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Gene Copy-Number Variation and Associated Polymorphisms of Complement Component C4 in Human Systemic Lupus Erythematosus (SLE): Low Copy Number Is a Risk Factor for and High Copy Number Is a Protective Factor against SLE Susceptibility in European Americans

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Interindividual gene copy-number variation (CNV) of complement component *C4* and its associated polymorphisms in gene size (long and short) and protein isotypes (C4A and C4B) probably lead to different susceptibilities to autoimmune disease. We investigated the *C4* gene CNV in 1,241 European Americans, including patients with systemic lupus erythematosus (SLE), their first-degree relatives, and unrelated healthy subjects, by definitive genotyping and phenotyping techniques. The gene copy number (GCN) varied from 2 to 6 for total *C4*, from 0 to 5 for *C4A,* and from 0 to 4 for *C4B*. Four copies of total *C4,* two copies of *C4A,* and two copies of *C4B* were the most common GCN counts, but each constituted only between one-half and three-quarters of the study populations. Long *C4* genes were strongly correlated with *C4A* ($R = 0.695$; $P < .0001$). Short *C4* genes were correlated with *C4B* ($R = 0.437$; $P < .0001$). In comparison with healthy subjects, patients with SLE clearly had the GCN of total *C4* and *C4A* shifting to the lower side. The risk of SLE disease susceptibility significantly increased among subjects with only two copies of total *C4* (patients 9.3%; unrelated controls 1.5%; odds ratio [OR] = 6.514; $P = .00002$) but decreased in those with $\geqslant 5$ copies of *C4* (patients 5.79%; controls 12%; OR = 0.466; $P = .016$). Both zero copies (OR = 5.267; $P = .001$) and one copy (OR = 1.613; $P = .022$) of *C4A* were risk factors for SLE, whereas $\geqslant 3$ copies of *C4A* appeared to be protective (OR = 0.574; $P = .012$). Family-based association tests suggested that a specific haplotype with a single short *C4B* in tight linkage disequilibrium with the −308A allele of *TNFA* was more likely to be transmitted to patients with SLE. This work demonstrates how gene CNV and its related polymorphisms are associated with the susceptibility to a human complex disease.

Systemic lupus erythematosus (SLE [MIM 152700]) is a complex, prototypic autoimmune disease that predominantly affects women of child-bearing age. The hallmark of SLE is the generation of autoantibodies that react with self nuclear and cytoplasmic antigens, culminating in immunologic attacks to body organs.[1–3] Although under intense investigations, the genetic basis of human SLE is still not well understood.[4–6]

Complement component C4 (with isotopes C4A [MIM 120810] and C4B [MIM 120820]) is an effector protein of the immune system. It plays a pivotal role in the activation of the classical and the lectin complement pathways that lead to cytolysis or neutralization of invading microbes, opsonization of targets for phagocytosis, clearance of immune complexes, disposal of apoptotic materials, and reduction of the threshold for activation of B lymphocytes.[7–11] The link between the total deficiency of complement component C4 and human SLE was first observed in 1974.[12] It is known that complete or homozygous deficiency in any of the early components for the classical activation pathway of the complement system, such as C1q (MIM 120550, 120570, and 120575), C1r (MIM 216950), C1s (MIM 120580), and C4, are among the strongest genetic risk factors associated with human SLE. More than 75% of human subjects completely deficient in C1 or C4 proteins have SLE or lupus-like disease.[13,14] Animal studies confirmed that homozygous deficiencies of C1q or C4 in mice can be causative genetic factors for

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SLE.[15–18] However, the prevalence of complete deficiency of C4 or subunit proteins of the C1 complex in humans is extremely rare: only 84 cases have been reported so far.[11,14,19,20] Much more common is the low plasma or serum protein levels of these complement proteins, particularly C4 or one of its isotypes, C4A.[21,22] These abnormalities are present in 30%–40% of patients with SLE.[20,23] Curiously, in many healthy subjects, the low range of C4 plasma protein concentrations overlaps that of patients with SLE. The complex genetics of human *C4* has precluded a simple interpretation of the mechanism for low C4 protein levels in health and SLE.

The polymorphic C4 proteins are categorized into two isotypes, C4A and C4B, each with multiple allotypes.[10,24,25] The C4A and C4B proteins exhibit marked differences in chemical reactivities to substrates, although they share >99% amino acid sequence identity. The isotype-specific residues are encoded by exon 26 and are located at amino acid residues 1101–1106, which are PCPVLD for C4A and LSPVIH for C4B (differences underlined).[26–28] The thioester bond present in the activated C4A has a longer half-life against hydrolysis (~10 s) than that of C4B (<1 s). The thioester carbonyl group of C4A effectively forms a covalent amide bond with amino group–containing substrates, such as protein antigens. The activated C4B is highly reactive because one of its isotypic residues, His 1106, catalyzes a nucleophilic attack of the thioester carbonyl group on hydroxyl group–containing substrates to form a covalent ester linkage.[29–32] Therefore, it is thought that C4A proteins are more important in immunoclearance and that C4B proteins are more relevant in the defense against microbes.

The impetus for the present study is that our earlier work in healthy European Americans revealed an interindividual variation in the *C4* gene copy number (GCN) and dichotomies of *C4* gene size and C4 protein isotypes.[33–38] In the central region of the human major histocompatibility complex (MHC) on each chromosome 6 (fig. 1), there can be 1, 2, 3, or 4 copies of *C4* genes. Theoretically, two to eight copies of *C4* genes can be present in a diploid genome of a given individual. The *C4* gene size has two forms, long and short. The long gene is 21 kb in length, and the short gene is 14.6 kb. The long gene is due to the integration of the endogenous retrovirus HERV-K(C4) into its ninth intron.[10,37,42] Our earlier analysis revealed that, among subjects with equal copy numbers of *C4* genes, subjects with short genes have C4 plasma protein levels relatively higher than those of subjects with long genes.[43] Irrespective of gene size, each *C4* gene can code for either an acidic C4A protein or a basic C4B protein.

The duplication or multiplication of *C4* genes is discretely modular. The breakpoint(s) of duplication are identical among duplicated modules.[33,34] Each duplicated module also includes three genes neighboring *C4*. In MHC haplotypes with a single *C4* gene, there are single and intact genes coding for the nuclear protein kinase RP1 (*RP1,* also known as "*STK19*" [MIM 604977]) at the 5′ region, the steroid cytochrome P450 21-hydroxylase (*CYP21B,* also known as "*CYP21A1*" [MIM 201910]), and the extracellular matrix protein tenascin-X (*TNXB* [MIM 600985]) at the 3′ region of *C4*. In MHC haplotypes with two or more *C4* genes, concurrently present with each duplicated *C4* gene are a complete sequence for *CYP21* and partial sequences for *TNX* (i.e., *TNXA*) and *RP* (i.e., *RP2*). The duplicated *C4* genes are usually functional, and they can code for either a C4A protein or a C4B protein. The additional *CYP21* gene can be a nonfunctional *CYP21A* (also known as *CYP21A1P*) with multiple sequence alterations, including three deleterious nonsense mutations, or a functional *CYP21B* gene (see GenBank for entries of sequences of specific genes).[33,34,44–46] This phenomenon is referred to as the "RCCX modular duplication."[34,35]

To investigate the complex genetic diversity of complement *C4,* we genotyped the RCCX modules and determined the gene copy-number variation (CNV) of total *C4, C4A, C4B,* and the long and short *C4* genes in patients with SLE, their first-degree relatives, and unrelated healthy controls. The genotype data were independently confirmed by phenotyping experiments that elucidated the C4A and C4B protein polymorphisms in EDTA plasma (i.e., plasma collected in EDTA). Our primary goal was to determine whether total *C4* (i.e., *C4A* plus *C4B*), *C4A,* and/or *C4B* CNVs are genetic risk factors for SLE. The results provide concrete data of gene CNV for an important immune-defense protein in health and disease, suggesting that low copy number is a risk factor for and high copy number is a protective factor against susceptibility to human SLE.

Material and Methods

Study Populations

The protocols for human subject recruitment and study were approved by institutional review boards of the Ohio State University, the Columbus Children's Hospital, and the University of California–Los Angeles. To avoid confounding factors affecting *C4* genetics among different racial and/or ethnic groups, the current report focuses primarily on patients with SLE who are Americans of European ancestry ($n = 233$), their first-degree relatives ($n = 362$), and unrelated healthy European Americans ($n = 517$) residing in central Ohio. The patients all satisfied four or more diagnostic criteria for SLE from the American College of Rheumatology.[47] The mean age ($\pm$SD) of the female patients with SLE ($n = 216$), female first-degree relatives ($n = 221$), male patients with SLE ($n = 17$), and male first-degree relatives ($n = 142$) was 41.2 ± 14.9 years, 57.7 ± 14.9 years, 38.2 ± 15.4 years, and 58.0 ± 15.6 years, respectively. The mean age of unrelated, healthy, European American female ($n = 389$) and male ($n = 128$) controls was 38.6 ± 11.1 years and 34.3 ± 12.1 years, respectively (table 1).

