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# Effects of Complement *C4* Gene Copy Number Variations, Size Dichotomy, and *C4A* Deficiency on Genetic Risk and Clinical Presentation of Systemic Lupus Erythematosus in East Asian Populations

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## Abstract

**Objectives—**Human complement *C4* is sophisticatedly complex with multiple layers of diversity. This study aims to elucidate the CNVs of *C4A* and *C4B* in disease risk of SLE, and compare the basis of race-specific *C4A*-deficiency in East-Asians (EA) and Europeans.

**Patients and Methods—**Our EA study-population included 999 SLE patients and 1,347 healthy subjects. Variations in gene copy-numbers (GCNs) for *total C4*, *C4A*, *C4B*, *long* and *short*

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AUTHOR CONTRIBUTIONS

JYC, YLW, EKC, YY, CSL and CYY designed project. JYC, CSL, YLL, CHS, YJJW, YY and MYM performed diagnosis and clinical studies. JYC, YLW, MYM, YY, YJJW, CMW, EKC, KEL, BZ, KJ, CHS, YLL, CSL and CYY recruited study subjects. YLW, YJJW, KEL, CMW, EKC, YY, BZ, HW, DY, AA, HNN, JYC and CYY performed experiments and data analyses. CYY, JYC, YLW, EKC and YY drafted the manuscript. All authors read and approved the manuscript.

genes were determined and validated rigorously by independent genotyping technologies. Genomic regions with C4B96 were investigated to determine the basis of the most basic C4B protein that is concurrent with C4A-deficiency.

**Results—**In EA, strong protective effects of high GCNs for *total C4* and *C4A* against SLE were notable; low and medium GCNs for *total C4* and *C4A,* and the absence of *short* genes were risk factors of SLE. Homozygous *C4A*-deficiency was infrequent but had an odds-ratio (OR) of 12.4 (p=0.0015). Patients who experienced very-low serum complement were associated with low GCNs of *total C4* (OR=3.27, $p=7.0\times10^{-7}$) and *C4B* (OR=2.55, $p=2.5\times10^{-5}$). Patients with low complement had high frequencies of anti-dsDNA (OR=4.96, $p=9.7\times10^{-17}$), hemolytic anemia (OR=3.89, $p=3.6\times10^{-10}$) and renal disease (OR=2.18, $p=8.5\times10^{-6}$). The *monomodular-short* haplotype with *C4A*-deficiency and in linkage-disequilibrium with *HLA-DRB1*0301* prevalent in European was scarce in EA. Instead, most EA-subjects with *C4A*-deficiency shared a recombinant haplotype with *bimodular-LS* encoding C4B1 and C4B96, which was linked to *HLA-DRB1*1501.* DNA sequencing revealed the E920K polymorphism for C4B96.

**Conclusion—***C4* CNVs and *C4A*-deficiency are important in the risk and manifestations of East-Asian and European SLE.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a female dominant autoimmune disease characterized by the generation of autoantibodies against nuclear antigens including double-stranded DNA (dsDNA) (1-3). The formation of immune complexes between autoantibodies and self-antigens activates complement, causing systemic tissue injuries and organ damages (4,5). Reduced serum levels or depressed hemolytic activities of complement are common clinical laboratory findings in patients with SLE and glomerulonephritis (4,6-9). Such phenomenon is generally interpreted as a result of consumption due to SLE disease activities. However, low serum complement can both be a *cause* and an *effect* of SLE. Human subjects with a homozygous genetic deficiency in anyone of the early components for the classical pathway of complement activation (Supporting Information: Figure S1), C1q, C1r, C1s and C4 were almost always inflicted with SLE irrespective of race, gender and HLA haplotypes (10-14). Under susceptible backgrounds, mice with genetic knockout of complement C4 or C1q manifested lupus-like phenotypes with high titers of antinuclear antibodies (ANA) and anti-dsDNA and high frequency of glomerulonephritis (15-18). Cumulated information suggests that complement deficiency contributes to pathogenesis of SLE through impaired handling and clearance of immune complexes, deficient scavenging of apoptotic cells, aberrant induction of peripheral tolerance because of inefficient elimination of self-reactive B cells to produce high affinity autoantibodies with class switching, and defective control of cytokine production including type I interferons (5,19,20).

While a homozygous genetic deficiency for complement C1 or C4 can be a causal factor of SLE, their prevalence is extremely rare as only 122 cases have ever been documented (4). Genome-wide association studies led to the identification of numerous single nucleotide polymorphisms (SNPs) associated with increased risk of SLE, but most of those SNPs are of low effect-size with odds ratios (OR) between 1.1 and 1.5 (1-3,21).

SLE affects all racial groups but patients of Asian, African and Hispanic ancestries/ethnicities often have severe disease with renal involvement (22,23). While SLE risk factors identified in Asians are also germane to Europeans, there are remarkable differences between races with respect to effect-sizes and frequencies of risk alleles (24). In a case-control study of *C4* gene copy-number variations (CNVs; online Figure S1) in SLE of European ancestry, we found that low GCNs of *total C4* and *C4A* were risk factors for, and high GCNs of *total C4* and *C4A* were protective factors against, SLE disease susceptibility (25). A common major histocompatibility (MHC, or HLA in human) haplotype with a single *short C4B* gene (mono-S) and the absence of *C4A* is prevalent among European SLE patients. This mono-S haplotype is in strong linkage disequilibrium (LD) with the HLA class II gene *DRB1* allele **0301 (*or *DR3)* (26,27). Although the increased risk for *C4A*-deficiency in Asians had been observed in earlier studies (28-30), the mechanism of *C4A*-deficiency and diversity associated with *C4*-CNVs have *not* been investigated meticulously. Here, we report an in-depth study to elucidate patterns of CNVs for complement *C4* and associated variants in large cohorts of East-Asian (Chinese) SLE and race-matched healthy controls. This study unravels the molecular basis of *C4A*-deficiency and fine details pertinent to *C4*-CNVs in East-Asian and European SLE disease susceptibility and pathogenesis.

