealed a role for histo-compatibility antigens, complement components, low-affinity receptors for immunoglobulin G (IgG) [4] and genes involved in the clearance of apoptotic cells [5–11], among others. Studies in SLE patients and in mouse models support the involvement of DNase I among the last category of genes. The first evidence was reported by Chitrabamrung et al. [12] more than two decades ago. These authors found decreased DNase I activity in patients with SLE. More recently, it has been shown that a DNase I knockout mouse develops a lupus-like syndrome [13] and a nonsense mutation on the DNASEI gene leading to a non-functional protein has been identified in two Japanese girls with SLE [14]. These girls had very low DNase I activity and high titres of anti-nucleosome and anti-double-stranded DNA (dsDNA) antibodies. Subsequent analysis of several series of SLE patients from different populations showed that this mutation is extremely rare. In recent times, we have described two Spanish SLE patients with very low serum DNase I activity harbouring three new mutations in the DNASEI coding sequence that account for the reduced enzymatic activity [15]. The frequency of these new mutations was below 1% both in SLE patients and in the population. We also found other DNASEI single-nucleotide polymorphisms (SNPs) but there was no evidence suggesting a functional role for them. These studies support the involvement of DNase I in the pathogenesis of SLE.

However, common polymorphisms in DNASEI affecting SLE susceptibility or phenotype had not been described until a recent study in Korean patients [16]. In this report the Gln244Arg SNP was associated with production of anti-ribonucleoprotein (RNP) and anti-dsDNA antibodies but not with SLE susceptibility. Previous studies had already considered this SNP and it was not associated with decreased DNase I activity [17, 18]. Therefore, it is unclear how this SNP could contribute to autoantibody production, because an alternative mechanism was not proposed in the study by Shin et al. [16].

In the present study, we investigated the relationship of the Gln244Arg DNase I SNP with SLE susceptibility, serum DNase I activity and the production of autoantibodies in a Spanish population.

Patients and methods

Patients

We studied 276 unrelated SLE patients and 368 healthy control subjects belonging to the Spanish population. Patients with SLE were classified according to the American College of Rheumatology (ACR) 1997 revised criteria [19]. Healthy controls were recruited from the general population, from laboratory and hospital personnel, from blood bank donors and from outpatients admitted to the hospital for minor surgery. We also studied 43 patients with osteoarthritis (OA) and 43 with rheumatoid arthritis (RA), according to ACR classification criteria. All participants were of Spanish ancestry and gave their written consent.

Received 5 September 2005; revised version accepted 16 December 2005.
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Genotype analysis

The +2373 A → G (Gln244Arg) SNP was genotyped by polymerase chain reaction (PCR) – restriction fragment length polymorphism analysis. Primers were designed to include two potential restriction sites in the amplified fragment: the site with the SNP and an invariant site, which was used as an internal control for the enzymatic digestion. Amplification was carried out with the primer pair 5’-TCCAAGGGCAGCCGTGACTC-3’ (forward) and 5’-TTCCAGCTGACATGGTGACTC-3’ (reverse). The PCRs were performed in a total volume of 10 μl containing 50 ng of genomic DNA, 200 μM of dNTPs, 0.1 μM of each primer, 1 U of Taq DNA polymerase (Roche), 1.5 mM MgCl2 and 1 × PCR buffer. Cycling conditions were as follows: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 1 min, and extension at 72°C for 1 min. Final elongation was done at 72°C for 10 min. The digestions were done with 3.5 U of SbfI (New England BioLabs) and the fragments were separated by electrophoresis in 3% agarose gels in TBE (45 mM Tris-Borate, 1 mM EDTA, pH 8.3). Genotyping results were verified by sequencing about 15% of the samples.

Sequencing

The system used for sequencing was the Big Dye Ready Reaction kit (Applied Biosystems, Madrid, Spain) in an ABI Prism 3100-Avant (Applied Biosystems) genetic analyser. Cycling conditions were as follows: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 96°C for 15 s, annealing at 50°C for 10 s and extension at 60°C for 3 min. Final elongation was done at 60°C for 10 min.