An independent replication study was performed using 128 patients with SLE: 99 European American patients (89 females with mean age 37.4 ± 11.6 years; 10 males with mean age 40.9 ± 13.0 years) from Los Angeles and 29 female patients with white European ancestry (mean age 46.6 ± 13.0 years) from the

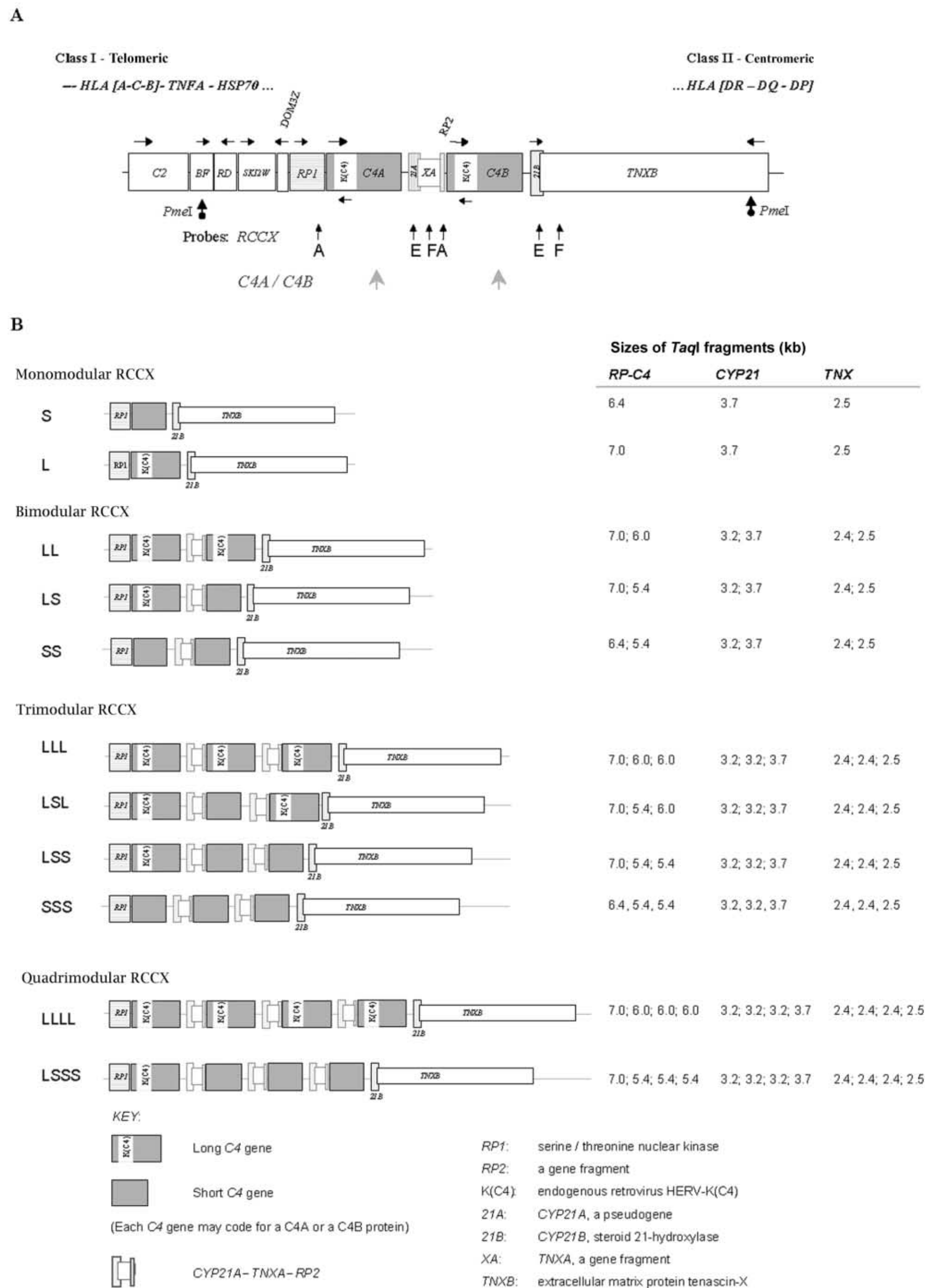


Figure 1. CNVs and size variations of complement *C4* genes and RCCX modules in the human MHC. *A,* Map showing the gene organization of the MHC-complement gene cluster with a bimodular LL haplotype for RCCX. Horizontal arrows represent gene transcriptional orientations. The *Pme*I restriction sites flanking the RCCX modules in *BF* and *TNXB* are shown as dotted arrows. Vertical arrows with letters depict the locations and names of DNA probes employed for genomic Southern-blot analyses. *B,* Structures and configurations of RCCX length variants observed in the white study populations. The fragment sizes of *Taq*I restriction fragments for *RP-C4, CYP21,* and *TNX* are shown on the right.[39–41]

Antiphospholipid Syndrome Registry Collaborative Registry (APSCORE). The APSCORE patients had SLE and antiphospholipid antibodies but did not receive a diagnosis of definite or expanded antiphospholipid syndrome. The clinical characteristics of the patients with SLE who are from Ohio and California are summarized in table 2.

Processing of Blood Samples

EDTA plasma was harvested by centrifugation and was stored at −86°C. Peripheral blood leukocytes were prepared via Ficoll gradient centrifugation for preparation of DNA plugs in low-gelling-temperature agarose and were used for long-range mapping ex-

Table 1. Demographic Data of Ohio SLE Cohort

Group	No. of Subjects	Age[a] (years)	BMI[a]
Female patients with SLE	216	41.2 ± 14.9	29.0 ± 7.4
First-degree relatives:			
Female	221	57.7 ± 14.9	29.6 ± 8.4
Male	142	58.0 ± 15.6	29.2 ± 5.7
Male patients with SLE	17	38.2 ± 15.4	29.1 ± 6.6

NOTE.—Cohort also included 517 unrelated, healthy European American controls (389 females and 128 males).

[a] Data are mean ± SD.

periments involving pulsed-field gel electrophoresis (PFGE). Genomic DNA samples for RFLP analyses were prepared from whole blood by use of Puregene kits (Gentra Systems).[39]

Determination of GCN and Gene Size of C4A, C4B, *and RCCX Modular Variations*

Elsewhere, we have described detailed procedures for genotyping and phenotyping of complement C4.[40,41] *Taq*I genomic RFLP was performed employing specific probes A, E, and F (fig. 1*A*), corresponding to the genomic regions containing *RP, C4, CYP21,* and *TNX*. Results of the *Taq*I Southern blots yielded information on the GCN and gene size of *C4* and the copy numbers of three other constituent genes in the *RP-C4-CYP21-TNX* (RCCX) modules in the central region of the MHC on chromosome 6p21.3. The relative GCN of *C4A* and *C4B* were determined by *Psh*AI-*Pvu*II genomic RFLP that distinguished the C4A and C4B isotypic sites.

Determination of RCCX Haplotype and Number and Size of C4 *Genes on Each Chromosome 6 by PFGE of* Pme*I-Digested DNA*

To confirm further the presence of quadrimodular, trimodular, bimodular, and monomodular RCCX structures present in each individual, human genomic DNA trapped in agarose plugs (to avoid shearing and mechanical breakage) was digested with the rare-cutter restriction enzyme *Pme*I, was subjected to PFGE, and then underwent Southern-blot analysis. The RCCX modular structures were represented as *Pme*I restriction fragments of the following sizes: monomodular short (S), 107 kb; monomodular long (L), 113 kb; bimodular short-short (SS), 133 kb; bimodular long-short (LS), 139 kb; bimodular long-long (LL), 146 kb; trimodular long-short-short (LSS), 165 kb; trimodular long-short-long (LSL) or long-long-short (LLS), 172 kb; trimodular long-long-long (LLL), 179 kb; quadrimodular long-short-short-short (LSSS), 192 kb; and quadrimodular long-long-long-long (LLLL), 211 kb.[41]

Determination of C4A and C4B Allotypes and Plasma Protein Concentrations

EDTA plasma samples were digested with neuraminidase and carboxyl peptidase B, followed by (nonreducing) high-voltage agarose-gel electrophoresis.[48,49] Complement C4A and C4B allotypes were revealed by immunofixation by use of goat anti-human C4 antibody and staining with Simple Blue dye (Invitrogen). Protein band intensities for C4A and C4B allotypes were quantified by ImageQuant software (Amersham). Plasma protein levels of total C4 were determined by single radial immunodiffusion (The Binding Site). C4A and C4B protein concentrations were calculated from their relative quantities revealed by immunofixation experiments.

Statistical Analyses

χ^2 analyses were performed to determine the differences in total *C4, C4A,* and *C4B* gene CNV among various groups. One-way analysis of variance (ANOVA) was used to compare the gene-copy indices of total *C4, C4A, C4B,* and long and short *C4* across three or more groups; analysis was adjusted for dependence based on data for families with SLE, and Tukey "Honestly Significantly Different" test, performed at the 0.05 significance level, was used to distinguish different clusters of groups. Two-group comparisons for these data were based on post hoc two-sample Student's *t* tests. Odds ratios (ORs) and 95% CIs were calculated by analysis of 2 × 2 tables, and Fisher's exact test was used for comparison. Statistical analyses were performed on SAS JMP version 6 (SAS Institute) and SPSS version 11.5 (SPSS) software. Transmission/disequilibrium tests of complement *C4A, C4B,* and RCCX haplotypes in families with SLE were performed using the computer program FBAT.[50]

Results

Genetic Mechanisms Leading to Lower Expression of C4A Protein Than of C4B Protein in Families with SLE

The prospective SLE study population consisted entirely of European Americans residing in Ohio. There were 233 patients with SLE, 362 first-degree relatives (parents and siblings) of the patients with SLE, and 517 unrelated healthy controls. The RCCX modular structures and the copy number of long and short *C4* genes were elucidated by Southern-blot analyses of *Pme*I-digested genomic DNA resolved by PFGE and by *Taq*I genomic RFLP (fig. 1*A*). The relative GCN of *C4A* and *C4B* were determined by ge-

Table 2. Clinical Features of Patients with SLE Related to American College of Rheumatology Criteria for Diagnosis of SLE

Diagnostic Criterion	Frequency of Criterion (%)	
	Ohio SLE Study (n = 233)[a]	California SLE Study (n = 99)[b]
Malar rash	50.9	59.6
Discoid rash	7.63	14.3
Photosensitivity	32.2	81.4
Oral ulcer	31.4	42.2
Arthritis	83.1	94.9
Serositis	31.4	55.2
Renal disorder	36.4	31.6
Neurologic disorder	12.7	12.4
Hematologic disorder:	37.3	53.8
Hemolytic anemia	8.05	10.4
Leukopenia	13.6	38.2
Lymphopenia	9.75	30.3
Thrombocytopenia	14.0	18.1
Immunologic disorder:	43.2	74.0
Anti-DNA	43.6	61.1
Anti-Sm	6.78	9.72
Antinuclear antibody	91.1	100

[a] Included 17 males and 216 females (males:females 1:12.7).

