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Research Article
Cq1 Exon Polymorphisms in Caucasian and African American Systemic Lupus Erythematosus patients

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Abstract

Background: C1q protein is composed of three protein chains (A, B and C) that are the products of separate genes. Genetic deficiencies in C1Q genes are important factors influencing the risk of systemic lupus erythematosus (SLE). Therefore, this study aimed to investigate the possible association of single nucleotide polymorphisms (SNPs) in the coding region of the C1Q genes with SLE. **Methods:** To search for potential SNPs in the encoding regions of C1q A, B and C chains, Cq1 exons were initially amplified and directly sequenced from leukocyte DNA from a subset of Caucasian and African American SLE patients and healthy controls. The sequences were analyzed by the Phrap and Phred software analysis system and the SNPs were identified by visual inspection. To test if any of these SNPs were linked to susceptibility to SLE, they were measured in 210 SLE patients ((59 African Americans and 151 Caucasians) and 129 matched healthy controls (55 African Americans and 74 Caucasians) by restriction fragment length polymorphism analysis. **Results:** The sequencing phase of the study identified three synonymous SNPs: Nucleotide 276G>A in C1QA, 66C>A in C1QB and 129G>A in C1QC. Statistically, no differences were found in genotype or allele frequencies between patients and controls for the 276G>A or 66C>A SNP. However, in Caucasians, the frequencies of the 129G>A genotypes were significantly different between SLE patients and controls ($P = 0.005$), specifically with the GG genotype being over represented in the controls ($P = 0.004$). **Conclusion:** The results show that the homozygous 129GG genotype is associated with protection against SLE onset. This protection is race dependent, being observed in Caucasians but not African Americans. The mechanism of this association is currently unclear.

Key words: Autoimmune disease, complement protein, genes, Lupus, synonymous SNPs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multi systemic and complex autoimmune disease that is due to the loss of tolerance to self-antigens, resulting in the production of autoantibodies and accumulation of immune complexes in human tissues[1,2]. In North America, the incidence of SLE is estimated to be about 40 per 100,000 people and about 90% of SLE cases occur in women of childbearing age (15-45 years of age). SLE is characterized by periods of disease remissions and flares, by variable clinical manifestations and by acute or chronic inflammation of several organs. These clinical manifestations include chronic, acute and subacute-cutaneous lupus erythematosus, photosensitivity and involvement of various organs such as the lung, central nervous system and kidney (lupus nephritis)[3,4].

Etiologically, SLE is thought to be a complex disease caused by both genetic and environmental factors[5-7]. Multiple chromosomal regions have been identified exhibiting strong linkage to SLE[8-10]. These studies have implicated many candidate genes as contributing susceptibility to SLE, including complement genes. However, the specific nature of these genetic associations remains largely unknown.

The association between complement and SLE has been well documented. Complement deficiencies in the classical pathway, especially C1q, represent one of the strongest risk factors for SLEonset[9-12]. It has been estimated that the frequency of SLE disease is 95% for all patients with C1q deficiency, 75% for all patients with C4 deficiency and 31% for all patients with C2 deficiency[1,8,13-15].

C1q is an essential component of the classical pathway. It is one of three sub components of complement protein 1 (C1) (along with C1s and C1r). C1q is composed of three chains (A, B and C), each encoded by separate genes located at chromosome 1p24.1-36.3[16]. C1q is a multifunctional protein that plays various roles involving both complement and non-complement functions. With regards to complement functions, C1q initiates the classical pathway activation through the binding of its globular heads to Fc domain of IgM or aggregated IgG. This causes a conformational change in C1q that leads to activation of the associated C1r and C1s, which represents the first cleavages of the classical pathway. Functions unrelated to complement activation include the binding of C1q to substrates such as DNA and apoptotic debris, a process that mediates the removal of these substrates. Furthermore, C1q can affect toll-like receptor-mediated cytokine production and immune complex-induced IFN-α production, providing further explanations for the increased SLE risk associated with inherited deficiencies of C1q[17,18].

The human genome exhibits several genetic variations ranging from microscopically visible chromosome abnormalities, variable number of tandem repeats, short tandem repeats, submicroscopic abnormalities and single nucleotide polymorphisms (SNPs)[19,20]. SNP analysis is increasingly used in the search for genetic markers to predict the risk of susceptibility to certain diseases and individual response to certain drugs and is important in studying diversity in the population and individuality. Previous studies have reported that SNPs are important in tracking the inheritance of disease genes within families such as type 2 diabetes, sickle cell anemia, cancers, autoimmune diseases and other diseases[21,22].

