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Concise Report

Associations of DNase IV polymorphisms with autoantibodies in patients with systemic lupus erythematosus

I. Kim[1], N. W. Hur[1], H. D. Shin[2], B. L. Park[2], H. S. Cheong[2] and S.-C. Bae[1]

Objectives. The aim of this study was to investigate genetic polymorphisms of DNase IV and their relationship with SLE and various autoantibodies present in SLE patients.
Methods. A total of 532 SLE patients and 521 healthy controls belonging to the Korean population were enrolled into this study. Sequencing of the entire coding region of the DNase IV gene (including the promoter region) was carried out using a DNA analyser. Autoantibodies including anti-Sm, anti-Ro, anti-La, anti-RNP and anti-dsDNA were determined either by indirect immunofluorescence or double immunodiffusion methods. Multiple logistic regression analysis was performed to examine the genetic association with SLE and autoantibodies.
Results. We found three single-nucleotide polymorphisms (SNPs): −2753G→A, +147T→G (Gly49Gly) and +1466G→T. The −2753G→A and +147T→G (Gly49Gly) SNPs were selected for larger scale genotyping based on linkage disequilibria and haplotype-tagging status. Although the −2753G→A SNP was more common than the +147T→G (Gly49Gly) SNP (frequencies: 0.330 and 0.002, respectively), its association with the risk of SLE was not statistically significant. However, −2753G→A SNP was significantly associated with the production of anti-Sm antibody [odds ratio (95% CI): co-dominant model, 1.89 (1.28–2.79); dominant model, 2.17 (1.20–3.90) and recessive model, 2.62 (1.33–5.17)].
Conclusions. We did not find significant relationships between DNase IV polymorphisms and the risk of SLE, but the association of the common −2753G→A allele in the promoter region with the production of anti-Sm antibody implicates DNase IV as a putative candidate gene of SLE.

Key words: DNase IV, Single-nucleotide polymorphism, Systemic lupus erythematosus.

Introduction

SLE is characterized by a broad variety of clinical profiles and the production of autoantibodies against intracellular and cell-surface antigens. The aetiology of SLE is unknown, but genetic factors are likely to be involved in its pathogenesis, disease expression and the production of autoantibodies [1]. DNase is an important enzyme involved in the metabolism and clearance of DNA, and has been suggested to play an important role in the pathogenesis of SLE. Decreased DNase activity has previously been observed in SLE patients [2]. Mutation analysis of DNase I was revealed to be associated with decreased enzyme activity, the production of anti-dsDNA and anti-RNP antibodies (Abs) [3, 4], and the risk of SLE [5]. Also, DNase II polymorphisms were reportedly associated with the risk of lupus nephritis [6]. DNase III knockout mice showed inflammatory myositis similar to autoimmune myocarditis in a murine model [7], and we previously found that polymorphisms of DNase III were negatively associated with the production of anti-Ro and anti-dsDNA autoantibodies among SLE patients [8].

DNase IV (also called flap endonuclease-1; FEN1) [9] is a multifunctional enzyme that exhibits structure-specific flap endonuclease activity in cleaving 5′-flaps of DNA or RNA, and its gene is located on chromosome 11q12 [10]. In addition to its 5′-flap endonuclease activity, DNase IV also exhibits 5′–3′ exonuclease and gap endonuclease activities. DNase IV acts as RNase H in Okazaki fragment maturation through the removal of RNA primers, and is involved in the repair of mismatches and long-patch base excisions [11]. Given that DNase IV plays an important role in DNA metabolism [11], genomic stability [12–14] and apoptosis [15, 16], it is reasonable to postulate that mutations of DNase IV can lead to abnormal DNA metabolism, genomic instability and increase susceptibility to apoptosis. This may induce autoantibody formation and autoimmune disorders such as SLE through an increased level of autoantigens. Therefore, DNase IV polymorphisms may contribute to the development of SLE and autoantibodies, similar to observations made for polymorphisms of DNases I–III. In the present study, we investigated genetic polymorphisms of DNase IV and their relationship to SLE and various autoantibodies including anti-Sm, anti-Ro, anti-La, anti-RNP and anti-dsDNA.