## STUDY POPULATIONS AND METHODS

### Study populations

Our study populations included 999 Chinese SLE patients and 1,347 race-matched healthy controls (Table 1). All patients fulfilled at least four of the eleven revised 1982-ACR diagnostic criteria for human SLE (31,32), The patients' female to male sex ratio was 11.6 to 1. Their mean age (±SD) at disease diagnosis was 30.61±11.45 years old. Besides the presence of ANA that was almost universal among SLE patients, the frequencies for SLE diagnostic disorders with details on hematologic and immunologic disorders are tabulated in *panel* B of Table 1. None of the race-match healthy controls reported to have an autoimmune disease. The IRB of the Nationwide Children's Hospital (USA), the ethics committees from the University of Hong Kong (Hong Kong) and the Chang Gung Memorial Hospital (Taiwan) approved the study. All blood donors provided written consent.

### Determination of *C4* gene CNVs

Processing of blood samples for plasma, peripheral mononuclear cells (PBMC) and genomic DNA were as described previously (33). The copy-numbers of *total C4*, *C4A* and *C4B* genes, and *long* and *short C4* genes were determined by TaqMan based, quantitative realtime PCR, and/or "hot-stop PCR" (33-35). Validation for CNV calls were achieved when GCNs of *total C4=C4A+C4B*. The RCCX haplotypes for the Ohio cohort were further determined by long-range mapping of *Pme*I and *Pac*I-digested genomic DNA and resolved by pulsed-field gel electrophoresis (PFGE), and *Taq*I, *Psh*AI/*Pvu*II restriction fragment length polymorphisms (RFLPs).

### Phenotyping of complement C4

Protein polymorphisms of C4A and C4B were determined by immunofixation of EDTA-plasma resolved by high-voltage agarose gel-electrophoresis (33,36,37). Ambiguous and intermediate allotypes migrating between C4B and C4A were further resolved by immunoblot analyses using monoclonal antibodies against Rodgers or Chido antigens (38,39). Interpretation of C4A and C4B allotypes were substantiated by their corresponding genotypes.

### Autoantibody assays

Autoantibody titers were determined by ELISA. ANA was considered as positive when serum titers were >1:80 by Hep-2 cell assay. Anti-ENA (Ro/SSA, La/SSB, Sm, and RNP) and anti-cardiolipin were assessed by ELISA according to vendor's instructions (Pharmacia Diagnostics).

### Statistics

Statistical analyses were performed using JMP Genomics version 6.0 (SAS Institute, Cary, NC) and Prism6 (GraphPad, San Diego, CA) software. Descriptive statistics were displayed as mean ± standard deviation (SD) for normally distributed data, and simple comparisons were made using Student's t-test for continuous data, or by $\chi^2$ analysis for categorical data. P-values were derived from Likelihood ratios. Odds ratios (OR) and 95% confidence levels were calculated by analysis of 2×2 tables through Fisher's exact test. For all analyses, $p \leq 0.05$ was considered significant.

## RESULTS

### CNVs of *C4A*, *C4B*, *long* genes, *short* genes and *total C4*

**CNV of C4A—**The number of *C4A* varied from 0 to 6 copies among different individuals (*panel* A, Fig. 1; Supporting Information: Table S1). There was a great difference in the distribution of GCN groups for *C4A* between SLE and controls ($p=8.0\times10^{-9}$). Homozygous deficiency of *C4A* (GCN=0) was found in nine patients and only one control, which translated into an OR of 12.4 (95% confidence interval, 1.57-97.9) and p=0.0015. The prevalence of heterozygous deficiency for *C4A* (GCN=1) was greater, 13.8% in SLE and 11.1% in controls, but its effect-size was modest: OR=1.28 (1.00-1.65), p=0.049. The most prevalent GCN group for *C4A* was two, which had a frequency of 67.8% in SLE and 61.9% in controls. Three and four copies of *C4A* had frequencies of 13.1% and 1.7%, respectively, in SLE; compared to 20.2% and 4.3%, respectively, in controls. For *very* high copy-number of *C4A* (GCN=5 and 6), the frequencies were low but similar between SLE and controls. The mean GCN (±SD) of *C4A* was 2.09±0.79 in SLE and 2.25±0.82 in controls ($p=3.4\times10^{-6}$, t-test).

**CNV of C4B—**The GCN of *C4B* varies from 0 to 5 copies among different individuals (*panel* B, Fig. 1). There was only slight difference in distribution of GCN groups for *C4B* between SLE and controls (p=0.045). The mean GCN of *C4B* was 1.85±0.68 in SLE and 1.88±0.71 in controls.

**CNV of long-C4 genes—**One to six copies of *long-C4* genes were present in a diploid genome among different individuals but the distribution patterns of *long C4* were similar between SLE and controls (*panel* C, Fig. 1). It is notable that no subject had an absence and only nine had one copy of *long* gene in the entire study population. The mean GCN of *long-C4* was 2.88±0.79 in SLE and 2.94±0.82 in controls.

**CNV of short-C4 genes—**Zero to six copies of *short-C4* genes was present and the distribution was different between SLE and controls (p=0.0008, $\chi^2$ analysis; *panel* D, Fig. 1). The absence of *short* gene existed in 29.7% of patients and 22.6% of controls; OR=1.48 (1.22-1.80), $p=8.3\times10^{-5}$. The mean copy-number of *short* genes was 1.05±0.97 in SLE and 1.17±0.94 in controls (p=0.005, t-test).

