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Association between the angiotensin-converting enzyme gene insertion/deletion polymorphism and susceptibility to systemic lupus erythematosus in the Indian population

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by intense inflammation and multiple organ damage (1). There is much evidence for genetic susceptibility to SLE and several candidate genes including that encoding the angiotensin-converting enzyme (ACE) have been identified (2). Biologically, ACE elevates the levels of angiotensin II, a vasoactive peptide, growth factor and potent pro-inflammatory modulator, which contributes to tissue remodelling (3, 4). The *ACE* gene contains a 287-base pair repeat insertion/deletion (I/D) polymorphism within intron 16 (5), and studies have shown that D/D homozygotes have an approximate two-fold higher level of tissue and plasma ACE as compared with I/I homozygotes (6). Although the D/D genotype and the concomitant increase in serum ACE and angiotensin II levels could contribute to the pathogenesis of SLE (3, 4), there are conflicting data with regard to the association of the *ACE* D/D genotype and susceptibility to the disease (7). The aim of the current study was to investigate *ACE* I/D gene polymorphisms and serum ACE levels in Indian SLE patients, and to determine any correlations to the clinical features of SLE.

One hundred and nine SLE patients (6 male, 103 female; mean age 27.8 ± 9.7 years with range 12-60 years) were recruited to this single-centre prospective observational study at the King Edward Memorial Hospital, Mumbai, Maharashtra, India, between January 2010 and January 2012. The clinical details of the SLE patient group are given in Supplementary Table 1. SLE was diagnosed according to the revised and updated criteria of the American Rheumatism Association for the classification of SLE (8, 9). Of the SLE patients, 39 were untreated at the point of inclusion in the study, and 70 were receiving corticosteroid treatment. A total of 100 unrelated healthy individuals without SLE or other autoimmune disease, or a history thereof, and matched for ethnicity, sex and age, were also included in the study as controls. Approval for the study was given by the institutional ethics committee, and written informed consent was

obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki Principles.

In a case-control study, the *ACE* I/D genotypes and serum ACE levels were determined for 109 SLE patients and 100 age and sex-matched controls using PCR amplification and an ELISA, respectively (Supplementary Material). The observed genotype frequencies of the controls (Table 1) did not differ significantly from the frequencies predicted by the Hardy-Weinberg equilibrium (P = 0.11). There was a strong statistical trend for an increase of the D/D and I/D genotypes in SLE, while there was a statistically significant increase in the D allele (Table 1). Serum ACE levels were significantly higher in the SLE patient group compared with controls (Table 1). In both SLE patients and controls, the D/D genotype correlated with the highest levels of serum ACE (Figure 1). Serum ACE levels in SLE patients were also increased significantly when compared with controls of the same *ACE* I/D genotype (Figure 1). There was no significant difference in the prevalence of *ACE* I/D genotypes or alleles or in the levels of serum ACE in relation to SLE activity, as measured by SLE disease activity indices (SLEDAI), or to the presence of vasculopathy or lupus nephritis (Table 1). SLEDAI scores did not differ significantly in relation to the D/D, D/I and I/I *ACE* genotypes: the median, 25th and 75th percentiles, and range were, respectively, 14, 11, 22, 6-51; 13, 10, 18, 3-34; and 10, 15, 21, 8-27 (P = 0.20, Kruskal-Wallis test), and no significant correlation was found between SLEDAI scores and serum ACE levels (r = -0.09 with 95% confidence intervals -0.28-0.11; P = 0.37, Spearman's rank correlation test).

Here, we provide evidence of an association between SLE and the D allele of the ACE I/D genotype, and between SLE and elevated levels of serum ACE in an Indian population. As reported before (6), the *ACE* D/D genotype was associated with significantly higher levels of

serum ACE. This has been suggested to result from either strong linkage disequilibrium of the I/D polymorphism with regulatory elements of *ACE* which are responsible for the variation in enzyme levels, or from alterations in mRNA stability or precursor mRNA splicing (6). Although how increased levels of serum ACE can result in SLE manifestations remains unestablished, elevated levels have been associated with an increased generation of reactive oxygen species which can lead to oxidative stress and subsequent tissue damage (10).

