Angiotensin-Converting Enzyme (Insertion/Deletion) and Endothelial Nitric Oxide Synthase Polymorphisms in Patients with Systemic Lupus Erythematosus

GLENN DOUGLAS, CHRISTOPHER REILLY, MARY ANNE DOOLEY, GRIER PAGE, GLINDA COOPER, and GARY GILKESON

ABSTRACT. ***Objective.*** Genetic polymorphisms in the angiotensin-converting enzyme (ACE) and endothelial nitric oxide synthase (eNOS) are linked with expression and/or progression of renal disease. We hypothesized that polymorphisms in the genes coding for ACE and eNOS may influence the development and/or progression of systemic lupus erythematosus (SLE) and lupus nephritis given their linkage with other renal diseases.

Methods. DNA from patients with SLE (n = 227) and their age and sex matched controls (n = 275) from the Carolina Lupus (CLU) Study cohort was assessed for ACE and eNOS polymorphisms. Seventy patients had biopsy-proven lupus nephritis. Two different eNOS polymorphisms (eNOS promoter T-786→C nucleotide substitution and eNOS 27 base pair tandem repeat in intron 4) and the ACE insertion/deletion (I/D) polymorphism in intron 16 were examined by restriction fragment length polymorphism-polymerase chain reaction.

Results. Allele frequency of the eNOS polymorphisms varied significantly between SLE patients and controls. There was no association of these polymorphisms with lupus within ethnic groups. We found no association of the polymorphism with the development of renal disease. No association was observed for the ACE I/D polymorphism with SLE or nephritis, or with ethnicity or sex.

Conclusion. eNOS genetic polymorphisms differed significantly across ethnic groups. There was no significant increased risk of SLE and/or lupus nephritis associated with eNOS or ACE polymorphisms in either the African American or Caucasian groups compared to ethnically matched controls. These studies emphasize the need to control for ethnicity when investigating genetic polymorphisms and disease. (J Rheumatol 2004;31:1756–62)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS NITRIC OXIDE GENETICS PREDISPOSITION
ANGIOTENSIN-CONVERTING ENZYME AFRICAN AMERICAN POLYMORPHISM

From the Medical Research Service, Ralph H. Johnson Veterans Administration Medical Center and Medical University of South Carolina, Charleston, South Carolina; Department of Physiology, Edward Via Virginia College of Osteopathic Medicine, Blacksburg, Virginia; Department of Medicine, University of North Carolina Affiliated Hospitals, Chapel Hill, North Carolina; Department of Biostatistics, University of Alabama at Birmingham, Birmingham, Alabama; and the National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina, USA.

Supported by the VA Career Development Award, VA REAP Award, the VA Medical Research Service, National Institutes of Health grants AR45476, AR39162 and AI41764, and the Medical University of South Carolina University Research Committee Award, Intramural Research Program of the National Institute of Environmental Health Sciences.

G.C. Douglas, MD, Research Fellow, Medical Research Service, Ralph H. Johnson VA Medical Center and Medical University of SC; C.M. Reilly, PhD, Discipline Leader for Physiology, Edward Via Virginia College of Osteopathic Medicine; M.A. Dooley, MD, Associate Professor of Medicine, Department of Medicine, UNC Affiliated Hospitals; G.P. Page, PhD, Assistant Professor, Department of Biostatistics, University of Alabama at Birmingham; G. Cooper, PhD, Adjunct Assistant Professor, National Institutes of Environmental Health Sciences; G.S. Gilkeson, MD, Professor of Medicine, Chief of Rheumatology, Ralph H. Johnson VA Medical Center.

Address reprint requests to Dr. G. Gilkeson, Department of Medicine, Division of Rheumatology, Medical University of South Carolina, 96 Jonathan Lucas Street, Suite 912, PO Box 250623, Charleston, SC 29425. E-mail: gilkeson@musc.edu

Submitted March 12, 2003; revision accepted March 3, 2004.

Systemic lupus erythematosus (SLE) is a multiorgan systemic autoimmune disease of uncertain etiology, although genetic factors influenced by environmental agents affect disease expression. Despite improved survival in patients with SLE recently, lupus nephritis remains a predominant cause of morbidity and mortality[1]. The renin-angiotensin system (RAS) and nitric oxide (NO) both make up part of the complex system that regulates vascular tone and inflammation, and therefore have been implicated to play a role in the progression of SLE and lupus nephritis.