Materials and methods

Patients

A total of 532 Korean patients who fulfilled the 1997 ACR criteria for SLE [17] were consecutively enrolled in the Hospital for Rheumatic Diseases, Hanyang University, Seoul, Korea. The following clinical and laboratory data were obtained: sex, age, age at first symptom onset and autoantibody status (anti-Sm, anti-RNP, anti-Ro, anti-La and anti-dsDNA). We included 521 healthy ethnically matched subjects as a control group in our examination of the genetic associations of polymorphisms with susceptibility to SLE and related phenotypes. Mean ages of SLE patients and normal controls are 34.4 and 35.4 yrs, respectively. Female subjects were dominant in both groups (15.6 : 1 and 4.6 : 1, irrespectively). In SLE patients, mean disease duration was 28.7 months and age of disease onset was 5.8 yrs. ANAs were tested by an indirect immunofluorescence technique using IT-1 cells; anti-dsDNA Abs by *Crithidia luciliae* assay and anti-Sm, anti-Ro, anti-La and anti-RNP Abs by double immunodiffusion. Written informed consent was obtained from each subject. The study was

[1]Division of Rheumatology, Department of Internal Medicine, Hanyang University College of Medicine and the Hospital for Rheumatic Diseases, Hanyang University and [2]Department of Genetic Epidemiology, SNP Genetics, Inc., Seoul, Korea.

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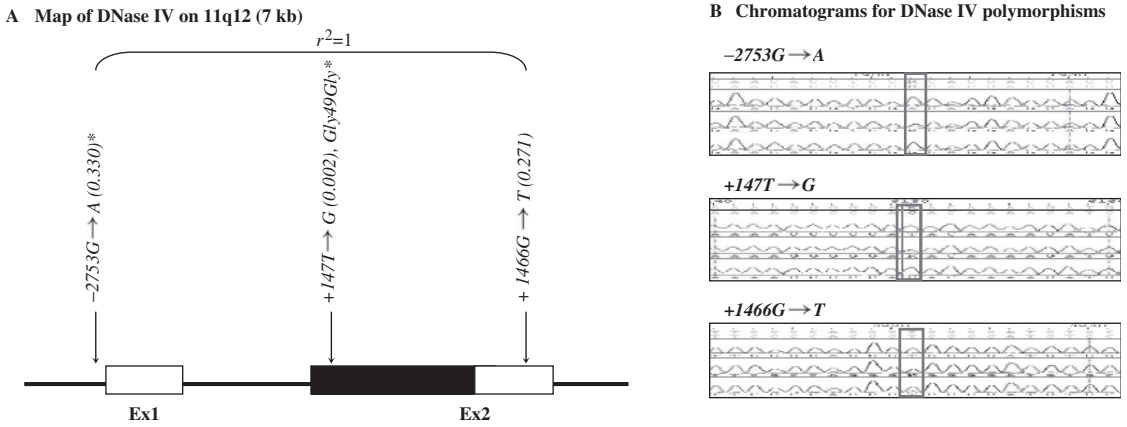
Correspondence to: S.-C. Bae, The Hospital for Rheumatic Diseases, Hanyang University Medical Center, Seoul 133-792, Korea. E-mail: scbae@hanyang.ac.kr



C Haplotypes in DNase IV

Hap.	−2753G→A	+147T→G	+1466G→T	Freq.
ht1	G	T	G	0.663
ht2	A	T	T	0.330
ht3	G	G	G	0.007

D LDs among DNase IV polymorphisms

		|D′|		
		−2753G→A	+147T→G	+1466G→T
r^2	−2753G→A	–	1	1
	+147T→G	0.004	–	1
	+1466G→T	1	0.004	–

FIG. 1. Gene map and haplotypes of DNase IV. Coding exons are marked by black blocks, and 5′ and 3′ untranslated regions (UTRs) by white blocks. The first base of the translational start site is denoted as nucleotide +1. Asterisks indicate polymorphisms genotyped in our total population ($n=1053$). The frequencies of polymorphisms not subjected to larger scale genotyping were based on sequence data ($n=24$). (**A**) Polymorphisms identified in DNase IV on chromosome 11q12 (reference GenBank sequence: NT_033903.7). (**B**) Chromatograms for DNase IV polymorphisms. (**C**) Haplotypes of DNase IV. (**D**) Linkage disequilibrium coefficients (|D′| and r^2) among DNase IV polymorphisms.