**CNV of total C4—**Two to eight copies of *total C4* in a diploid genome were detectable (panel E, Fig. 1; Supporting Information: Table S1). The most prevalent GCN group for *total C4* was four. Similar to *C4A*, there was a consistent shift for increased frequencies of the low and median copy-number groups, and reduced frequencies of the high copy-number groups in SLE ($p=3.7\times10^{-7}$, $\chi^2$ analyses). The mean GCN of total *C4* was 3.95±0.87 in SLE and 4.14±0.92 in controls ($p=8.9\times10^{-8}$, t-test).

Among the *C4* genes, the proportions for *C4A* and *C4B* were 53.2%/46.8% in SLE and 54.8%/45.2% in controls (p=0.011). For *long* and *short* genes, the proportions were 75.0%/25.0% in SLE; and 73.0%/27.0% in controls (p=0.017). SLE patients had consistent reduction in mean GCNs of *total-C4*, *C4A* and *short-C4* (Fig 1F, Supporting Information: Table S1). The reduction of GCNs for *total C4* or *C4A* was attributable to a reduction of *short* genes. The protective effects for high copy-numbers of *total-C4* and *C4A* against SLE were highly significant.

## Complement *C4* gene CNVs as risk factors for SLE diagnostic disorders

Intra-group analyses of *C4* CNVs were performed to investigate their associations with diagnostic disorders of SLE. Three different associations emerged (Table 2).

**a. *Potentially causal relationship*—**Nine SLE patients had a homozygous *C4A*-deficiency and all of them had malar rash (p=0.0016). Also, eleven SLE patients had homozygous *C4B*-deficiency and *none* of them had thrombocytopenia (p=0.0093). Thus, the absence of *C4A* could be a causal factor for malar rash, and the presence of *C4B* might be required for thrombocytopenia.

**b. *Risks associated with low GCNs*—**The ever presence of very-low serum complement levels were recorded in 79.4% of SLE patients. Very-low serum complement was strongly correlated with low GCNs of *total C4, C4B* and *long-C4*. Low GCN of *total C4* had the largest effect-size [OR=3.27 (1.94-5.52), $p=7.0\times10^{-7}$]. The OR for *C4B was* 2.55 (1.60-4.08), $p=2.5\times10^{-5}$, and for *long-C4* was 1.84 (1.24-2.72), p=0.0028. Low GCNs of *total C4* leading to very-low serum complement were attributable to low GCNs of *C4B*, which were likely *long* genes.

Low GCN of *C4B* was also associated with the presence of anticardiolipin-IgM [C4B=0 or 1, OR=1.89 (1.07-3.36), p=0.032]. The absence or low copy-numbers of *short-C4* were associated with discoid rash and arthritis with moderate effect-sizes [discoid rash: OR=1.64 (1.05-2.58), p=0.026; arthritis: OR=1.48 (1.07-2.03), p=0.016].

**c. *Risks associated with high GCNs*—**High GCN of *C4A* (GCN=3-6) appeared to be a risk factor for pericarditis [OR=2.26 (1.39-3.67), p=0.0017], hemolytic anemia [OR=1.84 (1.26-2.700, p=0.0019] and thrombocytopenia [OR=1.61 (2.08-2.40), p=0.022]. High GCN of *short-C4* genes (≥2) appeared as a risk factor of thrombocytopenia [OR=1.77 (1.24-2.52), p=0.0018].

## Associations of low serum C3/C4 levels with SLE diagnostic disorders

Besides low GCNs of *total C4*, *C4B* or *long* genes, low serum C3 and C4 levels could also reflect systemic complement consumption or SLE disease states (Table 3). Patients with very-low C3/C4 had an earlier age of disease-onset (mean age ± SD: 30.0±11.0 with low C3/C4 versus 33.3±13.8 without low C3/C4; p=0.0011). Very strong associations with large effect-sizes were found between very-low C3/C4 and (a) immunologic disorders such as the presence of anti-dsDNA [OR: 4.96 (3.41-7.22), $p=9.7\times10^{-17}$], anti-Sm [OR: 2.37 (1.54-3.64), $p=3.6\times10^{-5}$], and IgG-anticardiolipin [OR: 2.33 (1.41-3.87), p=0.0004]; (b) hematologic disorders such as hemolytic anemia [OR: 3.89 (2.42-6.27), $p=3.6\times10^{-10}$] and leukopenia [OR:1.92 (1.36-2.71), p=0.0002]; (c) renal disease [OR: 2.32 (1.63-3.31), $p=2.2\times10^{-6}$]; and (d) serositis including ascites [OR: 3.62 (1.10-11.8), p=0.011] and pericarditis [OR: 2.55 (1.29-5.01), p=0.0027]. Very-low C3/C4 also significantly associated with increased risks of serositis, thrombocytopenia, anti-RNP1, and anti-La/SSb, although their effect-sizes were modest.

## A specific Asian haplotype with C4B96 and *C4A*-deficiency

Immunochemical analyses of EDTA-plasma from four SLE patients with homozygous C4A deficiency and one patient with both C4A and C4B present were shown in *panels* A and B of Figure 2. Immunofixation experiment revealed that three out of four C4A-deficient patients possessed the slowest-migrating C4 allotype, C4B96, in addition to C4B1. The fourth C4A-deficient patient had C4B1 only (*panel* A). Immunoblot experiment revealed that C4B96 and C4B1 (and C4A1) but not C4A3 were associated with Chido blood-group antigens (*panel* B) (38,39). C4A-deficient patients had two to four copies of *C4* genes in a diploid genome and they *all* coded for C4B protein. Those *C4* genes either existed in (a) monomodular RCCX haplotype with a single *long* gene coding for C4B1, or (b) bimodular RCCX coding for C4B1-B96 or C4B1-B1. The limited accessibility of patient samples restricted further characterization of homozygous C4A deficiency.