DNase I assay

Serum DNase I activity was analysed by the SRED (single radial enzyme diffusion) method as described [20]. Briefly, duplicate serum samples were placed in wells on DNA-agarose plates containing 1.5% purified agarose D-1 (Promadisa, Spain) and 5 g/l of calf thymus DNA (Sigma Aldrich Chemical) in 0.05 M Tris–HCl, 0.025 M NaCl, 0.025 M CaCl2, pH 7.2. Serial dilutions of rhDNase I (Pulmozyme; Genetech, San Francisco, CA, USA) were used as standard. Samples were incubated 22 h at 37°C and gels were stained with Sybr Green I (Sigma Chemical) in TE (10 mM Tris–HCl, 0.05 M NaCl, 0.025 M CaCl2, pH 7.2). The area of the circles of hydrolysed DNA was quantified with an image analyser (Typhoon 9410; Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay (ELISA)

Anti-dsDNA and anti-ssDNA antibodies were assessed by ELISA as described elsewhere [21]. For dsDNA, microtitre plates (Nunc, Roskilde, Denmark) were treated with 1% solution of aqueous protamine sulphate (Sigma Chemical), and calf thymus DNA (Sigma Chemical) was adsorbed at 10 μg/ml. After washing, ssDNA was removed by digestion with 100 U/ml of S1 nuclease (Amersham Bioscience). After digestion, the plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween. For ssDNA, calf thymus DNA (Sigma) was boiled for 10 min and then cooled immediately on ice for at least 30 min; 5 μg/ml of ssDNA was coated in 0.15 M NaCl, 0.015 M sodium citrate buffer for 4 h at 37°C and washed in PBS. For both assays, the plates were blocked with PBS containing 1% bovine serum albumin and washed with PBS containing 0.05% Tween. Sera diluted 1:100 were then added and plates were incubated overnight at 4°C. After washing with PBS–0.1% Tween-20, peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch) was added to the plates for 1.5 h at 37°C. After washing, peroxidase-D-phenylendiamine substrate (Sigma Chemical) was incubated for 20 min at room temperature in the dark, and optical density was determined at 492 nm with a Multiskan Ex (Thermo Labsystems, Barcelona, Spain). Values greater than the mean ± 3 s.d. of the controls were considered positive.

For the quantitative determination of IgG antibodies to nucleosomes, an Immunometric Enzyme Immunoassay (Oxgentec Diagnostika, Germany) was used according to the manufacturer’s instructions. Sera samples were diluted 1:100 in sample buffer before the assay. Values greater than 20 U/ml were considered positive.

A Varelisa RNP Enzyme Immunoassay (Pharmacia Diagnostics, Germany) was used to quantify the serum levels of IgG anti-RNP antibodies. Serum samples were diluted 1:100 in sample buffer. Values greater than 9 U/ml were considered positive.

Statistical analysis

Concordance with Hardy–Weinberg equilibrium was determined with the χ2 test. Comparisons of allele frequencies were done using the χ2 test on a 2 × 2 contingency table. Genotypes were analysed by logistic regression, assigning a recessive genetic model (coding AA as 0, Aa as 0 and aa as 1). Comparison of DNase I activity was done with the Mann–Whitney U-test. Correlation between +2373 A → G genotypes and DNase I activity and antibodies was done with the Pearson correlation coefficient and χ2 test.

Results

We analysed the frequency of the +2373 A → G (Gln244Arg) SNP in 276 SLE patients (80.3% women) and 368 healthy controls (81.9% women) belonging to the Spanish population. Genotypes of controls and patients were discordant with Hardy–Weinberg equilibrium. The G allele of the +2373 A → G SNP was more common in SLE patients than in healthy controls (Table 1; P = 0.002). Analysis of the distribution of genotypes (Table 1) suggested a recessive genetic model with a higher frequency of GG genotypes in SLE patients (P = 0.002 after adjusting by sex).