A SNP is one of the most common types of genetic variation that is usually present in the genome of human being. SNPs usually occur once in very 500-1000 nucleotides. The presences of SNPs often do not affect the protein sequence (synonymous SNPs) and yet can play a role in altering the level of expression of a gene[23,24]. Some genetic association studies revealed that single synonymous SNP in the C1Q gene regions appear to be associated with SLE susceptibility[25-30]. Multiple clustered C1Q SNPs have also been shown to be significantly associated with SLE, especially in certain subset and haplotype analyses with sub-phenotypes of SLE such as subacute-cutaneous lupus, lupus nephritis and photosensitivity[30-33]. Accordingly, this study was undertaken to search for the presence of SNPs in C1Q A-, B-, C-gene coding regions and to explore if there were any associations between these SNPs and adult-onset of SLE in Caucasian and African American people.

MATERIALS AND METHODS

Study patients: Two hundred ten patients with SLE (151 Caucasian and 59 African American) were recruited for this study as part of the Ohio SLE Study (OSS), the focus of which was to identify genetic and clinical risk factors for SLE and lupus nephritis onset and progression. One hundred and twenty nine healthy controls (74 Caucasian and 55 African American) were recruited from central Ohio.

This study was approved by the human subjects Internal Review Board of the Ohio State University hospital in Columbus, Ohio, USA. All patients recruited at this study fulfilled at least 4 of the diagnostic criteria for SLE as proposed

Table 1: Primer sequences for the identification of three single nucleotide polymorphisms of C1Q genes

Genes	Primer names	Primer sequence 5′→3′	Annealing temperature (°C)	Size of PCR product (bp)
C1QA	AF1	GGAGGGGGTCCTTCCCTGGC	65	1926
	AR1	GTGGGAGCTGGGTGTGAGTG		
	AF2	GGCCCTCTGGAAACCCCGGCAA	65	315
		GGTGGGCTACCCAGCGCCCAGC		
	AR2	GGAGGAGACGATGGACAGGC		
C1QB	BF1	AACCTCTCACATTGTCTTCT	63	2011
	BR2	ATTCACCAAGCCCTACTGTG		
	BF2	AACCTCTCACATTGTCTTCT	65	390
	BR2	ATTCACCAAGCCCTACTGTG		
C1QC	CF1	GGGGAAGCAGATCTGAGGACATCT	68	500
	CR1	CCCCAAACCAGGCCAGCAGACTC		
	CF2	GGGGAAGCAGATCTGAGGACATCT	68	655
	CR2	CCCCAAACCAGGCCAGCAGACTC		
	CF3	TGCTGCTCCTGCTGCTGCTG	68	364
	CR3	CTGTTGGGTGCAGGGGGCTG		

by American College of Rheumatology 1982 classification and the updated criteria for SLE[3,34]. Informed consent was signed and obtained from all patients and controls.

DNA isolation, amplification and sequencing: Blood was collected in EDTA and genomic DNA was isolated from the buffy coat using the Puregene system DNA isolation protocol (Gentra Systems, Minneapolis, MN, USA). In a subset of individuals (10 SLE and 10 control, 5 Caucasian and 5 African Americans for each), each C1Q gene was amplified by the polymerase chain reaction (PCR) and sequenced using primers selected from published sequences[16]. The forward and reverse primers for amplification of C1QA-, B-, C-genes, annealing temperature and size of PCR products are listed in Table 1.

The PCRs were performed according to the manufacturer's instructions with some modifications (Invitrogen, Carlsbad, CA). Briefly, a total of 50 μL of reaction mixture was used per reaction. Each reaction mixture contained 5 μL of standard Taq reaction buffer (25 mM of $MgCl_2$), 22.5 μL of nuclease free H_2O, 5 μL of each primer (0.5 μM final concentration, Integrated DNA Technologies, Inc., Coravilli, Iowa), 2 μL of genomic DNA and 0.5 μL of Taq DNA polymerase (5 unit μL^{-1}). The PCR was conducted under the following conditions: After bringing the reaction mixtures to 80°C for 2 min, 10 μL of dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP) was added to each PCR mixture. Then, the reactions were cycled 35 times, with each cycle consisting of a 1 min denaturing at 95°C, a 30 sec annealing at 65°C (or as indicated in Table 1) and extension at 72°C for 3 min. The PCR reaction was ended with one cycle as a final extension at 72°C for 10 min. A negative control (lacked DNA) was run with each batch PCR reactions. To check the specificity of the PCR product for each sample, 1% a garose gel electrophoresis containing ethidium bromide was performed. A 100 bp DNA ladder (1 μg mL^{-1}) was included in each agarose gel.