Because it cannot be stored due to its short half-life, NO synthesis is tightly regulated by nitric oxide synthases (NOS). Three isoforms of NOS have been identified: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). In most studies of SLE patients and animal models of SLE, iNOS expression and NO metabolites are elevated[2-5]. Further, serum nitrate/nitrite and 3-nitrotyrosine concentrations correlate with disease activity[2,3,5-7]. NO production by eNOS, in contrast to NO production by iNOS, prevents smooth muscle cell proliferation, platelet adherence, and neutrophil activation and adhesion[8].

Endothelial NOS, through its antiinflammatory and

vasodilatory properties, may play an important role in protecting against the cardiovascular and renal complications commonly observed in patients with SLE. Loss of eNOS activity causes intravascular thrombosis, neutrophil activation, and diapedesis[9]. Further, glomerular eNOS expression is decreased in individuals with lupus nephritis and inversely correlates with glomerular injury[10]. However, it is unclear if the observed decrease in eNOS in SLE is primary or a result of disease. Genetic polymorphisms in the gene for eNOS may alter eNOS expression and thus contribute to cardiorenal morbidity in SLE.

Encoded on chromosome 7q35-36, eNOS is expressed mainly in endothelial cells, where it produces NO constitutively. A single T→C nucleotide substitution at position –786 in either allele in the promoter region of the eNOS gene results in a 52% reduction in promoter activity[11]. Presence of the "C" nucleotide has been associated with increased coronary spasm and advanced diabetic nephropathy[12]. Additionally, the eNOS gene contains a variable 27 base pair (bp) tandem repeat in intron 4 with 4 repeats (a), 5 repeats (b), or 6 repeats (c)[13]. The 4-repeat alleles are associated with coronary artery disease in smokers[14], lipid abnormalities[15], idiopathic recurrent miscarriages[16], advanced diabetic nephropathy[12], endstage renal disease in nondiabetics[17], and acute myocardial infarctions in a Russian population[18]. The effect of this 27 bp repeat on eNOS activity is unknown. Because of the link between eNOS polymorphisms and cardiorenal disease observed in these populations, we investigated these eNOS polymorphisms in patients with SLE.

In addition to the NO system, the RAS also helps regulate vascular function and may play a role in the development and expression of SLE and lupus nephritis. The use of angiotensin-converting enzyme (ACE) inhibitors in the treatment of renal and cardiovascular diseases has confirmed the central role the RAS plays in the pathogenesis and progression of these disorders. ACE converts angiotensin I into angiotensin II, a potent vasoconstrictor that helps maintain vascular tone and regulate renal perfusion. ACE also is involved in the degradation of bradykinin, a potent vasodilator that stimulates the release of prostacyclin and NO. Patients with renal diseases treated with ACE inhibitors and angiotensin II receptor blockers have decreased proteinuria, improved systemic and intraglomerular hypertension, reduced histological evidence of renal injury, and slower progression to endstage renal failure[19].

The ACE gene, located on chromosome 17q23, contains an insertion (I) and deletion (D) polymorphism within intron 16 consisting of the presence or absence of a 287 bp repeat sequence[20]. Of the 3 possible genotypes (DD and II homozygotes and ID heterozygote), the DD genotype is associated with roughly 2-fold higher tissue and plasma concentrations of ACE than the II genotype[20]. The D allele has been linked to SLE in a Slovak population[21], poorer response to ACE inhibitors in diabetic nephropathy[22], and increased mortality from coronary artery disease[23-25], carotid stenosis and ischemic stroke[26] and systemic sclerosis[27].

In light of these links between eNOS and ACE with autoimmunity and renal disease, we analyzed DNA samples from SLE cases and control subjects living in North Carolina and South Carolina (the Carolina Lupus Study, CLU) for the eNOS T-786→C, the eNOS 27 bp repeat in intron 4, and the ACE intron 16 I/D polymorphisms.

MATERIALS AND METHODS

Study populations. Subjects for this study were enrolled in the Carolina Lupus Study, a retrospective case-control study of genetic and environmental factors predisposing to SLE. Only Caucasian and African American individuals were recruited from the CLU cohort as there were insufficient numbers of other ethnic groups to obtain statistical significance. All 227 SLE patients lived in eastern North Carolina and South Carolina and met the 1997 revised American College of Rheumatology (ACR) classification criteria for SLE. All were diagnosed between January 1, 1995, and July 31, 1999. The CLU Study also included 275 population-based controls who were randomly selected from state driver's license registries and who were of similar ages and state distribution to the case group. Data collection included a 60 minute standardized in-person interview and a medical record review. Genomic DNA was extracted from blood samples obtained from SLE patients and controls at the time of the study interview.