[b] Included 10 males and 89 females (males:females 1:8.9).

nomic *Pvu*II-*Psh*AI RFLP that distinguished *C4A* and *C4B* isotypic sequences. The C4A and C4B protein polymorphisms were determined by immunofixation of EDTA plasma resolved by high-voltage agarose-gel electrophoresis.

In figure 2 and table 3, we present four examples by which the patient with SLE had a C4A plasma protein level lower than that of C4B, a general phenomenon observed in many European American patients with SLE. The results reveal that, if a subject is heterozygous and one of the RCCX haplotypes is bimodular (containing one *C4A* gene and one *C4B* gene), four different scenarios exist for the other haplotype that can lead to a lower effective GCN of *C4A* than of *C4B*: (*a*) monomodular S RCCX with a single, short *C4B* gene (families 4 and 183), (*b*) monomodular L RCCX with a single, long *C4B* gene (family 20), (*c*) bimodular RCCX haplotype that contains a mutant *C4A* and a functional *C4B* (family 183), and (*d*) multimodular RCCX haplotype with higher GCN for *C4B* than for *C4A* (family 109). Detailed results on the CNVs of total *C4, C4A, C4B,* long *C4,* short *C4,* and RCCX modules in all study subjects are summarized in table 4.

C4 *GCN in Female European American Patients with SLE, Female First-Degree Relatives, and Unrelated Healthy Female Controls*

Because our patients with SLE are predominantly female (females:males 12.7:1), our primary analyses were focused on female subjects.[51–53] We first examined the CNV for

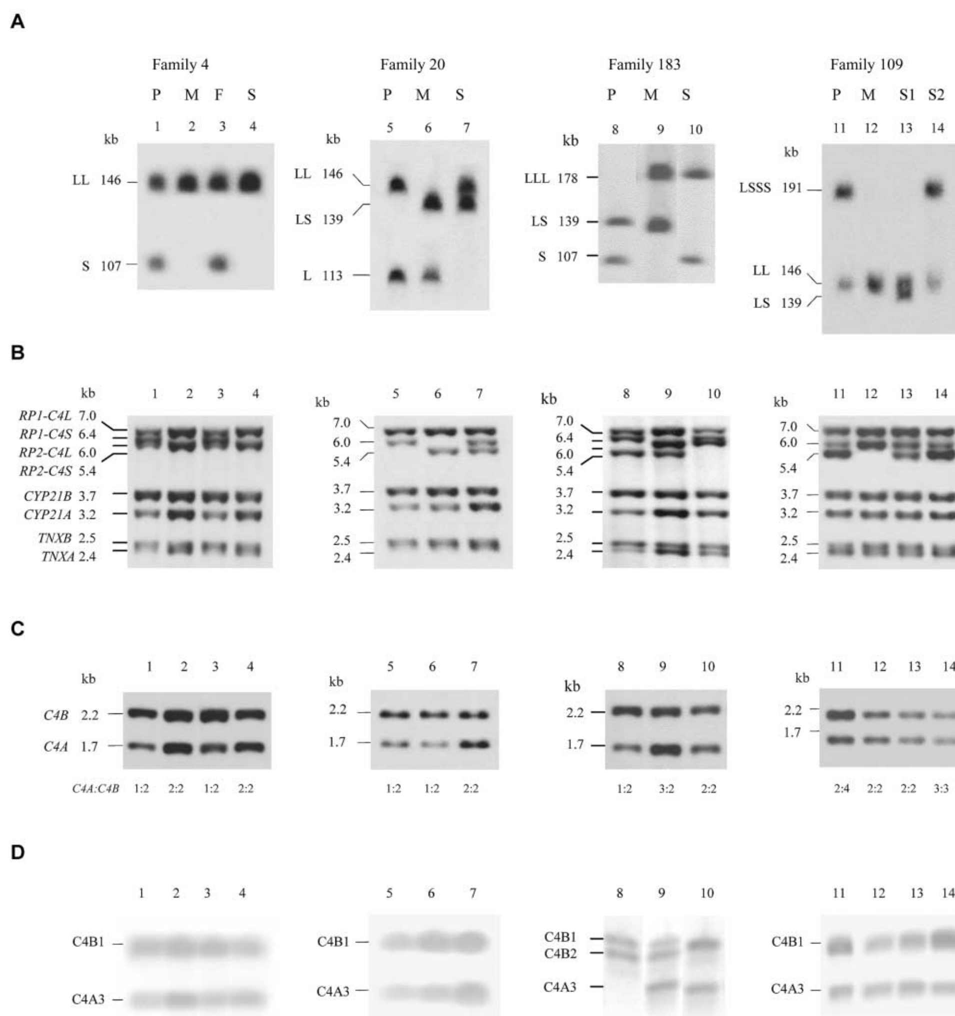


Figure 2. Different genetic mechanisms leading to lower expression of C4A protein than of C4B protein in four families with SLE. In each family, the RCCX modular structures were determined by PFGE of *Pme*I-digested genomic DNA, Southern blotting, and hybridization to a C4d-specific probe (*A*). *Taq*I genomic RFLP was applied to yield details of genomic structures for the RCCX constituents, including the dichotomies of *RP1* and *RP2, C4* long and *C4* short linked to *RP1* or *RP2, CYP21B, CYP21A, TNXB,* and *TNXA* (*B*). *Psh*AI-*Pvu*II RFLP was applied to determine the relative GCNs of *C4A* and *C4B* (*C*). The C4A and C4B protein polymorphisms were elucidated by immunofixation of EDTA plasma resolved by high-voltage agarose-gel electrophoresis (*D*). P = patient; M = mother; F = father; S = sibling (S1 = sibling 1; S2 = sibling 2).

Table 3. Summary of RCCX Modules, Gene CNV, and Protein Polymorphisms of Total *C4, C4A,* and *C4B* in Four Selected Families with SLE

Family and Subject	RCCX-1	RCCX-2	GCN *C4*	GCN *C4A*	GCN *C4B*	Allotypes C4A	Allotypes C4B	Concentration[a] (mg/dl) Total C4	Concentration[a] (mg/dl) C4A	Concentration[a] (mg/dl) C4B	*C4A* Mutation[b]
4:											
Patient	LL	S	3	1	2	A3	B1, B1	20.8	5.0	15.6	No
Mother	LL	LL	4	2	2	A3, A3	B1, B2	51.3	25.3	28.1	No
Father	LL	S	3	1	2	A3	B1, B1	36.0	16.6	19.4	No
Sibling	LL	LL	4	2	2	A3, A3	B1, B1	25.4	12.6	12.8	No
20:											
Patient	LL	L	3	1	2	A3	B1, B1	15.0	4.9	10.0	No
Mother	LS	L	3	1	2	A3	B1, B1	23.6	10.7	12.8	No
Sibling	LL	LS	4	2	2	A3, A3	B1, B1	38.9	18.6	20.3	No
183:											
Patient	LS	S	3	1	2	AQ0	B1, B2	22.5	0	22.5	Yes
Mother	LLL	LS	5	3	2	A3, A3, AQ0	B1, B2	34.3	17.1	17.3	Yes
Sibling	LLL	S	4	2	2	A3, A3	B1, B1	33.5	13.7	19.8	No
109:											
Patient	LSSS	LL	6	2	4	A3, A3	B1, B1, B1, B1	41.4	11.8	29.6	No
Mother	LL	LL	4	2	2	A3, A3	B1, B1	33.6	16.9	16.7	No
Sibling 1	LL	LS	4	2	2	A3, A3	B1, B2	40.8	19.0	21.7	No
Sibling 2	LSSS	LL	6	2	4	A3, A3	B1, B1, B1, B1	68.6	22.6	43.0	No

[a] Plasma protein concentrations.
[b] 2-bp insertion at codon 1213 in exon 29 of *C4A* (C4AQ0, mutant *C4A* without a protein product).

total *C4* genes. In the unrelated female controls (n = 389), the variation of *C4* GCN showed a pattern close to normal distribution. The majority (59.4%) had four copies of *C4* genes. The frequencies of subjects with two, three, five, and six copies of *C4* genes were 1.5%, 27.0%, 10.5%, and 1.5%, respectively (table 4 and fig. 3*A*).