Seven subjects in our American EA study-cohort contained C4B96 and Subject-89 was chosen for detailed analysis because of its relative simplicity (*panels* C-G, Fig. 2). *Pme*I-PFGE and *Taq*I-RFLP revealed that Subject-89 had heterozygous RCCX haplotypes with bimodular-LS and monomodular-L (*panels* D and E), and a total of two *C4B* and one *C4A* (*panel* F). The LS haplotype coding for C4B1-B96 was a recombinant between tenascin *TNXB* and *TNXA*, as characterized by the absence of pseudogene steroid 21-hydroxylase

*CYP21A*, and the presence of an 120-bp insertion in *TNXA,* or *XA+120*, as documented previously (Supporting Information: Fig. S2) (40-42). Examinations of other Asian subjects with C4B96 revealed that they all shared the same bimodular-LS haplotype encoding C4B1-B96, with two *CYP21B* (no *CYP21A*) and the presence of an *XA+120* recombinant. The coding sequences of *C4* genes in Subject-89 were amplified, sequenced to completion and compared with known *C4* sequences (Supporting Information: Figs. S2-S4; Table S2) (39,43). We identified a novel, non-synonymous G→A nucleotide change at exon 21 from the *short* gene coding for C4B96, which attributed to the E920K polymorphism. The negatively-charged glutamic acid-920 was changed to the positively-charged lysine-920 in C4B96. This basic residue is located at the MG7 domain of the C4 protein structure (*panel* J, Fig. 2) (44). The DNA sequence for K920 ablated a restriction site for *Eco*RI (GAATTC→AAATTC; *panel* H, Fig. 2). Thus, 1.7 kb PCR fragments spanning from intron 20 to exon 26 of *C4B* were generated from six subjects with C4B96 plus one control without this allotype. The PCR products were digested with *Eco*RI resolved by electrophoresis. All subjects with B96 displayed the 1.7 kb fragment in addition to the 1.5 kb from *C4B1* gene (*lanes* 1 and 3-7, panel I). C4 protein allotyping and/or *Eco*RI RFLP of PCR-amplified DNA revealed that 75% of EA-SLE with homozygous C4A-deficiency contained C4B96. *HLA-DRB1* genotyping revealed that all subjects with C4B96 had *DRB1*1501 (DR2)*.

## DISCUSSIONS

The phenomenon of common CNVs is gaining appreciation but their impacts on rheumatic diseases among different racial groups await accurate and in-depth investigations. Here we report the continuous variation in GCN of human complement *C4* and polymorphisms for *C4A*, *C4B*, *long* genes and *short* genes in association with SLE disease susceptibility and clinical manifestations, and compare them to those of European Americans.

In East-Asians, homozygous deficiency of *C4A* was present only in ~1% of SLE patients but it had a *very* large effect-size on disease susceptibility (OR=12.4). Heterozygous *C4A*-deficiency and low GCNs for *total C4* were more prevalent but their effects on SLE disease risk were modest (OR=1.28 and 1.45, respectively). On the other end, the protective effects for high copy-numbers *C4A* or *total C4* against SLE were conspicuous and highly significant. Over ¼ of healthy controls had high copy-numbers of *total C4* or *C4A*, and their frequencies were reduced in SLE ($p=8.8\times10^{-8}$ and $4.0\times10^{-9}$). Overall, there were reductions in SLE on the mean copy-numbers of *total C4* by 0.19, *C4A* by 0.15 and *short* genes by 0.12.

Compared with subjects of European ancestry, East-Asians have significantly higher GCNs of *total C4* and its associated variants (Table 4, Supporting Information: Fig. S5). In European-SLE, homozygous and heterozygous deficiencies of *C4A*, low GCNs of *total C4* and *long* genes were common and medium-to-high effect-size risk factors of SLE (25). Notably, 6.5% of had a homozygous *C4A* deficiency (OR=8.57), 27.6% had a heterozygous *C4A* deficiency (OR=1.97), 42.2% had low GCNs of *total C4* (OR=1.77). The variability of *total C4* or *C4A* in Europeans was driven by changes in copy-number of *long* genes instead of *short* genes as in East-Asians. Remarkably, 13.9% of European SLE patients had zero or

one copy of *long* genes, which were infrequent in Asians ($p=2.9\times10^{-19}$, Supporting Information: Fig. S5, panel H).

The main cause for *C4A*-deficiency among Europeans is the presence of mono-S coding for C4B1 and the absence of a *long C4A* gene in haplotypes with HLA-*DRB1*0301*. This mono-S haplotype has a frequency of 0.113 in healthy subjects and 0.169 in SLE (25). Strikingly, in our study cohort >2000 Asian subjects, none had a homozygous mono-S (which is a homozygous deficiency of *long* genes). A new and predominant mechanism for *C4A*-deficiency in Asians unraveled in this study is a bimodular-LS haplotype coding for C4B1-B96 with markers characteristic of an ancient recombination and is linked to *DRB1*1501* (or *DR2*).