The resulting PCR products of C1qA-, B-, C-chains of this subset were sequenced by ABI prism 3100 genetic analyzer using dRhodamine dye terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase, according to procedure described by Applied Biosystems Incorporation (850 Lincoln Centre Drive, Foster City, CA 94404. US). In order to confirm any novel polymorphism and to remove any potential errors in sequencing data, each PCR product was sequenced in both directions. The sequences were analyzed by the Phrap and Phred software analysis system and the SNPs were identified by visual inspection.

Restriction fragment length Polymorphism (RFLP): To interrogate the entire SLE and normal cohorts for the three SNPs identified from sequence analysis, RFLP analysis was performed using genomic DNA isolated from the buffy coat as described previously[5]. Table 1 shows the PCR primers used for amplification of each PCR product containing the SNP of interest and the expected size of each PCR product before digestion with restriction endonuclease.

Statistical analysis: Statistical analysis were performed with SPSS computer program for windows, version 13.0 (SPSS, Chicago, IL). Chi-square test was used to compare genotype frequencies and two-sided Fisher's exact test was used to compare carrier and allele frequencies, between the SLE cohort and control cohort. The p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

This study aimed to determine whether SNPs existed in the C1Q gene cluster that were associated with risk for SLE onset. The approach for this study was to completely sequence the encoding regions for the three C1Q genes in a representative sample of SLE patients and normals and then to interrogate a larger cohort of patients and normals for any identified SNP.

The sequencing phase of the study identified three synonymous SNPs, one in each gene. The SNP in the gene for the A chain was found at nucleotide 276 (numbering based on starting site of translation) in the second exon of this gene, where guanine (G) was replaced by adenine (A) (or cytosine (C) was replaced by thymine (T)) (Fig. 1a). This SNP was designated as C1QA276G>A. The 276G allele involved an Apa1 restriction enzyme site and a RFLP assay was developed based on the presence or absence of an ApaI cleavage site (Fig. 2a). In this assay, amplification using the primer pair AF2 and AR2 (Table 1) gave a 315 bp PCR product and digestion with ApaI yielded fragments of 260 and 55 bp for the product containing the 276G allele. Using this assay, the entire SLE and control cohorts were interrogated. The results of this analysis are shown in Table 2. While there were significant differences in both genotypes and allele frequencies between Caucasian and African Americans in both the