In female European American patients with SLE (n = 216), a shift of the *C4* GCN distribution to the low gene-dosage side was observed ($\chi^2 = 27.5$; df = 4; P = .000016). Slightly more than half the patients (51.9%) had four copies of *C4* genes. The frequency of subjects with two and three copies of *C4* genes increased to 9.3% and 32.9%, respectively. By contrast, the frequencies of subjects with five and six *C4* genes decreased to 5.1% and 0.9%, respectively. In comparison with female controls, the OR of patients with SLE who have only two copies of *C4* genes is 6.51 (95% CI 2.57–16.5; P = .00002); the OR of patients with SLE with five or six copies of *C4* genes is 0.466 (95% CI 0.25–0.88; P = .016) (fig. 3*D*).

We define gene-copy index as the mean copy number of a gene that manifests interindividual gene CNV in a selected population. The gene-copy index of *C4* was highest in the unrelated controls (3.81), lowest in the female patients (3.56; $P < .0001$ for FP vs. FC), and intermediate in the female first-degree relatives (3.67; P = .036 for FF vs. FC).

Low C4A *GCN in Female European Americans with SLE*

The reduction of *C4* GCN in patients with SLE can be the result of a decrease in *C4A, C4B,* or both. Therefore, we examined the distribution patterns of *C4A* and *C4B* among the female patients, first-degree relatives, and unrelated controls (figs. 3*B* and 3*C*).

Total absence of *C4A* genes (homozygous *C4A* deficiency) had a frequency of 6.5% in female patients with SLE, compared with 1.3% in unrelated controls (OR = 5.27; 95% CI 1.87–14.8; P = .001). Of the patients, 26% had only one copy of the *C4A* gene (i.e., heterozygous *C4A* deficiency), compared with 18.2% of unrelated controls (OR = 1.61; 95% CI 1.1–2.4; P = .022). Conversely, high *C4A* GCN (i.e., three, four, or five copies of *C4A* genes) was observed in 15.3% of the patient group, compared with 23.8% of the control group (OR = 0.57; 95% CI 0.37–0.89; P = .012). Overall, the pattern of *C4A* GCN distribution in female patients with SLE was significantly different from that in unrelated healthy controls (χ^2 = 25.1; df = 5; P = .00014). Similar to total *C4,* the distribution pattern of *C4A* GCN for the female first-degree relatives was also between the patterns for patients with SLE and unrelated controls (fig. 3*B*).

The gene-copy index of *C4A* among female unrelated controls, first-degree relatives, and patients with SLE was 2.05, 1.92 (P = .05 for FF vs. FC), and 1.81 (P = .0005 for FP vs. FC), respectively (table 5).

Unlike *C4A,* no significant differences in *C4B* GCN distribution patterns were observed among female patients, their first-degree relatives, and unrelated controls. Among the patients with SLE, 70.8% had the medium *C4B* gene dosage, with two copies of *C4B* genes (fig. 3*C*). The *C4B* gene-copy index in all three groups was similar (range 1.74–1.77) (table 3). Therefore, the main cause for the low GCN of total *C4* in European American patients with SLE was the low GCN of *C4A*.

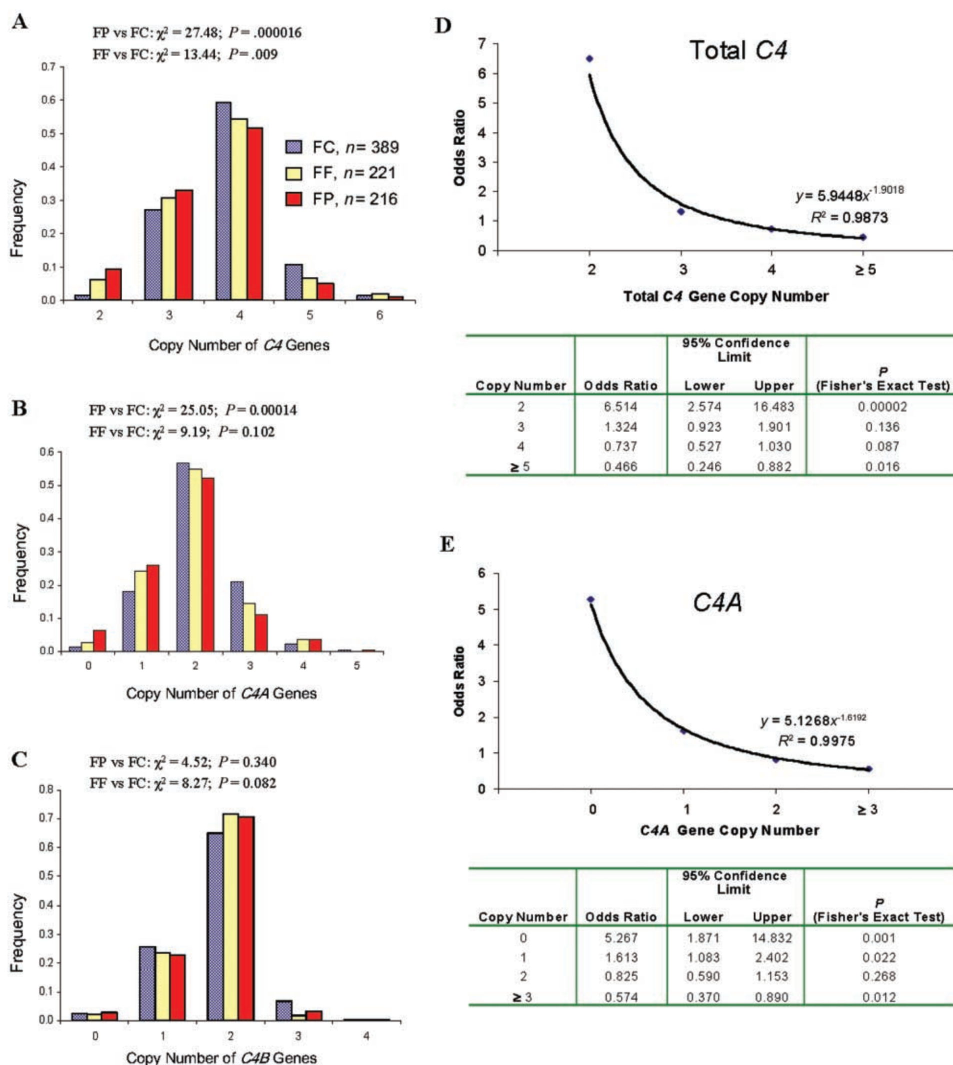
Table 4. CNV of *C4* and RCCX Modules among European American Female and Male Controls (FC and MC, respectively), Female and Male First-Degree Relatives (FF and MF, respectively), Female Patients with SLE from Ohio Study (FP), and Female Patients with SLE from a Replication Study (SP)

Genes or Haplotypes and Group	FC		MC		FF		MF		FP		SP	
	No.	Frequency	No.	Frequency	No.	Frequency	No.	Frequency	No.	Frequency	No.	Frequency
Total C4 genes:												
2	6	.015	1	.008	14	.063	10	.07	20	.093	3	.025
3	105	.27	30	.234	68	.308	48	.338	71	.329	45	.381
4	231	.594	81	.633	120	.543	71	.5	112	.519	62	.525
5	41	.105	12	.094	15	.068	13	.092	11	.051	4	.034
⩾6	6	.015	4	.031	4	.018	0	0	2	.009	4	.033
Total	389	…	128	…	221	…	142	…	216	…	118	…
C4A genes:												
0	5	.013	0	0	6	.027	6	.043	14	.065	3	.025
1	70	.182	19	.148	53	.241	37	.262	57	.264	39	.331
2	218	.566	71	.555	121	.55	77	.546	112	.519	63	.534
3	81	.21	30	.234	32	.145	20	.142	24	.111	11	.093
4	9	.023	8	.063	8	.036	1	.007	8	.037	1	.008
5	2	.005	0	0	0	0	0	0	1	.005	1	.008
Total	385	…	128	…	220	…	141	…	216	…	118	…
C4B genes:												
0	9	.023	5	.039	5	.023	5	.035	6	.028	3	.025
1	99	.257	39	.305	52	.236	27	.191	49	.227	16	.136
2	250	.649	75	.586	158	.718	102	.723	153	.708	90	.763
3	26	.068	9	.07	4	.018	7	.05	7	.032	6	.051
⩾4	1	.003	0	0	1	.005	0	0	1	.005	3	.025
Total	385	…	128	…	220	…	141	…	216	…	118	…
Long *C4* genes:												
0	4	.01	0	0	5	.023	3	.021	10	.046	0	0
1	26	.067	5	.039	18	.082	18	.127	20	.093	9	.101
2	100	.257	29	.227	60	.273	37	.261	63	.292	30	.337
3	145	.373	46	.359	78	.355	58	.408	68	.315	30	.337
4	98	.252	44	.344	53	.241	22	.155	51	.236	17	.191
5	14	.036	3	.023	5	.023	4	.028	3	.014	2	.022
6	2	.005	1	.008	1	.005	0	0	1	.005	1	.011
Total	389	…	128	…	220	…	142	…	216	…	89[a]	…
Short *C4* genes:												
0	130	.334	51	.398	64	.291	35	.246	70	.324	20	.225
1	170	.437	59	.461	109	.495	69	.486	96	.444	47	.528
2	80	.206	11	.086	42	.191	36	.254	43	.199	19	.213
3	8	.021	7	.055	4	.018	2	.014	6	.028	1	.011
4	1	.003	0	0	1	.005	0	0	1	.005	2	.022
Total	389	…	128	…	220	…	142	…	216	…	89[a]	…
RCCX haplotypes:												
L	33	.042	14	.055	31	.07	21	.074	32	.074	5	.028
S	88	.113	20	.078	55	.125	43	.151	73	.169	28	.157
LL	387	.497	139	.543	205	.466	117	.412	199	.461	76	.427
LS	208	.267	60	.234	123	.28	85	.299	105	.243	54	.303
SS	1	.001	0	0	1	.002	1	.004	4	.009	0	0
LSL	11	.014	3	.012	7	.016	5	.018	6	.014	2	.011
LSS	25	.032	8	.031	7	.016	6	.021	5	.012	6	.034
LLL	24	.031	11	.043	10	.023	6	.021	7	.016	6	.034
SSS	0	0	1	.004	0	0	0	0	0	0	0	0
LSSS	0	0	0	0	1	.002	0	0	1	.002	0	0
LLLL	1	.001	0	0	0	0	0	0	0	0	1	.006
Total	778	…	256	…	440	…	284	…	432	…	178[a]	…

[a] Copy numbers of long *C4*, short *C4*, and RCCX haplotypes in SP were calculated from California SLE cohort.