Among SLE patients who ever experienced very-low serum levels of C4 and C3, low GCNs of *total C4, C4B* and *long* genes were major risk factors. Low GCN of *total C4* would result in lower rate of biosynthesis and therefore a lower reservoir of C4 protein. During an active disease, high reactivity and high turnover of activated C4B protein would lead to fast depletion and therefore very low levels complement. Very-low serum complement levels were also strongly correlated with the presence of dsDNA autoantibodies, IgG-anticadiolipin, hemolytic anemia, renal disease and younger age of disease onset. The association between low C3/C4 and the presence of anti-dsDNA was remarkably strong, with OR=4.96 and $p=9.7\times10^{-17}$. Renal disease occurred in greater than half of East-Asian SLE patients. Among SLE patients with low GCN of *total C4* (GCN=2 or 3), low C3/C4 protein levels had greater effects on the occurrence of renal disease [OR=3.95 (1.37-11.4), p=0.0067] than those with medium and high GCNs of *total C4* [OR=1.45 (0.98-2.16), p=0.064]. Associations of low complement with renal disease and/or hematologic disease of SLE were also observed earlier in US patients (9,45). Other serologic factors that correlate with renal disease of SLE are anti-dsDNA and anti-C1q. In a recent multicenter study, it was found that simultaneous positivity of anti-C1q, anti-dsDNA and low complement associated with renal SLE with a combined odds ratio of 14.9 (5.8-38.4) (46). Measuring serum levels of C3 and C4 and anti-C1q, cell-bound levels of processed complement (e.g., erythrocyte-C4d) (47), and CNV genotyping of *C4A* and *C4B* are desirable laboratory tests to facilitate precision management of SLE and minimize renal disease.

The co-existence of *C4A*-deficiency with *HLA* risk alleles *DRB1*0301* in Europeans and *DRB1*1501* in East-Asians in SLE and other autoimmune diseases continue to be a fascinating topic on whether *C4-CNVs* or *C4A*-deficiency and *DRB1* genetic variants are independent, additive or confounding risk factors (4,48-50). The pathologic effects for genetic and/or acquired deficiencies of C4, C1q, C1r and C1s on SLE have been overwhelming (4). Thus, it would be appropriate to investigate the feasibility of complement-guided therapeutics for SLE.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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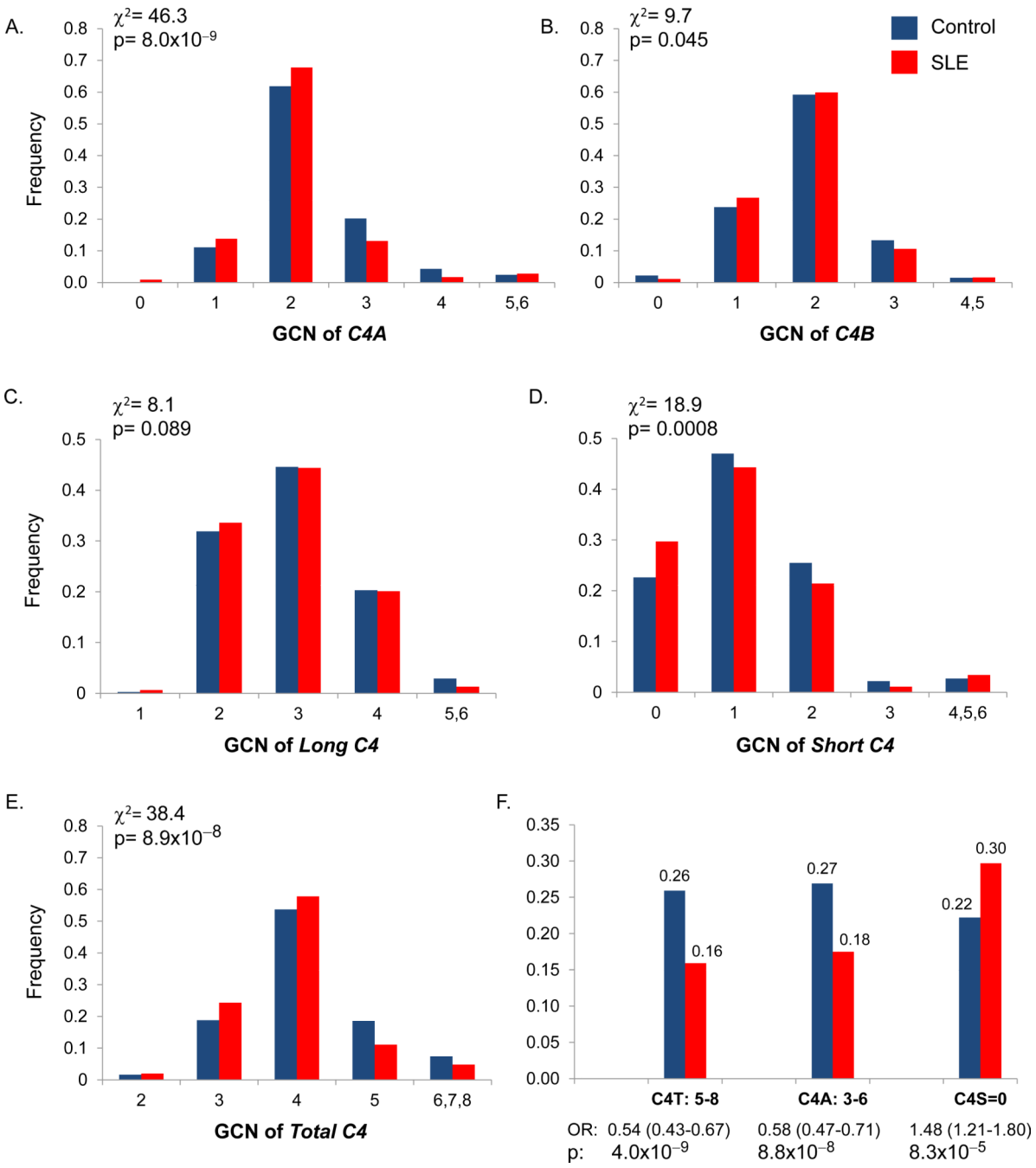


**Figure 1.**

Variations of complement *C4* gene copy-numbers (GCNs) in SLE patients (*red*) and controls (*blue*) of East-Asian ancestry. **A-E**, Distributions of GCN groups for *C4A* (**A**), *C4B* (**B**), *long* genes (**C**), *short* genes (**D**), *total C4* (**E**). **F**. A summary of *C4* genetic factors associated with SLE in East-Asians. High GCNs of *total C4* and *C4A* were strong protective factors and deficiency of *short* genes is a risk factor for SLE.