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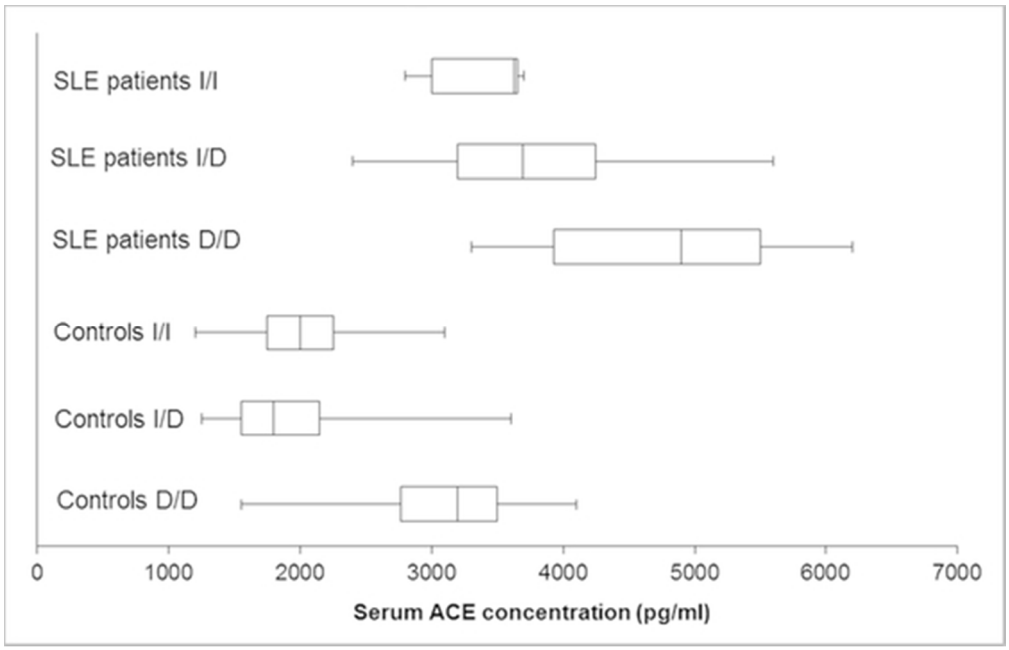
Figure Legend

Figure 1: Serum ACE levels in relation to *ACE* genotypes in SLE patients and controls. Serum ACE levels (pg/ml) are shown as the medians, 25th and 75th percentiles, and ranges for SLE patient and control groups. Serum ACE levels in SLE patients and controls with the *ACE* D/D genotype were significantly increased compared to those with other *ACE* I/D genotypes (*P* values were < 0.0001, Kruskal-Wallis test). Serum ACE levels of the SLE patients were significantly increased when compared with controls of the same *ACE* I/D genotype (*P* values were < 0.0001, Kruskal-Wallis test).

Table 1. Distribution and comparison of *ACE* I/D genotypes and alleles and of serum ACE levels in SLE patients and controls.