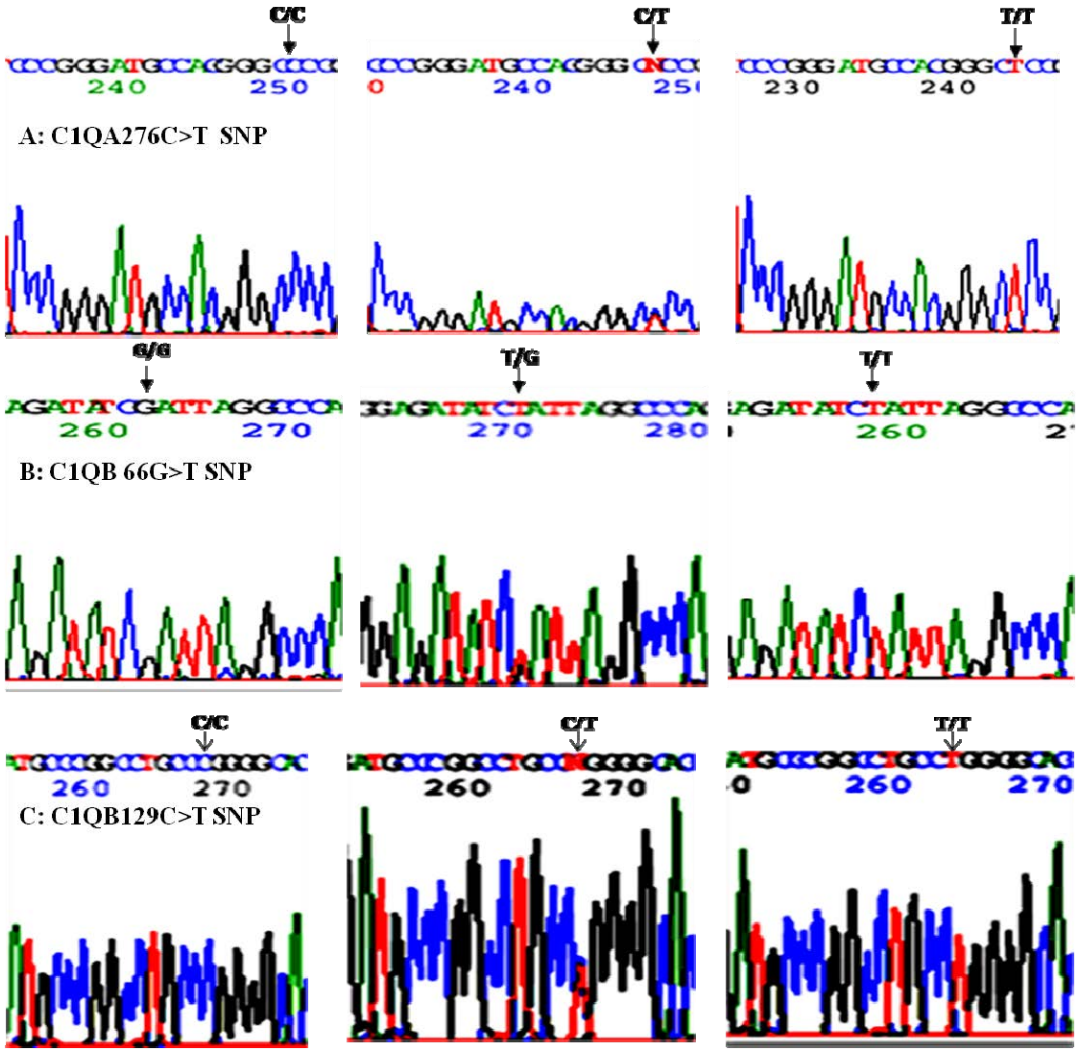

Fig. 1(a-c): Direct sequencing chromatography traces of PCR products showing the polymorphic sites of the single nucleotide polymorphisms in C1Qgenes. Representation of three automated homozygous and heterozygous chromatograms showed the sequence immediately surrounding the region of the three polymorphism sites: (A) C1QA276C>T SNP, (B) C1QB66 G>T SNP and (C) C1QC129C>T SNP