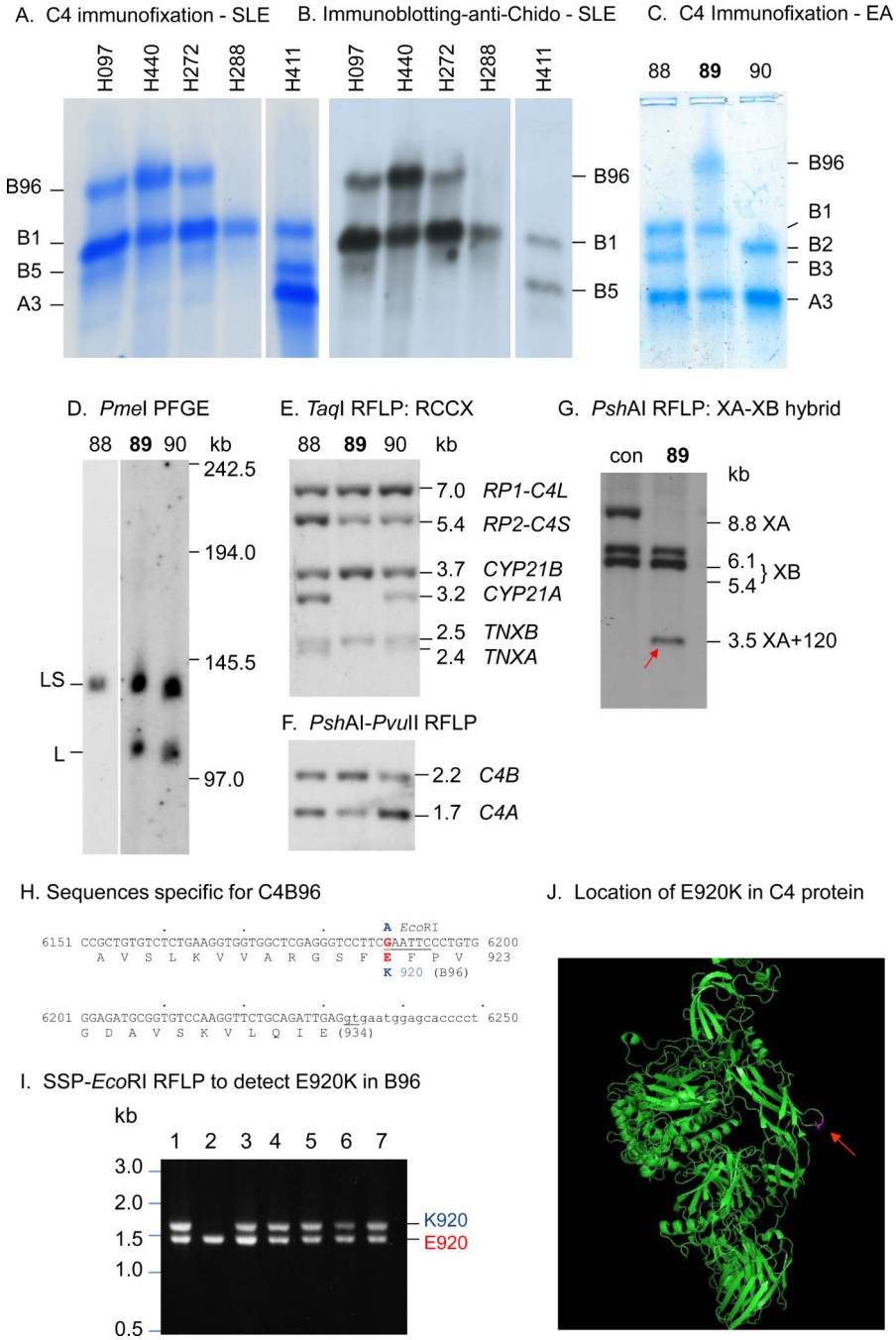


**Figure 2.**

*C4A*-deficiency in East-Asians. A. Immunofixation of C4 allotypes showing the homozygous deficiency of C4A protein in four SLE patients (H097, H440, H288 and H288) and one control (H411). **B**. Immunoblot analysis of C4 plasma protein from the same subjects shown in *panel* A using monoclonal antibodies against Chido antigens that is generally associated with C4B protein. **CH**, Characterization of C4B96 protein by immunofixation (**C**), RCCX haplotypes by long range mapping of *Pme*I-digested genomic DNA (**D**), *Taq*I RFLP of *RP-C4-CYP21-TNX* modules (**E**), *Psh*AI-*Pvu*II RFLP for relative

dosage of *C4B* and *C4A* genes (**F**) and rearrangement of RCCX in Subject-89 (**G**). A *red*-arrow indicates the presence of a *TNXA*-*XB* recombinant characterized by a 3.5 kb *Psh*AI restriction fragment after Southern blot analysis using a *TNX* 3' probe. **H**. DNA and amino acid sequences specific for C4B96 highlighting the E920K polymorphism with *Eco*RI RFLP. **I.** *Eco*RI RFLP of genomic PCR fragments from six subjects with C4B96 (lanes 1 and 3-7) and one subject *without* C4B96 (lane 2). **J**. The location of E920K in 3-dimensional structure of native C4 protein (indicated by a *red* arrow) (44). X-ray crystal structure of C4 was downloaded from RCSB PDB (http://www.rcsb.org/pdb/home/home.do; entry "4FXK", and visualized by PyMOL).