Polymerase chain reaction (PCR). Three polymorphisms were studied: (1) eNOS T-786→C substitution in the promoter region, (2) eNOS 27 bp tandem repeat insertion-deletion in intron 4, and (3) ACE I/D polymorphism in intron 16. PCR amplification of each genetic segment for the different polymorphisms was carried out using a BioRad iCycler (BioRad, Hercules, CA, USA) using 20 ng genomic DNA, 20 pM/µl of the forward primer, 20 pM/µl of the reverse primer, 2.5 mM of dNTP (Perkin-Elmer, Branchburg, NJ, USA), 0.025 U/µl Taq Hotstart DNA polymerase and buffer mix (Qiagen, Valencia, CA, USA). Primers and PCR conditions are described for each polymorphism.

eNOS promoter T-786→C polymorphism analysis. As defined by Nakayama, *et al*[11], 223 bp of DNA between residues 697 and 920 were amplified by PCR. Restriction fragment length polymorphism-PCR (RFLP-PCR) analysis of DNA from 502 CLU Study subjects was performed to determine if the eNOS T-786→C polymorphism was present in either allele of eNOS. The primers used for the eNOS T-786→C polymorphism were: sense primer 5' GCA TGC ACT CTG GCC TGA AGT G (located 697 to 719 bp), antisense primer 5' CAG GAA GCT GCC TTC CAG TGC (located 899 to 920 bp)[12]. 25 µl of the reaction and DNA mixture underwent denaturation for 10 min at 94°C followed by 40 cycles of the following PCR times: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed by a single extension step for 10 min at 72°C. Five microliters of the amplified product (223 bp) underwent restriction enzyme digestion with *Nae* I (0.5 U/µl; 10,000 U/ml; New England BioLabs) following the manufacturer's protocol as described[27]. The presence of the T-786→C polymorphism creates the *Nea* I restriction enzyme site and digests the 223 bp fragment to produce 118 bp and 105 bp fragments. Positive and negative controls were analyzed with each assay to confirm the effectiveness of the endonuclease reaction. Restriction fragments were separated by electrophoresis on agarose gel with ethidium bromide (Figure 1).

27 bp repeat eNOS intron 4 polymorphism analysis. For the eNOS 27 bp repeat in intron 4 polymorphism, DNA containing the repeat fragment was analyzed by PCR as described[14]. The sense primer was 5' AGG CCC TAT GGT AGT GCC TT (located 5111 to 5130 bp), antisense 5' TCT CTT AGT GCT GTG GTC AC (located 5511 to 5530 bp). Twenty-five microliters of the reaction and DNA mixture underwent denaturation for 10 min at 94°C

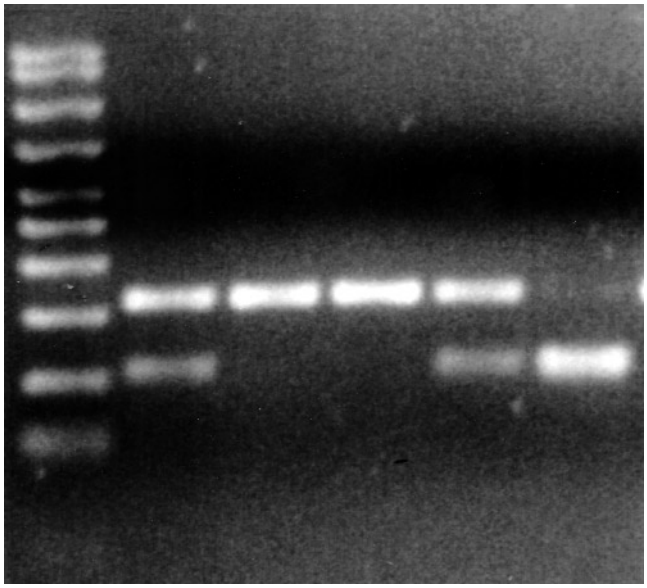

Figure 1. An agarose gel showing bands from patients with the eNOS T-786→C polymorphism. CC homozygotes have the restriction enzyme site and digest to produce 2 fragments, 118 and 105 bp, which overlap to appear as a single band (lane 6). TT homozygotes for polymorphism samples do not digest, and produce a single 223 bp band (lanes 3, 4). CT heterozygotes will digest and produce all bands of 223, 118, and 105 bp (lanes 2, 5). Lane 1: DNA ladder.

followed by 40 cycles of the following PCR times: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed by a single extension step for 10 min at 72°C. The number of 27 bp repeats on each allele was determined from the band length by separating the undigested PCR product using Biorad Criterion 15% TBE Precast Gels (Figure 2).