To explore whether this polymorphism influenced the functionality of the DNase I enzyme, we analysed enzyme activity in sera from 101 SLE patients, 38 healthy controls and 86 patients with other rheumatic diseases (43 patients with OA and 43 RA patients). Median DNase I activity was 4.2 ng/ml (interquartile range (IQR)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>SLE</th>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>174/368</td>
<td>165/276 (59.8)</td>
<td>1.66 (1.19–2.31)*</td>
</tr>
<tr>
<td>AG</td>
<td>167/368</td>
<td>98/276 (35.5)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>27/368</td>
<td>13/276 (4.7)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>515/736</td>
<td>428/552 (77.5)</td>
<td>1.48 (1.15–1.91)</td>
</tr>
<tr>
<td>A</td>
<td>221/736</td>
<td>124/552 (22.5)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are number of genotypes/number of subjects studied (%).

A Varelisa Aa versus AA as 0, Aa as 0 and aa as 1. Comparison of DNase I activity was done with the Mann–Whitney U-test. Correlation between +2373 A → G genotypes and DNase I activity and antibodies was done with the Pearson correlation coefficient and χ2 test.

Values are number of genotypes/number of subjects studied (%).

A 221/736 (30.0) 124/552 (22.5)

G 515/736 (70.0) 428/552 (77.5) 1.48 (1.15–1.91)

AA 27/368 (7.3) 13/276 (4.7)
3.2–5.2] in SLE patients, which was significantly lower than that observed in healthy controls (median 6.2 ng/ml, IQR 5.2–6.9; \( P = 4.1 \times 10^{-6} \)), OA patients (median 6.9 ng/ml, IQR 6.8–8.0; \( P = 2 \times 10^{-7} \)) and RA patients (6.8 ng/ml, IQR 6.9–7.5; \( P = 6.2 \times 10^{-10} \)).

Next, we analysed the link between the genotypes of the +2373 A → G SNP and DNase I activity, and did not find any evidence of relationship. There was no correlation between DNase I activity and genotypes in any of the groups \( r = 0.077 \) for SLE (Fig. 1); \( r = 0.0355 \) for healthy controls; \( r = 0.032 \) for the OA group; \( r = 0.191 \) for the RA group.

To further explore the functional involvement of the +2373 A → G SNP in SLE, we analysed the relationship between this SNP and the production of anti-RNP, anti-dsDNA, anti-ssDNA and anti-nucleosome antibodies. For the anti-RNP antibodies, the titres were very similar (Fig. 2A) in SLE patients with GG genotype (median 2.0, IQR 1.2–7.2) or with AA + AG genotypes (median 2.2, IQR 1.1–3.6). Also, the percentage of anti-RNP positives was similar in SLE patients with different genotypes: 20.3% among patients who were GG homozygotes and 18.9% in individuals with other genotypes. In the case of anti-dsDNA antibodies (Fig. 2B), there was also a lack of difference in antibody titres (median 0.4, IQR 0.2–0.7) in SLE patients with GG genotype; median 0.3, IQR 0.2–0.7 in those with the AA and AG genotypes. Also, the frequencies of SLE patients positive for anti-dsDNA were similar: 48.4% for GG homozygotes and 43.3% for the remaining patients.

Similar results were obtained for anti-ssDNA (Fig. 2C). The titres were as follows: median 0.73, IQR 0.47–0.93 in SLE patients with the GG genotype; median 0.63, IQR 0.46–0.95 in patients with the AA and AG genotypes. The frequency of SLE patients positive for anti-ssDNA was 67% among the GG homozygotes and 65% among the other two genotypes.

In the case of anti-nucleosome antibodies (Fig. 2D), the titres were comparable in SLE patients with GG genotype and those with the AA or AG genotype (median 96.4, IQR 26.1–169.35 and median 55.7, IQR 2.2–167.2, respectively). However, the frequency of SLE patients positive for anti-nucleosome antibodies was significantly different among SLE patients with different genotypes: 79% of SLE patients bearing the GG genotype had anti-nucleosome antibody in their sera, whereas the percentage was 55.2% among patients with the AA or AG genotype \( (P = 0.03) \). This difference did not remain significant after correcting for multiple testing.