## Table 1

Clinical characteristics of East-Asian SLE patients

| | Hong Kong | | Taiwan | | Combined | |
|---|---|---|---|---|---|---|
| Age of onset (yr) | 30.27±10.70 | | 30.68±11.62 | | 30.61±11.45 | |
| Female/Male (N) | 172 / 8 | | 748 / 71 | | 920 / 79 | |
| | N (yes/no) | frequency | N (yes/no) | frequency | N (yes/no) | frequency |
| Malar rash | 113 / 67 | 0.628 | 467 / 352 | 0.570 | 580 / 419 | 0.581 |
| Discoid rash | 39 / 141 | 0.217 | 158 / 661 | 0.193 | 197 / 802 | 0.197 |
| Photosensitivity | 62 / 118 | 0.344 | 189 / 630 | 0.231 | 251 / 748 | 0.251 |
| Oral ulcer | 23 / 157 | 0.128 | 224 / 595 | 0.274 | 247 / 752 | 0.247 |
| Arthritis | 147 / 33 | 0.817 | 520 / 299 | 0.635 | 667 / 332 | 0.668 |
| Serositis | 35 / 145 | 0.194 | 229 / 590 | 0.280 | 264 / 735 | 0.264 |
| Renal Disease | 52 / 128 | 0.289 | 464 / 355 | 0.567 | 516 / 483 | 0.517 |
| Neurologic disease | 11 / 169 | 0.061 | 137 / 682 | 0.167 | 148 / 851 | 0.148 |
| Immunologic disorder | 150 / 30 | 0.833 | 600 / 219 | 0.733 | 750 / 249 | 0.751 |
| Hematologic disease | 118 / 62 | 0.656 | 598 / 221 | 0.730 | 716 / 283 | 0.717 |
| leukopenia <3.5K | | | 471 /348 | 0.575 | | |
| hemolytic anemia | | | 263 / 556 | 0.321 | | |
| thrombocytopenia | | | 216 / 602 | 0.264 | | |
| anti-dsDNA | | | 624 / 179 | 0.771 | | |
| anti-RNP-1 | | | 295 / 374 | 0.441 | | |
| anti-Sm | | | 262 / 408 | 0.391 | | |
| anti-Ro (Ssa) | | | 362 / 189 | 0.657 | | |
| anti-La (SSb) | | | 148 / 404 | 0.268 | | |
| ACA-IgG | | | 188 / 461 | 0.290 | | |
| ACA-IgM | | | 55 / 544 | 0.092 | | |
| Low C3/C4 | | | 641 / 166 | 0.794 | | |
| Pericarditis | | | 101 / 718 | 0.123 | | |
| Pleuralitis | | | 159 / 660 | 0.194 | | |
| Ascites | | | 43 / 776 | 0.053 | | |

Healthy controls included 765 subjects (396 females and 369 males) from Taiwan, 371 subjects (204 females, 162 males, 5 unknown sex) from Hong Kong, and 211 subjects (122 females and 89 males) from central Ohio, USA.

## Table 2

CNVs of complement *C4* as risk factors for SLE diagnostic disorders

| | | Yes | No | | |
|---|---|---|---|---|---|
| **Disorders** | **Group** | **N (freq.) / GCI** | **N (freq.) / GCI** | **OR (95% CI)** | **p** |
| *a. Potentially causal relationship* | | | | | |
| Malar Rash | *C4A* = 0 | 9 (.016) | 0 (0) | na[1] | 0.0016 |
| | *C4A* ≥ 1 | 547 (.984) | 407 (1.0) | | |
| | *C4A GCI* | 2.05±.82 | 2.16±.75 | | 0.034 |
| Thrombocytopenia | *C4B* = 0 | 0 (0) | 11 (.019) | na[2] | 0.0093 |
| | *C4B* ≥ 1 | 205 (1.0) | 564 (.981) | | |
| | *C4B GCI* | 1.83±.63 | 1.82±.69 | | ns |
| *b. Reduced GCN as a risk factor* | | | | | |
| Low C3/C4 | *C4-total* = 2-3 | 183 (.306) | 18 (.118) | 3.27 (1.94-5.52) | $7.0\times10^{-7}$ |
| | *C4-total* ≥ 4 | 416 (.695) | 134 (.882) | | |
| | *C4-total GCI* | 3.88±.89 | 4.16±.83 | | 0.0003 |
| | *C4B* = 0-1 | 194 (.324) | 24 (.158) | 2.55 (1.60-4.08) | $2.5\times10^{-5}$ |
| | *C4B* = 2-5 | 405 (.676) | 128 (.842) | | |
| | *C4B GCI* | 1.77±.67 | 2.03±.65 | | $1.3\times10^{-5}$ |
| | *C4-Long = 1-3* | 490 (.801) | 107 (.686) | 1.84 (1.24-2.72) | 0.0028 |
| | *C4-Long = 4-6* | 122 (.199) | 49 .314) | | |
| | *C4-Long GCI* | 2.87±.78 | 3.06±.83 | | 0.0084 |
| Anticardiolipin-IgM | *C4B* = 0-1 | 23 (.426) | 145 (.282) | 1.89 (1.07-3.36) | 0.032 |
| | *C4B* = 2-5 | 31 (.574) | 370 (.718) | | |
| | *C4B GCI* | 1.63±.73 | 1.84±.68 | | 0.029 |
| Discoid rash | *C4-Short* = 0-1 | 124 (.821) | 475 (.736) | 1.64 (1.05-2.58) | 0.026 |
| | *C4-Short* = 2-6 | 27 (.179) | 170 (.264) | | |
| | *C4-Short GCI* | 0.83±.87 | 1.08±1.03 | | 0.0060 |
| Arthritis | *C4-Short* = 0 | 174 (.344) | 76 (.261) | 1.48 (1.07-2.03) | 0.016 |
| | *C4-Short* ≥ 1 | 326 (.656) | 214 (.738) | | |
| | *C4-Short GCI* | 0.97±.96 | 1.15±1.06 | | 0.017 |
| *c. Increased GCN as a risk factor* | | | | | |
| Pericarditis | *C4A* = 3-6 | 28 (.292) | 106 (.154) | 2.26 (1.39-3.67) | 0.0017 |
| | *C4A* = 0-2 | 68 (.708) | 581 (.846) | | |
| | *C4A GCI* | 2.27±.79 | 2.08±.78 | | 0.027 |
| Hemolytic anemia | *C4A* = 3-6 | 58 (.234) | 76 (.142) | 1.84 (1.26-2.70) | 0.0019 |
| | *C4A* = 0-2 | 190 (.766) | 459 (.858) | | |
| | *C4A GCI* | 2.22±.88 | 2.05±.74 | | 0.0061 |
| Thrombocytopenia | *C4-Short* = 2-6 | 67 (.328) | 124 (.216) | 1.77 (1.24-2.52) | 0.0018 |
| | *C4-Short* = 0-1 | 137 (.672) | 449 (.784) | | |
| | *C4-Short GCI* | 1.27±1.17 | 0.94±.93 | | $5.8\times10^{-5}$ |
| | *C4A* = 3-6 | 46 (.224) | 88 (.153) | 1.61 (1.08-2.40) | 0.022 |
| | *C4A* = 0-2 | 159 (.776) | 489 (.848) | | |