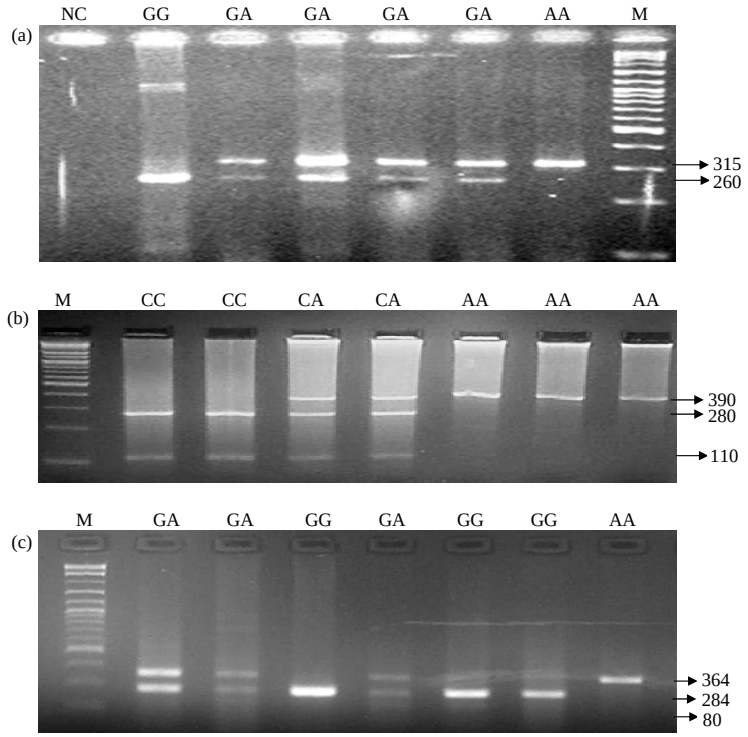

Fig. 2(a-c): Agarose gel electrophore grams of PCR-RFLP analysis of C1Q exon polymorphisms in Caucasian and African American SLE patients and controls were as follows: (A) Image of PCR products of C1QA 276G>A SNP after digestion by restriction endonuclease ApaI, 276G variant was cut, whereas the 276A variant was uncut, (B) Image of PCR products of C1QB 66 C>A SNP after digestion by restriction endonuclease Taq$^{\alpha}$1. The 66C variant was cut, whereas the 66A variant was uncut and (C) Image of PCR products of C1QC 129G>A SNP after digestion by restriction endonuclease Sma1. The 129G variant was cut, whereas the 129A variant was uncut. Lane M is 100 bp molecular weight marker. The size of PCR products were indicated. Lane NC is a negative control

control and the SLE cohorts, there were no significant differences between control and SLE cohorts.

The SNP in the gene for the B chain was found at nucleotide 66 (numbering based on starting site of translation) in the signal peptide-encoding region of this gene, where C was replaced by A (or G was replaced by T) (Fig. 1b). This SNP was designated as C1QB66C>A. The 66A allele involved a Taq$^{\alpha}$1 restriction enzyme site and a RFLP assay was developed based on the presence or absence of this cleavage site (Fig. 2b). In this assay, amplification using the primer pair BF2 and BR2 (Table 1) gave a 390 bp PCR product and digestion with Taq$^{\alpha}$1 yielded fragments of 280 and 110 bp for the product containing the 66A allele. Table 2 shows the results of interrogating the entire SLE and control cohorts using this RFLP. In contrast to the C1QA276G>A, the A allele of C1QB66C>A SNP is much less common, appearing in 1% or less of Caucasian cohorts and 5% or less in the African American cohorts. The 66A allele appears to be slightly more frequent in African Americans compared to Caucasians ($P = 0.076$ for Controls, $P = 0.017$ for SLE patients). Moreover, statistical data analysis of C1QB66C>A SNP revealed that there were no significant differences between control and SLE cohorts.

The SNP in the gene for the C chain was found at nucleotide 129 in the first exon of this gene, where G was replaced by A (or C was replaced by T) (Fig. 1c). This SNP was designated as C1QC129G>A. The 129G allele involves a Small restriction enzyme site. Amplification across this region, using the primer pair CF3 and CR3 (Table 1), gave a 364 bp product and digestion with Small yielded fragments of 284 bp and 80 for the 129G variant (Fig. 2c). Interrogation of the entire SLE and control cohorts revealed that this relatively common SNP was significantly different in genotypic frequency between normal Caucasians and normal African Americans ($p<0.014$) with the GG genotype appearing more frequently in Caucasians (Table 2). No statistically significant difference was seen between Caucasian and African Americans in the SLE cohort. Interestingly, a significant difference was found in

Table 2: Genotype distributions and allele frequencies of three single nucleotide polymorphisms in C1Q exons in Caucasian and African American with SLE and controls

	Normal healthy			SLE patients		
C1Q polymorphism	Ca (N = 74)	AFA (N = 55)	Total (129)	Ca (N = 151)	AFA (N = 59)	Total (210)
C1QA276G>A genotypes						
GG	12 (16.2)[a]	25 (45.5)[a]	37 (28.7)	25 (16.6)[a]	30 (50.8)[a]	55 (26.2)
GA	36 (48.7)	25 (45.5)	61 (47.3)	76 (50.3)	23 (39.0)	99 (47.1)
AA	26 (35.1)[a]	5 (9.0)[a]	31 (24.0)	50 (33.1)[a]	6 (10.1)[a]	56 (26.7)
C1QA alleles						
G	0.41[a]	0.68[a]	0.52	0.42[a]	0.71[a]	0.50
A	0.59[a]	0.32[a]	0.48	0.58[a]	0.29[a]	0.50
C1QB66C>A genotypes						
AA	0 (0.0)	0 (0.0)	0 (0.0)	1(0.7)	2 (3.4)	3 (1.4)
CA	0 (0.0)	3 (5.5)	3 (2.3)	1 (0.7)	2 (3.4)	3 (1.4)
CC	74 (100.0)	52 (94.5)	126 (97.7)	149 (98.6)	55 (93.2)	204 (97.1)
C1QB alleles						
A	0.0	0.03	0.01	0.01	0.05	0.02
C	1.0	0.97	0.99	0.99	0.95	0.98
C1QC129G>A genotypes						
GG	41 (55.4)[b,c]	18 (32.7)[b]	59 (45.7)	52 (34.4)[c]	27 (45.7)	79 (37.6)
GA	23 (31.1)[b,c]	31 (56.4)[b]	54 (41.9)	80 (53.0)[c]	24 (40.7)	104 (49.5)
AA	10 (13.5)	6 (10.9)	16 (12.4)	19 (12.6)	8 (13.6)	27 (12.9)
C1QA alleles						
G	0.71	0.61	0.67	0.61	0.66	0.62
A	0.29	0.41	0.33	0.39	0.34	0.38