Patient or control group	Number of subjects	Number of alleles in the group (%)		*P* value[a]	Odds ratio (95% confidence interval)	*P* value[a]	Number of subjects with genotype (%)			*P* value[a]	*P* value[a]	Serum ACE levels in pg/ml (median) and range	*P* value[b]	*P* value
		D	I				D/D	I/D	I/I					
Healthy controls	100	101 (51)	99 (49)	NA	NA	NA	22 (22)	57 (57)	21 (21)	NA	NA	2000; 1200-1400	NA	NA
SLE patients	109	133 (61)	85 (39)	0.04	1.53 (1.04-2.26)	NA	36 (33)	61 (56)	12 (11)	0.06	NA	3800; 2400-6200	< 0.0001	NA
SLEDAI 1-8	16	16 (50)	16 (50)	0.96	0.98 (0.46-2.07)	0.30	2 (13)	12 (75)	2 (16)	0.40	0.33	3800; 2800-5600	< 0.0001	0.45[c]
SLEDAI 9-18	63	77 (61)	49 (39)	0.08	1.54 (0.98-2.42)		21 (33)	35 (56)	7 (11)	0.13		4000; 2500-6200	< 0.0001	
SLEDAI > 18	30	40 (67)	20 (33)	0.04	1.96 (1.07-3.59)		13 (43)	14 (47)	3 (10)	0.05		3645; 2400-6200	< 0.0001	
Lupus nephritis	34	42 (62)	26 (38)	0.14	1.58 (0.90-2.78)	0.99	13 (38)	16 (47)	5 (15)	0.17	0.42	3900; 2900-6200	< 0.0001	0.55[b]
No lupus nephritis	75	91 (61)	59 (39)	0.08	1.51 (0.98-2.32)		23 (31)	45 (60)	7 (9)	0.08		3700; 2400-6200	< 0.0001	
Class I/ II/III lupus nephritis[d]	10	15 (75)	5 (25)	0.06	0.34 (0.12-0.97)	0.57	6 (60)	3 (30)	1 (10)	**0.03**	0.87	4550; 3000-5500	< 0.0001	0.40[c]
Class IV lupus nephritis[d]	18	23 (64)	13 (36)	0.19	0.58 (0.28-1.20)		8 (44)	7 (39)	3 (17)	0.13		3950; 3000-6200	< 0.0001	
Class V/VI lupus nephritis[d]	6	7 (58)	5 (42)	0.81	0.73 (0.22-2.37)		2 (33)	3 (50)	1 (17)	0.81		3600; 2900-6000	0.0006	
Vasculopathy	15	18 (60)	12 (40)	0.44	0.68 (0.31-1.49)	0.90	4 (27)	10 (67)	1 (7)	0.42	0.65	3690; 2400-5200	< 0.0001	0.13[b]
No vasculopathy	94	115 (61)	73 (39)	0.04	0.65 (0.43-0.97)		32 (34)	51 (54)	11 (12)	0.08		3850; 2690-6200	< 0.0001	

NA, not applicable.
SLEDAI, SLE disease activity index.
P values < 0.05 considered significant.
[a]Chi-square test with Yate's correction for contingency tables comparing SLE patients with controls or comparing SLE patient sub-groups. The Bonferroni-adjusted *P* values were not significant in multiple comparisons of lupus nephritis and vasculopathy in relation to I/D genotypes.
[b]Mann-Whitney test comparing SLE patients with controls.
[c]Kruskal-Wallis test comparing SLE patient sub-groups.
[d]Class I, minimal mesangial lupus nephritis; class II, mesangial proliferative lupus nephritis; class III, focal lupus nephritis; class IV, diffuse lupus nephritis; class V, membranous lupus nephritis; and class VI, advanced sclerosing lupus nephritis



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Supplementary Material

Materials and Methods

ACE gene I/D polymorphism analysis

High molecular weight genomic DNA was prepared from EDTA-collected venous blood samples (10-20 ml) using a Cyclo-Prep™ Genomic DNA Isolation Kit (Genetix Biotech Asia, New Delhi, India) according to the manufacturer's instructions and was stored in Tris-EDTA buffer (pH 8.0) at -20°C. Genomic DNA was subjected to PCR amplification using oligonucleotide primers 5'-CTGGAGACCACTCCCATCCTTTCT-3′ (forward) and 5'-GATGTGGCCATCACATTCGTCAGAT-3' (reverse) (Eurofins Genomics, Bangalore, India) in order to amplify the I/D polymorphic 287-base pair repetitive sequence (rs1799752) in intron 16 of the *ACE* gene (Reference sequence AF118569, repeat region 14094-14381; National Center for Biotechnology Information, Bethesda, MD, USA).

PCR amplifications were performed on 100 ng of genomic DNA in 25-µl reactions containing: 0.5 units of *Taq*I DNA polymerase (Chromos Biotech, Bengaluru, India), 1 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Chromos Biotech), 1.5 mM magnesium chloride (Chromos Biotech) and 0.2 volumes of 5x buffer (50 mM Tris-hydrochloride, pH 8.5; 0.05% (w/v) gelatin; 250 mM potassium chloride; 0.5% (v/v) Tween 20; and 0.5% (v/v) Nonidet P-40) (Chromos Biotech). The PCR amplifications were performed in an Eppendorf Mastercycler® gradient thermo cycler (Eppendorf North America, Inc., Hauppauge, NY, USA) under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of amplification

at 94℃ for 1 min, 58℃ for 1 min and 72℃ for 1 min; and a final extension at 72℃ for 7 min.