CNV of Long and Short C4 *Genes and Their Correlations with* C4A *and* C4B

We then examined the variations of long and short *C4* genes. In all three groups of female subjects, the copy number of long genes varied from 0 to 6, whereas that of short genes varied from 0 to 4. Three copies of long *C4* genes and one copy of a short *C4* gene were the most prevalent counts among all study groups (figs. 4*A* and 4*B*). The gene-copy index of long *C4* was 2.66 in patients with SLE and 2.91 in unrelated controls ($P = .006$, by Student's *t* test) (table 5). No significant difference was observed for



Copy Number	Odds Ratio	95% Confidence Limit Lower	Upper	P (Fisher's Exact Test)
2	6.514	2.574	16.483	0.00002
3	1.324	0.923	1.901	0.136
4	0.737	0.527	1.030	0.087
≥ 5	0.466	0.246	0.882	0.016

Copy Number	Odds Ratio	95% Confidence Limit Lower	Upper	P (Fisher's Exact Test)
0	5.267	1.871	14.832	0.001
1	1.613	1.083	2.402	0.022
2	0.825	0.590	1.153	0.268
≥ 3	0.574	0.370	0.890	0.012

Figure 3. *C4* gene CNVs in female patients with SLE, first-degree relatives, and unrelated healthy subjects. *A–C,* Distribution patterns of total *C4, C4A* and *C4B* GCN groups among white female patients with SLE (FP [*red bars*]), their female first-degree relatives (FF [*yellow bars*]), and unrelated female controls (FC [*blue bars*]). *D* and *E,* GCN-dependent variation in OR and SLE disease susceptibility for total *C4* (*D*) and *C4A* (*E*).

the gene-copy index of short *C4,* which was 0.94 in patients with SLE and 0.93 in unrelated female controls.

The next question we asked was about the degree of correlation between long or short *C4* genes and *C4A* or *C4B* (table 6). Among three female population groups, the Pearson's coefficients of correlation (*R*) between long *C4* genes and *C4A* were 0.77, 0.69, and 0.64 for patients with SLE, female first-degree relatives, and unrelated female controls, respectively. The overall correlation coefficient between long genes and *C4A* was 0.70 ($P < .0001$). The Pearson's coefficient of correlation between short *C4* genes and *C4B* was 0.48, 0.38, and 0.44 for the patients with SLE, female first-degree relatives, and female unrelated controls, respectively. The overall coefficient of correlation between short genes and *C4B* was 0.44 ($P < .0001$).

Inverse correlations between long genes and *C4B* and between short genes and *C4A* were observed in most groups. Such inverse correlations implied that the greater the copy number of long *C4* genes in an individual, the less likely there will be an increase in copy number of *C4B,* and the greater the number of short *C4* genes, the less likely there will be an increase in the copy number of *C4A.*

Increase in Monomodular RCCX and Decrease in Bimodular and Trimodular RCCX in SLE

The *Pme*I-PFGE and the *Taq*I genomic RFLP data allowed us to examine the constituents of the RCCX haplotypes and the configurations of long and short *C4* genes in the RCCX. Seven common (L, S, LL, LS, LSL, LSS, and LLL; frequency for each haplotype >1%) and four rare (SS, SSS,

Table 5. Comparison of Gene-Copy Indices for Total *C4, C4A, C4B,* Long *C4,* and Short *C4* among Patients with SLE, Their First-Degree Relatives, and Unrelated Healthy Controls

Cohort and Group or Variable	*n*	Gene-Copy Index ± SD				
		Total *C4*	*C4A*	*C4B*	Long *C4*	Short *C4*
Ohio SLE Cohort:						
FC	389	3.81 ± .75	2.05 ± .79	1.77 ± .61	2.91 ± 1.03	.93 ± .80
FF	221	3.67 ± .77	1.92 ± .80	1.74 ± .55	2.80 ± 1.07	.93 ± .75
FP	216	3.56 ± .77	1.81 ± .89	1.76 ± .58	2.66 ± 1.14	.94 ± .82
MC	128	3.83 ± .83	2.21 ± .77	1.65 ± .68	3.11 ± .94	.80 ± .82
MF	141	3.62 ± .75	1.81 ± .76	1.79 ± .58	2.63 ± 1.05	1.04 ± .75
MP	17	3.53 ± .72	1.71 ± .77	1.82 ± .53	2.53 ± 1.07	1.00 ± .87
P value:						
FP vs. FC	…	.0001	.0005	…	.005	…
FF vs. FC	…	.036	.05	…	NS	…
MP vs. MC	…	NS	.015	…	.033	…
MF vs. MC	…	.05	.0002	…	.001	…
MC vs. FC	…	NS	.045	…	NS	…
Overall	…	.002	.0001	NS	.002	NS
Replication cohort:						
SP	118	3.68 ± .77	1.75 ± .76	1.92 ± .66	2.73 ± 1.03	1.08 ± .83
P value:						
SP vs. FC	…	NS	.0007	.015	NS	NS
SP vs. FP	…	NS	NS	.019	.04	.009
Overall[a]	…	.0003	.0001	.035	.001	.04

NOTE.—Gene-copy index is the mean copy number of a specific gene in a selected population. FC = female unrelated controls; FF = first-degree female relatives of patients with SLE; FP = female patients with SLE; MC = male unrelated controls; MF = first-degree male relatives of patients with SLE; MP = male patients with SLE; NS = not significant; SP = female patients with SLE in the replication study.

[a] The overall *P* value was determined by the ANOVA *F* test after dependence adjustment. Student's post hoc *t* test was used to calculate *P* values for each individual pair.

LSSS, and LLLL; frequency for each haplotype <1%) RCCX length variants were detected (fig. 1*B* and table 4).

The frequencies of monomodular haplotypes with a single short *C4* gene or a single long *C4* gene was 15.5% in unrelated female controls and 24.3% in female patients with SLE ($\chi^2 = 14.0$; $P = .0002$). By contrast, the frequency with trimodular/quadrimodular RCCX haplotypes was 7.8% in the unrelated control group compared with 4.4% in the patient group ($\chi^2 = 5.33$; $P = .02$). Overall, the distributions of RCCX haplotypes between patients with SLE and unrelated controls were significantly different ($\chi^2 = 15.9$; $P = .0032$), but those between female first-degree relatives and unrelated controls were not ($\chi^2 = 6.92$; $P = .14$) (fig. 4*C*).

Bimodular LL and bimodular LS were the most common RCCX haplotypes among all European American subjects. A modest difference in the frequency of bimodular structures between patients with SLE (70.4%) and controls (76.5%) was observed ($\chi^2 = 5.43$; $P = .02$).

Association of TNFA *−308G→A Polymorphism with SLE, Secondary to the Monomodular S RCCX Haplotypes with Absence of* C4A

The −308A allele (also known as "*TNF2*") of the human tumor necrosis factor-α gene (*TNFA*) has been suggested to be associated with autoimmune diseases, including SLE.[54–56] Because *TNFA* is located 403.5 kb telomeric to complement *C4* in the MHC class III region, we compared the frequencies of *TNF2* among female European American patients with SLE, their female first-degree relatives, and healthy female controls. Overall, the allelic frequency of *TNF2* was 0.17 in unrelated healthy controls and 0.231 in the patients with SLE ($\chi^2 = 4.5$; $P = .039$; OR = 1.48).

The roles of *TNF2* and low *C4A* GCN as risk factors of SLE are compared by two methods. First, we categorized the study subjects according their *C4A* GCN—low (GCN of zero or one), medium (GCN of two), or high (GCN of three, four, five, or six)—and then compared the frequency of *TNF1* and *TNF2* in each group. Second, we segregated the study subjects into *TNF1* (−308GG) and *TNF2* (−308GA or −308AA) carriers and then compared the frequencies of low, medium, and high *C4A* GCN in each group (table 7). When the *C4A* GCN groups were controlled for, there was no consistent increase in the frequency of *TNF2* in the patients with SLE. No significant difference was observed for the distribution of the *TNF1*- and *TNF2*-carrier frequencies between the healthy controls and the patients with SLE among the subjects with low, medium, or high *C4A* GCN. When *TNF1* and *TNF2* carriage was controlled for, there was a consistent increase in the frequencies of low *C4A* GCN and a consistent decrease in the frequencies of high *C4A* GCN in the patients with SLE, compared with controls. In particular, a great difference in frequencies of low *C4A* GCN was observed