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Sequencing and genotyping analysis of the DNase IV gene

We sequenced all exons and their boundaries of the DNase IV gene, including the promoter region (~1.5 kb), to discover genetic variants in 24 Korean unrelated DNA samples, which were composed of 12 SLE patients and 12 healthy controls, using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems) were used for comparative sequencing in accordance with the recommendations of the manufacturer. Twenty-three primer sets of DNase IV for the amplification and sequencing analysis were designed based on GenBank sequences (No. NT_033903.7). For genotyping of polymorphic sites, amplification and extension primers were designed for single-base extension [18]. Primer extension reactions were performed with the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems). To clean up the primer extension reaction, one unit of shrimp alkaline phosphatase (SAP) was added to the reaction mixture, and the mixture was incubated at 37°C for 1 h, followed by 15 min at 72°C for enzyme inactivation. The DNA samples, containing extension products, and Genescan 120 Liz size standard solution were added to Hi-Di formamide (Applied Biosystems) according to the recommendation of the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then electrophoresis was performed using the ABI Prism 3100 Genetic Analyzer. The results were analysed using ABI Prism GeneScan and Genotyper programs (Applied Biosystems).

Statistics

Chi-square tests were used to determine whether individual polymorphisms were in Hardy–Weinberg equilibrium. Heterozygosity for each locus with allele frequencies p and $q=1-p$ is given by $H=1-p^2-q^2=2p(1-p)$. We examined the linkage disequilibrium (LD) expressed as Lewontin's D' ($|D'|$), where $D'=D/|D_{\max}|$, and the LD coefficient r^2 between all pairs of biallelic loci [19]. The haplotypes of each individual were inferred from the PHASE (version 2.1) program using an algorithm developed by Stephens *et al.* [20], which uses a Bayesian approach incorporating *a priori* expectations of haplotypic structure based on population genetics and coalescence theory.

The relationship between genotype distributions of DNase IV polymorphisms [−2753G→A, +147T→G (Gly49Gly) and ht1] were compared between the SLE patients and healthy controls using multiple logistic regression models to calculate odds ratios (ORs), 95% CIs and corresponding P-values whilst controlling for age (continuous variable) and sex (male = 0, female = 1) as covariates. Because −2753G→A was almost equivalent with ht2 (Fig. 1C), the ht2 was not analysed in our study. The genotype distributions of DNase IV polymorphisms [−2753G→A, +147T→G (Gly49Gly) and ht1] and the production of autoantibodies (anti-Sm, anti-RNP, anti-Ro, anti-La and anti-dsDNA Abs) were also analysed using multiple logistic regression models to calculate ORs, 95% CIs and corresponding P-values using age, sex and disease duration (continuous variable) as covariates among SLE patients. To correct type I error, we applied Bonferroni correction [corrected P-value corresponding to $P<0.05$ was calculated as follows: $P<0.05/(3\times5)=0.0033$]. ORs and P-values of co-dominant, dominant and recessive models were also calculated for anti-Sm and anti-RNP Abs.

TABLE 1. Logistic analysis of DNase IV polymorphisms with the risk of SLE using age, disease duration and sex as covariates in the total population

		Frequency		Co-dominant		Dominant		Recessive	
Loci	Allele	Positive ($n=530$)	Negative ($n=517$)	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
−2753G→A	A	0.332	0.33	1.03 (0.86, 1.23)	0.76	1.07 (0.84, 1.37)	0.58	0.96 (0.66, 1.40)	0.84
+147T→G	G	0.001	0.004	0.20 (0.02, 1.76)	0.14	0.20 (0.02, 1.76)	0.14		
ht1	ht1	0.668	0.657	1.03 (0.86, 1.23)	0.76	1.17 (0.80, 1.69)	0.42	0.99 (0.77, 1.26)	0.91

The ht2 was not analysed because −2753G→A was almost equivalent with ht2. Logistic regression models were used to calculate ORs, 95% CIs and the corresponding *P*-values for SNP sites whilst controlling for age and sex as co-variables. The common alleles were used as the reference genotype to the heterozygote and homozygote of the minor allele. *P*-values of co-dominant, dominant and recessive models are also given.