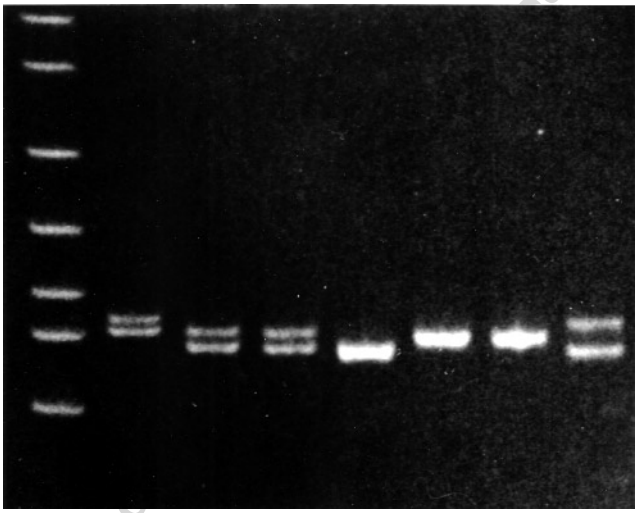

Figure 2. An agarose gel showing polymorphisms for the 27 bp repeat in intron 4 of eNOS. Intron 4 of the eNOS gene contained 4, 5, or 6 tandem 27 bp repeats labeled a, b, and c, respectively. A b/c heterozygote, band at 420 and 447 bp, respectively, is shown in lane 2. Two b/a heterozygotes are shown in lanes 3 and 4. An a/a homozygote (band at 393 bp) is shown in lane 5, whereas b/b homozygotes are shown in lanes 6 and 7. A c/a heterozygote is shown in lane 8. Lane 1: DNA ladder.

ACE intron I/D polymorphism analysis. DNA from 493 CLU Study subjects was analyzed for the presence of the insertion (I) or deletion (D) of the 287 bp long repeat sequence within intron 16 of the ACE gene as described[21]. The sample size differed between this and the eNOS polymorphism analysis because DNA was not available from all subjects for both analyses. The sense primer sequence was 5' CTG GAG ACC ACT CCC ATC CTT T, and antisense sequence was 5' GAT GTG GCC ATC ACA TTC GT. Twenty-five microliters of the reaction and DNA mixture underwent denaturation for 10 min at 94°C followed by 30 cycles of the following PCR times: 1 min at 94°C, 1 min at 58°C, 2 min at 72°C, followed by a single extension step for 10 min at 72°C. The amplified product was separated by electrophoresis on agarose gel. The presence of the I allele produced a 490 bp band, whereas the D allele produced a 190 bp band (Figure 3).

Confirmation of polymorphism by direct sequencing. Amplified, undigested cDNA segments from selected patients for each polymorphism were sequenced using an automated ABI DNA sequencer to confirm the presence of the different polymorphisms and the effectiveness of the PCR procedure.

Statistical analysis. In the CLU cohort case-control comparisons, only unrelated cases and controls were investigated. The frequencies of the eNOS T-786→C, 27 bp intron 4 repeat, and ACE I/D polymorphism genotypes were compared between SLE cases and controls using contingency tables stratified by race and sex. P values were assessed using Fisher's exact test with a p value < 0.05 considered significant. The odds ratio and 95% confidence interval were calculated as a measure of association. Allele frequencies in control and case groups were compared using standard chi-squared tests.