### Discussion

Several lines of evidence suggest that defects in DNase I activity play a role in SLE pathogenesis. Mutations affecting its function have been described in several SLE patients but they are rare and seem to account for only a small proportion of the SLE subjects with very low DNase activity \( [15, 22] \). Based on previous reports \( [17, 18] \), the common polymorphisms in the coding sequence appeared irrelevant for DNase I function, including the +2373 A → G SNP. However, the recent description of an association between this SNP and anti-RNP and anti-dsDNA antibodies but not SLE susceptibility in Korean patients \( [16] \) required confirmation. Our study has shown association between the GG allele of the +2373 A → G SNP and SLE susceptibility, but no association with the majority of antinuclear antibodies (anti-dsDNA, anti-ssDNA and anti-RNP) and no effect on DNase I activity. We found a trend indicative of a relationship between GG genotype of +2373 A → G SNP and the percentage of patients with antinucleosome antibodies, but this result should be taken with caution as there was no difference in the antibody titres and because the result did not remain significant after correction for multiple testing. Therefore, we have found association with SLE susceptibility but not with antibodies, whereas the previous Korean study found association with autoantibodies but not with susceptibility. This discrepancy could be related either to heterogeneity between the populations or to technological differences in antibody determination. Indeed, clinical heterogeneity of SLE between different ethnic groups is a well-known phenomenon and some examples of differences between Asians and Caucasians have been reported \( [23] \). Also, differences in the genetic makeup of populations are a frequent cause of discrepancies in genetic studies (see below). Methodological differences in antibody determination between the studies could also be involved. In this study, ELISA was used to determine anti-dsDNA and anti-RNP antibodies, whereas C. luciliae and double immunodiffusion assays, respectively, were used in the study of Shin et al. \( [16] \).

Previous studies with DNase I electrophoresis phenotypes indicate that the +2373 A → G SNP is irrelevant for enzymatic activity \( [17, 18] \). We confirmed the lack of relationship between the +2373 A → G SNP and serum DNase I activity. The result was observed consistently in samples from SLE patients and from different groups of controls. Additionally, the SNP did not explain the low SLE DNase I activity found in SLE patients. Therefore, there are no clues about what could be the mechanism of action of the +2373 A → G SNP in its contribution to SLE. However, it is important to note in this regard that discrepancies may exist between DNase I activity as detected in the serum and as found in specific tissues.

The lack of effect of the +2373 A → G SNP on DNase I activity invites prudence in the interpretation of the association we have observed between the GG genotype and SLE susceptibility, given that the involvement of DNase I in SLE seems to depend on its enzymatic activity on DNA. Prudence is also called for with respect to the discrepant results between Spanish and Koreans discussed above, which may be related to heterogeneity in gene variation, as evidenced by the different frequency of the G allele of the +2373A → G SNP (70% in Spanish and 44% in Koreans). Contradictory results, probably related to genetic heterogeneity between Asians and Europeans, have also been reported in relation to SLE (\( CTLA4 [24, 25] \)), RA (\( PADI4 [26–28] \)) and OA (asporin \( [29, 30] \)). Therefore, it seems plausible that the +2373 A → G SNP of \( DNASEI \) will be in linkage disequilibrium with other polymorphisms affecting DNase I activity and SLE susceptibility.
In addition, it cannot be excluded that the causal SNP will be in a nearby gene; for example, TRAPI, which has been implicated in apoptosis induced by reactive oxygen species [31]. There is strong linkage disequilibrium between $+2373\ A \rightarrow G$ (rs1053874) and a TRAPI SNP, though TRAPI and DNASEI lie in different haplotype blocks (HapMap and Perlegen databases). However, functional evidence for the involvement of TRAPI in SLE is lacking and an SNP affecting DNase I activity seems more likely.

In conclusion, both this study and that of Shin et al. [16] reinforce interest in exploring DNase I activity in relation to susceptibility to SLE and the SLE phenotype in order to discover the molecular basis of the relationship and to define the role that low DNase I has in SLE susceptibility and evolution.

### Acknowledgements

We thank the patients for their contribution. This work was supported by Fondo de Investigación Sanitaria, Instituto de Salud Carlos III (Spain) (grants 01/3054, 02/0490 and 02/0531) with contribution from FEDER funds (European Union), and by grants from the DXID (Xunta de Galicia). A.B. is supported by FIS (02/0490); C.C. and A.G. are Researchers from the FIS Program (Instituto de Salud Carlos III, Ministerio de Sanidad).

The authors have declared no conflicts of interest.

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