PCR amplification products were analysed in 1.5% (w/v) agarose gels along with pUC Mix Marker 8 DNA fragment size markers (Thermo Fisher Scientific Inc., Waltham, MA, USA). Following staining with ethidium bromide, gels were visualised under UV light and genotyped according to the following pattern: the presence of a single PCR amplification product of 480 base pairs represented an I/I homozygous individual, the presence of a single PCR amplification product of 193 base pairs represented a D/D homozygous individual, and the presence of both PCR amplification products represented a I/D heterozygous individual. The accuracy of the genotyping protocol was verified by repeating the PCR analysis on 20 randomly selected DNA samples. All initial results were verified by this re-analysis.

Enzyme-linked immunosorbent assay for serum ACE level measurement

Venous blood samples (10-20 ml) were collected from patients and controls, serum prepared and stored at -80℃ until required. Enzyme-linked immunosorbent assay (ELISA) kits for measuring serum ACE levels in patient and control sera were purchased from Bio Vendor-Laboratoni Medicina a.s (Brno, Czech Republic) and used according to the manufacturer's protocol. Sera were analysed in triplicate in two experiments, and ACE levels were reported as pg/ml. Intra- and inter-assay coefficients of variation were < 10%.

Statistical analyses

Statistical analysis was carried out using GraphPad InStat 3 software (GraphPad Software Inc., La Jolla, CA, USA). The frequencies of the *ACE* I/D genotypes and alleles in SLE patient and control groups were compared using the Chi-square test with Yate's correction for contingency tables. Odds ratios with 95% confidence intervals were calculated according to a previous method (1). Expected genotype counts were calculated according to the Hardy-Weinberg equilibrium (2), and compared with observed genotypes in the Chi-square test. Comparison of continuous variables was carried out using Mann-Whitney or Kruskal-Wallis tests, as appropriate. Bonferroni-adjusted *P* values were calculated for multiple comparisons of variables. Spearman's rank (*r*) correlation test was used to measure the statistical dependence between two variables. In all tests, *P* values (two-tailed) < 0.05 were regarded as significant.

Supplementary Table 1. Clinical details of SLE patients included in the study

Clinical feature	Number (%) of SLE patients or detail of SLE patient group
Mean age at SLE onset ± SD	25.7 ± 9.2 years (range 11-67 years)
Mean SLE duration ± SD	2.1 ± 2.9 years (range < 1-20 years)
SLE activity[a]:	
Mean SLEDAI ± SD	15.3 ± 7.6 years (range 3-51 years)
SLEDAI 1-8	16 (14.7)
SLEDAI 9-18	63 (57.8)
SLEDAI > 18	30 (27.5)
Clinical manifestations:	
Lupus nephritis	34 (31.2)
Arthritis	68 (62.4)
Alopecia	76 (69.7)
Myositis	11 (10.1)
Urinary casts	27 (24.8)
Hematuria	30 (27.5)
Proteinuria	32 (29.4)
Raynaud's phenomenon	11 (10.1)
Cutaneous vasculitis	6 (5.5)
Photosensitivity	40 (36.7)
Mucosal ulcers	58 (53.2)
Malar rash	49 (45.0)
Leukopenia	7 (6.4)
Thrombopenia	8 (7.3)
Pleurisy	13 (11.9)
Fever	50 (45.9)
Seizures	5 (4.6)
Psychosis	5 (4.6)
Organic brain syndrome; cranial nerve disorder; lupus headache; new onset of cerebrovascular accident(s) excluding arteriosclerosis	3 (2.8)
New skin rash	6 (5.5)
Serological analysis:	
Anti-nuclear autoantibodies	109 (100)
Anti-DNA autoantibodies	100 (91.7)
Renal pathology[b]:	
Class I, Minimal mesangial lupus nephritis	2/34 (5.9)
Class II, Mesangial proliferative lupus nephritis	4/34 (11.8)
Class III, Focal lupus nephritis	4/34 (11.8)
Class IV, Diffuse lupus nephritis	18/34 (52.9)
Class V, Membranous lupus nephritis	4/34 (11.8)
Class VI, Advanced sclerosing lupus nephritis	2/34 (5.9)

[a]Disease activity was assessed using the SLE disease activity index (SLEDAI) (3).
[b]Histological findings in renal biopsies were classified according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 criteria (4).

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