RESULTS

Case-control study. Characteristics of the CLU study groups are summarized in Table 1. All cases (n = 227) met ACR criteria for SLE. Mean (± SD) age in patients with SLE was 39.4 ± 15.0 years. There were 205 women and 22 men; 142 were African American and 85 were Caucasian. Of the SLE

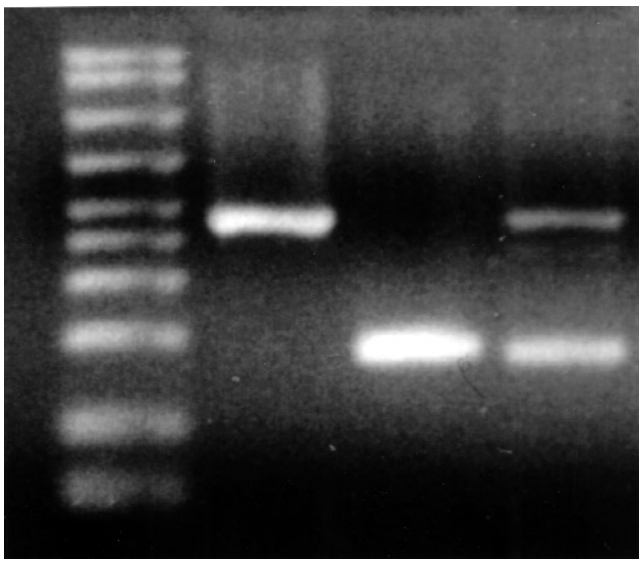

Figure 3. An agarose gel showing bands from patients with the ACE I/D polymorphism. II homozygotes have 2 alleles with the 287 bp insertion and produce a single band at 490 bp length (lane 2). DD homozygotes lack the 287 bp fragment and produce a single band at 190 bp (lane 3). ID heterozygotes will have a band at both 190 and 490 bp (lane 4). Lane 1: DNA ladder.

Table 1. Demographic characteristics of cases and controls in the CLU study.

	Cases, n = 227 n (%)	Controls, n = 275 n (%)
Female	205 (90)	247 (90)
Male	22 (10)	28 (10)
Age, yrs	39.4 ± 15.0	40.3 ± 14.5
Race		
African American	142 (63)	73 (27)
With nephritis	56 (39)	
Caucasian	85 (37)	202 (73)
With nepritis	14 (17)	

patients, 70 had lupus nephritis diagnosed by renal biopsy. Unrelated healthy controls (n = 274) were of similar age, sex, and geographic location.

Due to reports that the eNOS T-786→C polymorphism reduces eNOS activity and is linked to cardiovascular and renal disease, we sought to determine if allelic differences may enhance the risk of developing SLE and/or lupus nephritis[11,12]. After PCR amplification of the target DNA, we used RFLP to cut the 223 bp fragment to produce 118 and 105 bp fragments (Figure 1). When all lupus cases and controls were analyzed, there was a significant association between SLE and the T allele ($p < 0.001$). However, further analysis showed that this association was due to a difference in allele frequency in African Americans as compared to Caucasians ($p < 0.001$; Table 2).

African American cases showed a predominance of the TT genotype (70%), while the Caucasians showed a TC combination as the predominant genotype (47%). In both groups the CC allele combination was the least-observed genotype (3% and 13%, respectively). When the controls and lupus patients were evaluated by ethnicity, there was no significant difference between the SLE cases and controls in either the African American or Caucasian cohorts, indicating that the C nucleotide at eNOS 786 does not contribute to SLE, but is linked to ethnicity.

Next, we examined the 27 bp tandem repeat in the eNOS gene due to its link with renal and cardiovascular disease. Genotype analysis revealed that intron 4 of the eNOS gene contained 4, 5, or 6 tandem 27 bp repeats (labeled a, b, and c, respectively, Figure 2). We calculated the allele frequencies of the 27 bp repeats from African Americans and Caucasians with and without disease (Table 3). The results show that the b/b genotype was the most frequent in both cases and controls among Caucasians (68% and 73%, respectively). The b/a genotype was most commonly observed in the African American cases (45%), whereas the b/b was more frequent in African American controls (47%). The differences in allele or genotype frequency within both Caucasian and African American groups were not statistically significant between the SLE case and control cohorts. These data suggest that the 27 bp tandem repeats in intron 4 of the eNOS gene do not contribute to SLE. However, allele frequencies in the African Americans were significantly different from the Caucasians ($p < 0.001$).

Due to a reported association of the ACE intron 16 I/D polymorphism with renal disease and SLE, we examined this polymorphism in our lupus cohort[21-25]. PCR amplification of the region containing the polymorphism in the ACE gene produced a 490 bp fragment in the presence of the insertion allele and a 190 bp fragment in its absence (Figure 3). When the results were calculated, we found that the I/D allelic combination was the most prevalent, while the I/I combination was the least prevalent in both African Americans and Caucasians (Table 4). There was no significant difference in allelic frequency between the African Americans and Caucasians or within the cases and controls, indicating that the I/D polymorphism of intron 16 of the ACE gene is similar in African Americans and Caucasians.