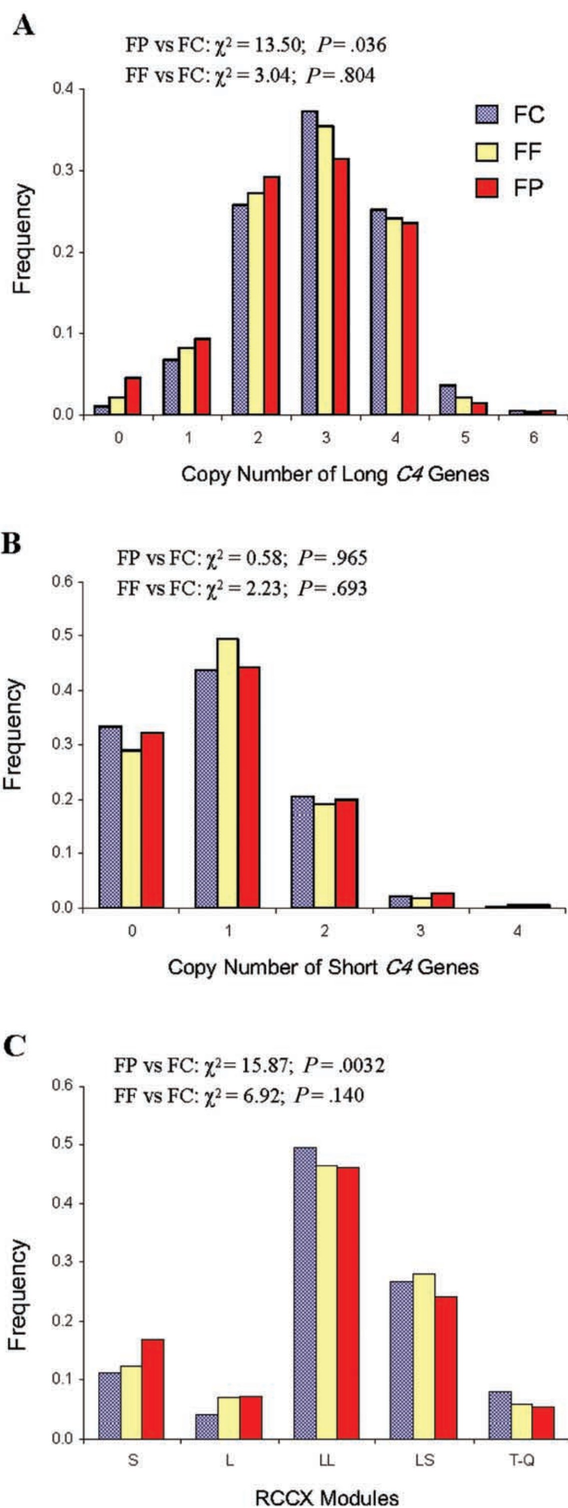


Figure 4. Distribution patterns of long and short *C4* genes and RCCX haplotype structures among female patients with SLE (FP [*red bars*]), their female first-degree relatives (FF [*yellow bars*]), and unrelated female controls (FC [*blue bars*]). *A,* Gene CNV of long *C4*. *B,* Gene CNV of short *C4*. *C,* Haplotype variation of RCCX structures. T-Q = trimodular and quadrimodular.

between the control group (39.6%) and the patients with SLE (70.1%) among the subjects who are *TNF2* carriers ($\chi^2 = 16.0$; $P = .0003$), suggesting that low *C4A* GCN is a strong risk factor for SLE among the subjects who are *TNF2* carriers. Of equal importance is the substantial reduction in the frequency of subjects with high *C4A* GCN (8.05%) in patients with SLE, compared with healthy controls (30.2%). These analyses suggest that *C4A* gene CNV is likely a more important risk factor for human SLE than is the *TNFA* −308G→A polymorphism.

Among the *TNF2* alleles in the unrelated control group, slightly less than half were linked to the monomodular S haplotype of RCCX with the presence of a single short *C4B* gene (i.e., with the absence of a *C4A* gene). This haplotype is abbreviated as *S-TNF2*. A segregation analysis of *TNF2* haplotypes in the *S-TNF2* group and the *nonS-TNF2* group revealed two relevant features (fig. 5). First, the frequency of the *S-TNF2* group in the patients with SLE was 2.1 times greater than that in the healthy controls (patients with SLE 0.164; controls 0.079). Second, the frequency of the *nonS-TNF2* group in the patients with SLE was less than that in the healthy controls (patients 0.067; controls 0.091) ($\chi^2 = 13.7$; $P = .003$). This observation further underscores the importance of complement C4A deficiency as a primary genetic risk factor in the MHC class III region associated with SLE.

Family-Based Association Test (FBAT) Reveals Monomodular S RCCX C4B with C4A Deficiency and TNFA *Promoter Polymorphism as Risk Factors and C4A6 as a Protective Factor for SLE*

FBAT was applied, to analyze whether haplotypes with specific *RCCX* length variants, C4A and C4B protein allotypes, and *TNFA* promoter polymorphisms are genetic risk factors for SLE. It is anticipated that a SLE risk factor would be transmitted more frequently from parents to offspring who develop SLE. The analytical technique FBAT works robustly against the bias caused by genetic admixture and stratification.[50]

As shown in table 8, the monomodular RCCX structures with either a long or short *C4* gene had a higher-than-expected frequency of transmission to the patient population, with a *P* value of .014. Further analysis demonstrates a highly significant increase in the transmission of the monomodular S haplotype to the patient ($P = .005$).

There were 46 different combinations of C4A and C4B protein allotypes in the Ohio SLE families. Among them, 12 haplotypes each had a frequency >1%. C4 protein haplotypes without a C4A protein (i.e., C4B1, C4AQ0-B1, and C4AQ0-B2) were more frequently transmitted to patients ($P = .013$), supporting a probable role of C4A deficiency as a risk factor for SLE development. In addition, the haplotype with C4A6-B1 was less frequently transmitted to patients ($P = .03$), suggesting a possible protective role of C4A6 against SLE disease susceptibility.

We further analyzed the transmission of specific RCCX

Table 6. Pearson Coefficient of Correlation (*R*) of Long and Short *C4* Genes with *C4A* and *C4B* in Female Patients with SLE, Female First-Degree Relatives, and Unrelated Female Controls

	Female Patients with SLE (*n* = 216)		Female First-Degree Relatives (*n* = 220)		Unrelated Female Controls (*n* = 387)		All Three Groups (*n* = 823)	
Correlation	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Long *C4* with:								
C4A	.765	<.0001	.688	.688	.642	<.0001	.695	<.0001
C4B	−.230	.0007	−.028	−.028	−.135	.0079	−.133	.0001
Short *C4* with:								
C4A	−.435	<.0001	−.285	−.285	−.301	<.0001	−.335	<.0001
C4B	.482	<.0001	.380	.380	.442	<.0001	.437	<.0001

haplotypes, C4 protein phenotypes, and the *TNFA* promoter polymorphism −308G→A to patients with SLE. The most common RCCX haplotype with a C4A deficiency, monomodular S coding for C4B1 in linkage to the *TNF2* allele (i.e., *S(C4B1)-TNF2*), was significantly increased in transmission to the patients with SLE ($P = .004$). By contrast, C4A6-B1 linked to bimodular LS of RCCX and *TNF1* allele (i.e., *LS(C4A6-B1)-TNF1*) was significantly decreased from the expected transmission frequency in the patient population ($P = .011$). These results are consistent with results observed in case-control studies suggesting that C4A deficiency is a risk factor for SLE. On the other hand, RCCX haplotypes coding for C4A6 appear to be protective.

Gene CNV of Complement C4 in an Independent European American Patient Population with SLE

To validate the association of low GCN of total *C4* and/or *C4A* deficiency with SLE in European Americans, we examined gene CNV of *C4* in an independent group of patients with SLE. These patients were recruited from two sources. The first source was from Southern California, consisting of 99 European American patients (89 females and 10 males). *C4* genotyping experiments were performed by genomic Southern-blot analyses that allowed elucidation of copy numbers for total *C4, C4A, C4B,* long *C4* genes, short *C4* genes, and the RCCX modules. The second source was APSCORE, providing 29 female patients with SLE who were *not* symptomatic for thrombosis or recurrent spontaneous abortions. A novel series of real-time PCR strategies were applied to determine the GCNs of total *C4, C4A,* and *C4B* (Y.L.W., S.L.S., Y.Y., B.Z., B.H.R., D.J.B., H.N.N., L.A.H., and C.Y.Y., unpublished data). The *C4* gene CNV results for these patients with SLE were compared with those of unrelated, healthy female controls from Ohio ($n = 389$).

Because of the smaller patient sample size for the replication study, we categorized the *C4* GCN groups into low, medium, and high. The results showed that 40.6% of the female replication patients with SLE had low copy number of total *C4* (i.e., two or three copies), which was similar to the 42.2% of patients in the Ohio SLE study (tables 4 and 9). The OR for low *C4* gene dosage in female replication patients was 1.67 (95% CI 1.09–2.55; $P = .024$). The OR for high total *C4* gene dosage (i.e., five, six, or seven copies of total *C4*) in female replication patients was 0.52, although the difference from controls was not statistically significant.

The frequency of subjects with homozygous or heterozygous *C4A* gene deficiency (i.e., zero or one copy of the *C4A* gene) was 35.6% in the female replication patients with SLE, which was similar to the 32.5% in Ohio patients with SLE. The OR for low *C4A* GCN was 2.12 (95% CI 1.35–3.33; $P = .001$), and the OR for high *C4A* GCN was 0.39 (95% CI 0.21–0.73; $P = .002$) in female replication patients. The OR in the Ohio SLE cohort for low *C4A* GCN was 2.02 (95% CI 1.38–2.96; $P = .0003$) and, for high *C4A* GCN, was 0.57 (95% CI 0.37–0.89; $P = .012$) (table 9).