Results

Polymorphisms of DNase IV

One of the three single-nucleotide polymorphisms (SNPs) in DNase IV was in the 5′ flanking region of exon 1 (−2753G→A), and the other two were in exons 2 [+147T→G (Gly49Gly) and +1466G→T]. Pairwise comparisons among SNPs revealed absolute LD ($|D'|=1$ and $r^2=1$, Fig. 1A) between −2753G→A and +1466G→T, and complete LD ($|D'|=1$ and $r^2\neq 1$) was also found (Fig. 1C). Two of the DNase IV SNPs [−2753G→A, and +147T→G (Gly49Gly)] were selected for larger scale genotyping (532 SLE patients and 521 healthy controls) based on LD and haplotype-tagging status. The −2753G→A SNP is located in the promoter region (Fig. 1A) and was more common (frequency 0.330) than the +147T→G SNP (frequency 0.002).

DNase IV polymorphisms and the risk of SLE

Our analysis of the associations between DNase IV polymorphisms [−2753G→A, +147T→G (Gly49Gly) and ht1] and the risk of SLE using multiple logistic regression models, including co-dominant, dominant and recessive models whilst controlling for age and sex as covariates, revealed no significant associations between SNPs and the risk of SLE (Table 1).

DNase IV polymorphisms and the production of autoantibodies among SLE patients

Our examination of the associations between DNase IV polymorphisms and the production of autoantibodies (anti-Sm, anti-RNP, anti-Ro, anti-La and anti-dsDNA Abs) with multiple regression analysis using age, sex and disease duration as covariates in SLE patients revealed that the common −2753G→A SNP—which is located in the promoter region—was significantly associated with an increased risk of anti-Sm Ab and anti-RNP Ab formation (Table 2). The ht1 [G–T] SNP exerted a protective effect on the production of anti-Sm Ab (Table 2). The productions of other autoantibodies were not significantly associated with DNase IV SNPs. After applying Bonferroni correction to correct type-I errors in multiple comparisons, the statistical significances of SNPs [−2753G→A and ht1] for the risk of anti-Sm Ab remained, but the significance of −2753G→A for the risk of anti-RNP Ab disappeared (Table 2). Dominant and recessive models were then applied to examine the relationship between DNase IV SNPs and anti-Sm and anti-RNP Abs. The production of anti-Sm Ab was positively associated with −2753G→A and negatively associated with ht1 in co-dominant, dominant and recessive models [−2753G→A: ht1, OR (95% CI): codominant model 1.89 (1.28–2.79):0.55 (0.37–0.81); dominant model 2.17 (1.20–3.90):0.43 (0.21–0.85) and recessive model 2.62 (1.33–5.17):0.47 (0.26–0.85)]. However, the statistical significances in dominant and recessive models disappeared after applying Bonferroni correction. The production of anti-RNP Ab was also positively associated with −2753G→A in co-dominant and recessive models [OR (95% CI): co-dominant model 1.34 (1.00–1.79); dominant model 1.24 (0.83–1.86) and recessive model, 2.04 (1.16–3.59)], but all of the statistical significances disappeared after applying Bonferroni correction.

TABLE 2. Logistic analysis of DNase IV polymorphisms according to autoantibodies (anti-Sm, anti-RNP, anti-Ro, anti-La and anti-dsDNA Abs) using age, disease duration and sex as covariates in SLE patients