Values for the alleles are the frequency, all other values are the number and (%). SLE: Systemic lupus erythematosus, Ca: Caucasian and AFA: African American. [a]p<0.001 for genotype and allele frequencies between Caucasian and African Americans. [b]p<0.02 for genotype frequency between Caucasian normals and African American normals. [c]p<0.005 for genotype frequency between Caucasian normals and Caucasian SLE patients

genotypic frequency between Caucasian normals and Caucasian SLE patients (P = 0.005), which again was driven by a higher frequency of the GG genotype in the normals.

Studies have reported several SNPs across the genomic area of the C1Q genes[25-36]. For instance, Namjou *et al.*[27] genotyped 17 SNPs across 37 kb of C1Q gene areas in African American and Hispanic SLE patients. Similarly, Trouw *et al.*[35] studied 13 SNPs across 54 kb of the C1Q gene areas in a Dutch cohort. Neither study found any association between a SNP and SLE onset. However, some of these SNPs were found to be associated with some SLE manifestations, including reduce levels of C1q in subacute-cutaneous, photosensitivity, low C3 and CH50 and lupus nephritis[25-30,35,36]. Of the three SNPs identified in the present study, only the C1QA276G>A SNP (also known as rs172378) has been previously reported[25,27,36]. Interestingly, this SNP has been found to be associated with decreased levels of C1q in patients with subacute-cutaneous lupus. We were unable to test this association due to a lack of these clinical data.

The finding in the present study of an association between the 129GG genotype and protection against SLE onset raises the question as to the mechanism of this protection. Being a synonymous SNP, this association may be due to a difference in C1q levels through as effects on transcription rates or stability or possibly translation. C1q levels in these patients were not determined and thus such a mechanism was not explored in this study. Another mechanism may be through linkage with another C1q SNP affecting C1q function, though this does not appear to be the case based on the analysis of the complete encoding sequence of C1q that was done in the subset cohort in whom the 129G>A was identified. Regardless of the mechanism, it is interesting that this protection appears to require the homozygous 129GG genotype.

It is worth noting that the sample size of this study is relatively small and the findings reported herein require replication in another preferably larger cohort. Also of worth noting is that the protective effect of C1QC129GG genotype is race dependent, being observed in Caucasians but not African Americans. Other racial and ethnic populations require study to determine if the C1QC129G>A or the other two SNPs identified in this study are associated with SLE susceptibility.

CONCLUSION

Three silent SNPs were detected, one each in the coding regions of C1QA, B and C genes in the SLE patients and control group of Caucasians and African Americans. These three SNPs, designated as C1QA276G>A, C1QB66C>A and C1QC129G>A are synonymous, having no effect on the protein sequence of C1q A-, B- and C-polypeptides.

Statistically, no significant differences in the distribution of the genotype or allele frequencies between patients and controls were observed for the C1qA chain or C1qB chain SNP. However, the C1qC chain SNP, specifically the 129GG genotype, was associated with protection against SLE but only in the Caucasian cohort. Future studies are needed to confirm this association in separate normal and SLE Caucasian cohorts, to determine if such an association exists with other racial/ethnic cohorts and to explore the mechanism of this association.

SIGNIFICANCE STATEMENT

This study discovered the existence of the C1QC129G>A SNP as a C1QC gene polymorphism that might play a protection role against SLE disease but only in Caucasians. This finding can be beneficial by providing new insight into the influence of C1q in SLE pathogenesis. As such, this study could offer a new avenue for researchers to explore with regards to the mechanism by which this C1QC gene variant contributes protection against SLE. Thus, a new theory on the role of complement in SLE susceptibility may be revealed.

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