The gene-copy indices for total *C4* and *C4A* were 3.68 and 1.75, respectively, in the female replication patients with SLE. In the Ohio SLE cohort, the corresponding gene-copy indices were 3.56 and 1.81 (in healthy controls, gene-copy indices were 3.81 for total *C4* and 2.05 for *C4A*) (table 5). These results are consistent with the notion that low total *C4* or low *C4A* GCNs are more prevalent, whereas high *C4A* GCN is less frequent in both groups of patients with SLE.

Discussion

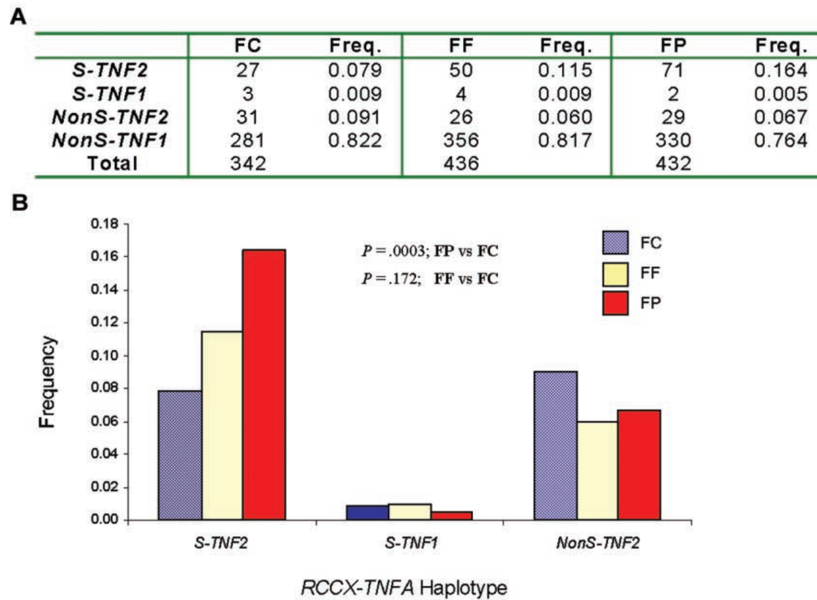
Two recent genome-scan meta-analyses identified similar genomic regions—namely, 6p21.1-6p22.3 and 6p21.1-6q15—that have the most consistent evidence of linkage with human SLE.[6,57] The human MHC (also known as "HLA") is implicated as an SLE susceptibility locus in both studies. Indeed, multiple linkage or association studies have suggested that HLA haplotypes with *DR3, DR2* (i.e., *DRB1*1501*) (HLA-DRB1 [MIM 142857]), or *B8* (HLA-B [MIM 142830]); deficiencies of MHC class III gene complement *C4A* or complement *C2*; and the promoter polymorphism of *TNFA* (−308G→A) are risk factors for SLE.[19,53,58–60] A limitation for most epidemiologic studies on MHC and associated diseases is that, although poly-

Table 7. CNV of *C4A* and the *TNFA* −308 G→A Polymorphism in European American Female Patients with SLE and Controls

Factor	No. (%) of Female Controls	No. (%) of Female Patients	χ^2	*P*	OR (95% CI)
TNF2 and *TNF1*					
TNF2 allele	58 (17.0)	100 (23.1)	4.501	.039	1.48 (1.03–2.11)
TNF1 allele	284 (83.0)	332 (76.9)			
Total	342 (100)	432 (100)			
TNF2 carriers	53 (31.2)	87 (40.3)	3.41	.07	1.49 (.98–2.27)
TNF1 only	117 (68.8)	129 (59.7)			
Total	170 (100)	216 (100)			
C4A GCN:	...	...	14.5	.0002	2.59 (1.57–4.28)
Low *C4A* (<2)	27 (15.9)	71 (32.9)			
Medium/high *C4A* (⩾2)	143 (84.1)	145 (67.1)			
Total	170 (100)	216 (100)			
Controlling for *C4A* GCN:					
Low *C4A* (<2):	...	...	.95	.366	
TNF1	6 (22.2)	10 (14.1)			
TNF2 carrier	21 (77.8)	61 (85.9)			
Total	27 (100)	71 (100)			
Medium *C4A* (2):	...	...	0	1.000	
TNF1	78 (83.0)	93 (83.0)			
TNF2 carrier	16 (17.0)	19 (17.0)			
Total	94 (100)	112 (100)			
High *C4A* (>2):	...	...	1.28	.321	
TNF1	33 (67.4)	26 (78.8)			
TNF2 carrier	16 (32.7)	7 (21.2)			
Total	49 (100)	33 (100)			
Controlling for *TNFA* polymorphism:					
TNF2 carrier:	...	...	16.0	.0003	
Low *C4A*	21 (39.6)	61 (70.1)			
Medium *C4A*	16 (30.2)	19 (21.8)			
High *C4A*	16 (30.2)	7 (8.05)			
Total	53 (100)	87 (100)			
TNF1 only	...	...	2.57	.277	
Low *C4A*	6 (5.13)	10 (7.75)			
Medium *C4A*	78 (66.7)	93 (72.1)			
High *C4A*	33 (28.2)	26 (20.2)			

morphic variants, SNPs, or microsatellites of MHC identified yield consistent results to establish linkage or identify haplotypes with higher risk of disease susceptibility, they do not necessarily provide insights on the causative allele(s). The strong linkage disequilibrium (LD) of MHC class I, II, and III alleles in many MHC ancestral haplotypes[59,61] imposes great difficulty for identifying which candidate gene or genes are the most relevant in MHC-associated diseases, including SLE. In addition, there had been a lack of accurate data on the gene CNVs of complement *C4A* and *C4B* in healthy controls and patients with SLE. There is also no knowledge of microsatellites or tagging SNPs that can serve as surrogates of *C4* gene CNVs. The recent advance of the International HapMap Project has yielded large quantities of information on the SNPs and LD blocks in human genomes from three racial groups, including those in the MHC.[62,63] This information will provide a foundation for future deliberate analyses of causal variants in MHC-associated disease.[64] Through a preliminary analysis of LD blocks of SNPs in the MHC in European Americans from the centromeric class II *DR* region through the central class III region to the telomeric class I region, it appears that the RCCX modules with complement *C4A* and *C4B* are present in different major LD blocks from the class II *DR* region and the *TNFA* region close to the class I genes (UCSC Genome Browser).

To date, complete DNA sequences for three HLA consanguineous cell lines corresponding to haplotypes *A1-B8-Cw7-DR3-DQ2* (cell line COX),[65] *A3-B7-Cw7-DR15-DQ6* (cell line PGF),[65] and *A26-B18-Cw5-DR3-DQ2* (cell line QBL)[66] have been published (MHC Haplotype Project). Two regions in the human MHC are confirmed to have gene CNVs. The first CNV region is in the class II region between *DRB1* (a functional gene) and *DRB9* (a nonfunctional gene segment) that houses zero or one additional *DRB* functional gene (*DRB3, DRB4,* or *DRB5*), and zero, one, or two copies of *DRB* pseudogenes (*DRB2, DRB6, DRB7,* or *DRB8*). The second CNV region is the RCCX modular variation at the class III region, as described in the present article. Similar to our observations of family



A

	FC	Freq.	FF	Freq.	FP	Freq.
S-TNF2	27	0.079	50	0.115	71	0.164
S-TNF1	3	0.009	4	0.009	2	0.005
NonS-TNF2	31	0.091	26	0.060	29	0.067
NonS-TNF1	281	0.822	356	0.817	330	0.764
Total	342		436		432	

Figure 5. Significant increase in the frequency of monomodular S RCCX haplotype with C4A deficiency linked to the −308A allele of the *TNFA* gene (*S-TNF2*) in SLE. *A,* Number and frequency (Freq.) of each haplotype in unrelated female controls (FC), female first-degree relatives (FF), and female patients with SLE (FP). *B,* Differences between the three groups. *NonS-TNF2* = *TNF2* allele not linked to monomodular S haplotype of RCCX; *NonS-TNF1* = −308G allele of the *TNFA* gene not linked to monomodular S haplotype of RCCX.

C008 published elsewhere,[36] the *A1-B8-Cw7-DR3-DQ2* haplotype has *DRB2* (pseudogene) and *DRB3* (functional gene) between *DRB1* and *DRB9* in the class II region and monomodular S RCCX with a single short *C4B* gene in the class III region; the *A3-B7-Cw7-DR15-DQ6* haplotype has *DRB5* and *DRB6* (pseudogene) between *DRB1* and *DRB9* in the class II region and a bimodular long-long (LL) RCCX haplotype coding for C4B1-A3 in the class III region.[36] Intriguingly, the order of *C4A* and *C4B* genes with respect to *C2*/factor *B* and *DR* are reversed (i.e., *C4B-C4A* instead of *C4A-C4B*) in this *B7-DR15* haplotype.[65] For the *A26-B18-Cw5-DR3-DQ2* haplotype, *DRB2* and *DRB3* are present in the class II region, and the monomodular L with a single *C4A* gene is present in the class III region. The presence of three different RCCX/*C4* GCN and length variants in three HLA haplotypes underscores the structural complexity of complement *C4A* and *C4B*.