			Frequency			
Auto-Ab	Loci	Allele	Positive ($n=60$)	Negative ($n=424$)	OR (95% CI)	*P*
Anti-Sm	−2753G→A	A	0.467	0.320	**1.89 (1.28, 2.79)**	**0.001**
	+147T→G	G	0.000	0.001		0.99
	ht1	ht1	0.542	0.679	**0.55 (0.37, 0.81)**	**0.003**
			($n=137$)	($n=346$)		
Anti-RNP	−2753G→A	A	0.383	0.318	1.34 (1.00, 1.79)	0.05
	+147T→G	G	0.000	0.001		0.99
	ht1	ht1	0.621	0.681	0.77 (0.57, 1.03)	0.07
			($n=185$)	($n=298$)		
Anti-Ro	−2753G→A	A	0.324	0.344	0.88 (0.67, 1.16)	0.36
	+147T→G	G	0.003	0.000		0.99
	ht1	ht1	0.677	0.656	1.14 (0.86, 1.50)	0.37
			($n=46$)	($n=437$)		
Anti-La	−2753G→A	A	0.348	0.335	1.04 (0.67, 1.62)	0.87
	+147T→G	G	0.000	0.001		0.99
	ht1	ht1	0.652	0.665	0.96 (0.62, 1.50)	0.86
			($n=372$)	($n=127$)		
Anti-dsDNA	−2753G→A	A	0.332	0.346	0.95 (0.70, 1.28)	0.73
	+147T→G	G	0.001	0.000		0.99
	ht1	ht1	0.667	0.659	1.03 (0.76, 1.39)	0.86

The ht2 was not analysed because −2753G→A was almost equivalent with ht2. Bold face indicates statistically significant values (only associations with $P<0.0033$, corresponding to Bonferroni-corrected *P*-value <0.05). NS: not significant ($P>0.0033$). Logistic regression models were used to calculate ORs, 95% CIs and the corresponding *P*-values for SNP sites whilst controlling for age, sex and disease duration as covariables. The common alleles were used as the reference genotype to the heterozygote and homozygote of the minor allele.

Discussion

Our study identified three SNPs [−2753G→A, +147T→G (Gly49Gly) and +1466G→T] in DNase IV. Although none of these SNPs were associated with the risk of SLE, the common −2753G→A allele was positively associated with the formation of anti-Sm autoantibody, while ht1 was negatively associated with the production of anti-Sm Ab. Applying Bonferroni correction removed the statistical significance of −2753G→A for the increased risk of anti-RNP Ab formation, whereas that for anti-Sm Ab remained.

The mechanisms underlying the genetic effects of DNase IV polymorphisms on the formation of anti-Sm and anti-RNP Abs are unknown. One possible explanation is that mutation in the promoter region of DNase IV, which plays important roles in Okazaki fragment maturation [11], induces structural and functional changes in DNase IV that consequently result in inefficient removal of initiator RNA from RNA/DNA hybrids. Such impaired RNA degradation combined with decreased clearance of apoptotic cell debris in SLE might stimulate development of autoantibodies against RNA or RNA protein such as anti-Sm and anti-RNP Abs. In addition to DNase IV functioning during Okazaki fragment maturation and repair of mismatched DNA, apoptotic proteins such as endonuclease G also interact with

DNase IV. Reduction of *Crn-1* (a *Caenorhabditis elegans* FEN-1 homologue) activity by RNA interference resulted in cell death and suggests that CRN-1/FEN-1 may play a critical role in switching the state of cells from DNA replication/repair to DNA degradation during apoptosis through initiating and facilitating DNA fragmentation [16], which indicates that DNase IV plays an important role in apoptosis.

Although DNase IV polymorphism in promoter lesion (−2753G→A) was associated with the formation of anti-Sm and -RNP Abs in our study, the question of whether −2753G→A really decreases enzyme activity still remains because the enzyme activity was not evaluated. Also, despite high specificity of double immunodiffusion test in detecting autoantibodies, the status of anti-Sm and -RNP Abs positivity may vary depending on different tests (i.e. immunoblot assay, double immunodiffusion or ELISA). To confirm the association of DNase IV polymorphisms observed in our study with anti-Sm Ab, methodological differences need to be taken into consideration.

In summary, we have identified three polymorphisms in the human DNase IV gene, with one common polymorphism in the promoter region, −2753G→A, being significantly associated with the production of anti-Sm Ab in SLE patients. The association of this common allele in the promoter region with the production of anti-Sm Ab implicates DNase IV as a putative candidate gene of SLE. Further biological and functional data are required to confirm the association of the common −2753G→A allele in DNase IV with the production of autoantibodies.

Rheumatology key message

- The common −2753G→A SNP of the DNase IV gene is associated with the production of anti-Sm autoantibodies in SLE patients.

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