Table 2. eNOS promoter T-786→C allele frequency in different racial groups. Genotype and allele frequencies for the eNOS T-786→C polymorphisms differed significantly between case and control groups. Among both SLE cases and controls, African Americans were significantly more likely to have the eNOS T allele than Caucasians ($p < 0.001$). Distribution of genotypes among SLE patients compared to ethnically matched controls showed no significant difference.

eNOS T-C	Total		African American, n = 215		Caucasian, n = 287	
	Cases, n = 228	Controls, n = 274	Cases, n = 143	Controls, n = 72	Cases, n = 85	Controls, n = 202
Genotype, n (%)						
TT	134 (59)	120 (44)	100 (70)	52 (72)	34 (40)	68 (34)
TC	79 (34)	120 (44)	39 (27)	19 (27)	40 (47)	101 (50)
CC	15 (7)	34 (12)	4 (3)	1 (1)	11 (13)	33 (16)
Allele Frequency						
T	0.76*	0.66	0.84	0.85	0.59	0.63
C	0.24*	0.34	0.16	0.15	0.41	0.37

* $p < 0.001$.

Table 3. eNOS intron 4 insertion deletion allele frequency in different racial groups. Three alleles for the 27 base pair (bp) tandem repeat in intron 4 of the eNOS gene were identified: 4 repeats (a), 5 repeats (b), or 6 repeats (c). The eNOSb allele was more frequent among SLE cases compared to controls (p = 0.003). However, within ethnic groups, there was no significant difference in genotype or allele frequency. In both SLE cases and controls, African Americans were significantly more likely to have the eNOS 4b allele than Caucasians (p < 0.001).

eNOS Intron 4	Total		African American, n = 211		Caucasian, n = 287	
	Cases, n = 226	Controls, n = 267	Cases, n = 141	Controls, n = 70	Cases, n = 85	Controls, n = 197
Genotype, n (%)						
bb	102 (45)	176 (66)	44 (31)	33 (47)	58 (68)	143 (73)
ba	89 (39)	71 (27)	63 (45)	24 (34)	26 (31)	47 (24)
aa	20 (9)	14 (5)	19 (13)	8 (11)	1 (1)	6 (3)
bc	10 (4)	5 (2)	10 (7)	4 (6)	0	1 (0.5)
ac	4 (2)	1 (0.4)	4 (3)	1 (1)	0	0
cc	1 (0.4)	0	1 (0.7)	0	0	0
Allele Frequency						
b	0.67*	0.80	0.57	0.67	0.84	0.85
a	0.29*	0.19	0.37	0.29	0.16	0.15
c	0.04	0.01	0.06	0.04	0	0.003

* p = 0.003.

Table 4. ACE (I/D) allele frequency in CLU subjects. Genotype and allele frequency for the ACE insertion/deletion polymorphism did not differ significantly between SLE cases and controls or among ethnic groups. No significant association with either the I or D allele was observed among ethnic groups.

ACE I/D	Total		African American, n = 210		Caucasian, n = 286	
	Cases, n = 225	Controls, n = 271	Cases, n = 140	Controls, n = 70	Cases, n = 85	Controls, n = 201
Genotype, n (%)						
II	38 (17)	55 (20)	25 (18)	13 (18)	13 (15)	42 (21)
ID	110 (49)	127 (47)	66 (47)	32 (46)	44 (52)	95 (47)
DD	77 (34)	89 (33)	49 (35)	25 (36)	28 (33)	64 (32)
Allele Frequency						
I	0.41	0.44	0.41	0.41	0.41	0.45
D	0.59	0.56	0.59	0.59	0.59	0.55

Furthermore, there was no correlation with SLE disease expression with any combination of allele frequencies.

Frequency of all polymorphisms followed a Hardy-Weinberg distribution for both SLE and control groups and did not differ between men and women (data not shown). Of the 70 SLE patients with biopsy-proven glomerulonephritis, there was not a significant association with any of the polymorphisms examined (data not shown). Moreover, no linkage disequilibrium was observed between the 2 eNOS and/or ACE I/D polymorphisms.