In the present study, we establish strong correlations of gene CNV of complement *C4* (total *C4*) and its associated polymorphisms (*C4A* and long *C4* genes) with human SLE. Typical of many susceptibility genes associated with an autoimmune disease, the risk factor (i.e., low copy number

Table 8. FBAT for RCCX, *C4,* and *TNFA* Haplotypes in the Ohio SLE Cohort

Haplotype	Frequency	No. of Families	Observed Score	Expected Score	*P*
RCCX:					
S and L	.209	105	82	68.4	.014
S	.127	81	61	47.7	.005
C4:					
B1	.129	82	61	48.1	.005
A6B1	.041	31	11	17.1	.03
AQ0X	.164	98	75	62.0	.013
TNFA:					
TNF2	.191	88	109	118	.078
RCCX*(C4)-TNFA*:					
S(C4B1)-*TNF2*	.112	58	54	42.5	.004
LS(C4A6B1)-*TNF1*	.032	25	8.5	14.8	.011

Note.—All markers as a group—including RCCX, *C4, TNFA,* and RCCX*(C4)-TNFA* but not *TNFA*—were statistically significant. The individual haplotypes in each marker group were then tested and are summarized above. AQ0X represents bimodular RCCX structure with a mutant *C4A* gene with a 2-bp insertion in exon 29 (C4AQ0) and a functional *C4B* gene that can code for a C4B1 or C4B2 protein.

Table 9. Replication Study to Validate Low GCN of Total *C4* or *C4A* as Risk Factor for and High GCN as Protective Factor against SLE in European Americans

Gene, Patients, and GCN	OR (95% CI)	P[a]
Total *C4*:		
Ohio[b]:		
2 or 3	1.823 (1.287–2.583)	.001
4	.737 (.527–1.030)	.087
5 or 6	.466 (.246–.882)	.016
SP[c]:		
2 or 3	1.666 (1.087–2.554)	.024
4	.783 (.518–1.184)	.289
5 or 6	.520 (.239–1.132)	.129
C4A:		
Ohio[b]:		
0 or 1	2.024 (1.384–2.959)	.0003
2	.825 (.590–1.153)	.268
3, 4, or 5	.574 (.370–.890)	.012
SP[c]:		
0 or 1	2.121 (1.352–3.326)	.001
2	.926 (.612–1.400)	.752
3, 4, or 5	.391 (.210–.729)	.002

[a] *P* value calculated by Fisher's exact test.
[b] Prospective European American patients with SLE who are from Ohio.
[c] Independent European American patients with SLE who were part of a replication study.

of total *C4* or *C4A*) is present in the general population, but the prevalence is significantly increased in the patient population (with SLE). Importantly, a dose-dependent phenomenon from increased SLE disease risk at low GCN to protection against disease susceptibility at high GCN is observed for both total *C4* and *C4A*. The RCCX modular variation reflects differences in the copy number and configurations of long and short *C4A* or *C4B* genes in a haplotype. This study shows that monomodular RCCX structure with a short *C4B* gene and the absence of *C4A* is a relatively common haplotype among European American patients with SLE. By contrast, trimodular RCCX haplotypes with three functional *C4* genes are less frequent among patients with SLE.

We employ the concept of gene-copy index (i.e., the mean copy number of a specific gene in a selected population) to facilitate a quantitative comparison of gene CNV among groups. The results provide a quantitative basis for gene CNV of total *C4* or *C4A* as a risk factor for or a protective factor against SLE disease susceptibility. The gene-copy indices of total *C4* and *C4A* in the female first-degree relatives are lower than those in unrelated healthy controls but are higher than those in patients with SLE. This suggests that first-degree relatives of patients with SLE are harboring some genetic risk factor for autoimmune disease, but most of them are healthy probably because of the weak penetrance of genetic risk factors involved in complex diseases and/or because their milieus of genetic and environmental risk factors have not reached the threshold to initiate the disease process. Nevertheless, the lower total *C4* and *C4A* GCNs in first-degree relatives than in unrelated healthy controls would offer a logical explanation for the higher incidence and clustering of autoimmune diseases in family members of patients with SLE.[1,4] A replication study involving an independent patient cohort reaffirmed the role of low total *C4* and *C4A* GCNs in increased risk of SLE susceptibility.

Besides the SLE disease activities, our data also revealed that gene CNV of *C4* is the predominant genetic factor that mediates the plasma or serum C4 protein concentrations[23,43] (Y.Y., Y.L.W., H.N.N., B.H.R., D.J.B., L.A.H., and C.Y.Y., unpublished data). Therefore, a comprehensive elucidation of the *C4* gene CNV and quantitative variations of plasma or serum C4 protein levels plus their activation and/or inactivation split products (C4a and/or C4d) would yield highly relevant information to help diagnose and manage SLE.[21,23,67,68]

Historically, interindividual gene CNV has not been considered to be a source of inherent genetic diversity. However, recent advances in global genomewide analysis have revealed multiple and variable segmental duplications of genomic regions among different individuals.[69–71] A public database on human genome diversity lists 3,463 possible loci, including complement *C4,* that can have CNV (Human Genome build 35, Database of Genomic Variants). The data source was mainly derived from varying fluorescence intensities on microarray chips after comparative hybridization with labeled genomic DNA from different human subjects. More-refined studies will be necessary to elucidate the distribution patterns of interindividual and interpopulation gene CNVs, their related polymorphisms, and possible physiologic roles in health and disease.[72–74] This is because, although duplicated genomic segments involved in CNVs are highly homologous, they may not be totally identical in DNA sequences. Case-by-case examination often reveals secondary genetic events that yield new markers or polymorphisms and sometimes add novel functions to existing gene products. Although sequence exchanges among variants of duplicated sequences promote diversity, they can also carry a genetic burden by harboring deleterious mutations. Of the RCCX modules, multiple mutations accumulated in the *CYP21A* can be transferred to *CYP21B,* which can become allelic when a monomodular RCCX pairs with a bimodular RCCX and thus abolishes or diminishes the enzymatic activity of the steroid 21-hydroxylase. Mutations or the absence of *CYP21B* are the main cause of congenital adrenal hyperplasia.[34,35,75] Moreover, the acquisition of the 120-bp deletion from the *TNXA* gene fragment by the intact tenascin-X gene *TNXB* is responsible for a form of Ehlers-Danlo syndrome (MIM 130020).[76]

For complement *C4,* secondary genetic diversifications led to the emergence of two forms of *C4* genes (long and short) and two isotypes of C4 proteins (C4A and C4B). C4A is probably a newly evolved protein that acquires a novel function by binding effectively to amino groups of

protein substrates or immune complexes, thereby enhancing immune-complex clearance that would otherwise promote autoimmunity. Gene CNV permits the coexistence and quantitative variation of C4A and C4B proteins and the emergence of new protein variants or allotypes in each group. C4A6 is a variant allotype that dissociates the lytic pathway from the classical or lectin activation pathway of the complement system, as the R458W polymorphism in C4A6 disables the binding of C5 and abrogates the C5 convertase activity of C4b2a3b.[77,78] This study suggests that C4A6 could be a protective factor against SLE. One possibility is that this allotype could attenuate the deleterious effects of complement lytic pathway–mediated tissue injury during an autoimmune process.

In addition to C4A deficiency, we have also examined two other genetic risk factors in MHC class III region—namely, the deficiency of complement C2[79,80] and the −308A allele of *TNFA* (i.e., the *TNF2* allele)—as susceptibility factors for SLE.[81–83] Complement C2 deficiency appeared infrequently in our patients with SLE, since only one homozygous and four heterozygous C2 deficiencies were present among 233 Ohio patients with SLE. On the other hand, a significant increase in the frequency of *TNF2* in SLE was observed (patients 0.231; controls 0.170; OR = 1.48; $P = .039$). In healthy subjects, close to half of the *TNF2* alleles are linked to monomodular S of RCCX, *S-TNF2*. In SLE, the increase of *TNF2* frequency appeared to be secondary to the increase of monomodular S with C4A deficiency, which had a haplotype frequency of 0.164 in patients with SLE and 0.079 in unrelated healthy controls. By contrast, the frequency of *TNF2* alleles not associated with monomodular S (i.e., *nonS-TNF2*) was reduced in patients with SLE (patients 0.067; controls 0.091). The relevance of the *S-TNF2* haplotype in SLE was further substantiated by FBAT analysis.

The strength of this work lies on the high resolution and the comprehensiveness by which the diversities of C4A and C4B genotypes and phenotypes in healthy subjects and in patients with SLE are elucidated. Recently, association between gene CNV and human disease has been documented in three other incidences: an increased risk of HIV infection and progression to severe disease for individuals with low GCN of *CCL3L1,*[84] low copy number of beta-defensin 2 in inflammatory bowel disease,[85] and possibly low GCN of immunoglobulin receptor *FCGR3B* in glomerulonephritis.[86] Further detailed analysis of gene CNV and associated genetic diversities in human populations promises to increase our understanding of complex diseases and quantitative genetic traits.[87,88]

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Database of Genomic Variants, http://projects.tcag.ca/variation/ (for human genomic loci with possible gene CNV [build 36])

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human *C4A* coding sequence [accession number NM_007293], human *C4B* coding sequence [accession number NM_001002029], human endogenous retrovirus in long *C4* gene, HERV-K(C4) [accession number U07856], monomodular S RCCX coding for C4B [accession number AL662849], monomodular L RCCX coding for C4B [accession number NG_005163], and bimodular LL RCCX coding for C4B-C4A [accession number AL64592])

International HapMap Project, http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/

MHC Haplotype Project, http://www.sanger.ac.uk/HGP/Chr6/MHC/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SLE, C4A, C4B, C1q, C1r, C1s, *STK19, CYP21A1, TNXB,* HLA-DRB1, HLA-B, and Ehlers-Danlo syndrome)

UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgTracks (for the RCCX region in the human MHC, chromosome 6, position 32040000–32220000)

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