DISCUSSION

We examined the frequency of 3 different polymorphisms: ACE insertion/deletion in intron 16, eNOS T-786→C, and eNOS insertion/deletion in intron 4 polymorphisms in patients with SLE and matched controls. To our knowledge, this is the first study analyzing eNOS T-786→C and eNOS intron 4 polymorphisms in a population with SLE. Overall, we observed a significant difference in allele frequency between SLE case and control groups for both eNOS T-786→C and eNOS intron 4 polymorphisms. However, no significant difference in allele frequency was seen in case and control groups when controlling for ethnicity. The significant variation in allele frequency in the eNOS polymorphisms between ethnic groups completely accounted for the observed differences between case and control groups. These data emphasize the importance in selecting appropriate control cohorts that are matched for ethnicity.

The allele frequencies for the eNOS T-786→C polymorphism in our analysis were similar to those previously reported[11,12,28]. Individuals with CC homozygous genotypes have a reported greater risk of advanced diabetic nephropathy[12], but we did not observe an increased risk of lupus nephropathy among CC individuals. Instead, individuals with SLE were more likely to have the TT genotype than controls. Although one Japanese study found a positive

association between the C allele and coronary spastic angina[10], another study found no correlation with essential hypertension in 2 independent Japanese populations[28]. Our data failed to show a link between the eNOS T-786→C polymorphism and SLE or lupus nephritis, suggesting that it is unlikely this polymorphism is associated with an increased risk for developing SLE and/or nephritis in African Americans or Caucasians.

We also found no statistically significant association of the eNOS4a/b or a/a genotype with SLE. Although the presence of the a deletion has been associated with various diseases[12,14-18,29], other studies showed no significant link of the eNOS4a deletion with cardiovascular or renal disease[15,30-35]. The effect of the eNOS4a insertion/deletion polymorphism on eNOS activity, expression, and NO production has not been investigated to date. We identified a third allele in our population with 6 tandem repeats (c insertion) for eNOS intron 4 that is rare, but has been described[34]. The significance of this polymorphism is currently unknown. Further studies with a larger patient population are needed to determine if the eNOSc allele may be linked to disease or affects eNOS expression and NO production.

As eNOS produces NO constitutively, it is unlikely that the polymorphisms we studied account for the measurable differences in NO production seen in SLE patients and different ethnic groups[2]. Instead, NO production from iNOS, which produces several-fold more NO than eNOS, is more likely responsible. We reported a significant association of iNOS promoter polymorphisms with SLE in African American women[36].

The presence of the D allele in ACE intron 16 is associated with increased serum ACE concentrations and various diseases, including SLE[21,37]. Although currently unproven, the presence of the 287 bp I allele may downregulate transcription of the ACE gene, thereby decreasing ACE levels. However, we found no significant association of either the ACE I or D allele with SLE, lupus nephritis, or ethnicity. Similarly, other studies failed to show any association of the ACE polymorphism with SLE[38,39], cardiovascular disease[40], antiphospholipid syndrome[41], or diabetic nephropathy[35,42,43].

Allele frequency for the ACE I/D polymorphism may differ considerably between ethnic groups. In a study by Tassiulas, *et al*, the prevalence of the D allele was higher in African Americans than Caucasians (0.73 vs 0.63)[38]. In the same study, SLE patients were more likely to have the I allele than the D allele. However, we found no statistically significant difference in ACE I/D allele frequency among African Americans and Caucasians.

To date, roughly half the studies investigating eNOS and ACE polymorphisms in different diseases have shown significant associations; however, many reports are contradictory. Our study clearly emphasizes the importance of appropriate control cohorts when investigating genetic polymorphisms and disease. The numerous conflicting reports linking genetic polymorphisms and different diseases point to the multigene interactions and environmental factors that likely define a complex disease such as SLE. We used a candidate gene approach in these studies, based on prior reports. Newer technologies utilizing microarray analysis provide a genome-wide look at gene expression, providing new potential links to the pathogenesis of SLE and other complex autoimmune illnesses.

Although we observed no significant correlation between SLE and the eNOS and ACE polymorphisms, a correlation with other disease manifestations may still exist in the CLU population. We are prospectively following the CLU cohort to determine if an association of these polymorphisms exists with hypertension, coronary artery disease, diabetes, lipid concentrations, or renal disease progression and response to therapy in individuals with SLE and controls.

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