| | | Yes | No | | |
|---|---|---|---|---|---|
| **Disorders** | **Group** | **N (freq.) / GCI** | **N (freq.) / GCI** | **OR (95% CI)** | **p** |
| | *C4A GCI* | 2.26±.98 | 2.05±.70 | | 0.0012 |

GCI, gene copy-index – the mean of gene copy-number in a population with standard deviation; OR, odds ratio p-values for continuous data were calculated by t-tests; p-values and odds ratios of categorical data were derived by $\chi^2$ analyses.

na[1], odds ratio not applicable; all nine SLE patients with homozygous C4A deficiency had malar rash; na[2], odds ratio not applicable; no subjects with thrombocytopenia had a homozygous C4B-deficiency; ns, not significant

## Table 3

Associations of low serum complement protein levels (C4/C3)* with clinical and immunologic features of EA-SLE.

| | Yes (N=641) frequency | No (N=166) frequency | OR (95% CI) | p |
|---|---|---|---|---|
| Malar rash | 0.577 | 0.530 | | ns |
| Discoid rash | 0.184 | 0.217 | | ns |
| Photosensitivity | 0.225 | 0.259 | | ns |
| Oral ulcers | 0.270 | 0.295 | | ns |
| Arthritis | 0.619 | 0.693 | | 0.08 |
| Neurologic disorder | 0.181 | 0.120 | | 0.055 |
| Immunologic disorders** | | | | |
| Anti-dsDNA | 0.846 | 0.524 | 4.96 (3.41-7.22) | $9.7\times10^{-17}$ |
| Anti-Sm | 0.429 | 0.241 | 2.37 (1.54-3.64) | $3.6\times10^{-5}$ |
| Anti-RNP-1 | 0.474 | 0.314 | 1.97 (1.32-2.94) | 0.0007 |
| Anti-Ro/SSa | 0.676 | 0.600 | | ns |
| Anti-La/SSb | 0.843 | 0.748 | 1.80 (1.10-2.98) | 0.016 |
| Aca-IgG | 0.320 | 0.168 | 2.33 (1.41-3.87) | 0.0004 |
| Aca-IgM | 0.097 | 0.068 | | ns |
| Hematologic disorders | | | | |
| Hemolytic anemia | 0.373 | 0.133 | 3.89 (2.42-6.27) | $3.6\times10^{-10}$ |
| Leukopenia ≤3.5k | 0.607 | 0.446 | 1.92 (1.36-2.71) | 0.0002 |
| Thrombocytopenia | 0.281 | 0.206 | 1.50 (0.99-2.27) | 0.048 |
| Renal disease | 0.608 | 0.416 | 2.18 (1.54-3.09) | $8.5\times10^{-6}$ |
| Serositis | | | | |
| Ascites | 0.062 | 0.018 | 3.62 (1.10-11.8) | 0.011 |
| Pericarditis | 0.140 | 0.060 | 2.55 (1.29-5.01) | 0.0027 |
| Age of onset (yrs±SD) | 29.98±10.95 | 33.29±13.75 | | 0.0011 |

ns, not significant

* Low C3 was defined as a documentation of serum C3 concentration <700 mg/L; low C4 was defined as a documentation of serum C4 concentration <100 mg/L.

** As proposed by SLICC in 2012, low C4/C3 is one of the diagnostic factors for an immunologic disorder in SLE.

## Table 4

CNVs of complement *C4* as a risk or protective factor in human SLE: a comparison between East-Asian and European-American.[¶]

| | East-Asian | European-American[*] |
|---|---|---|
| ***Total C4*** | | |
| SLE - GCI: | 3.95 ± 0.87 | 3.56 ± 0.78 |
| Controls - GCI: | 4.14 ± 0.92 | 3.83 ± 0.69 |
| difference | −0.19 | −0.27 |
| p | $3.7\times10^{-7}$ | $3.6\times10^{-6}$ |
| C4T=2+3, OR: | 1.45 (1.20-1.77) | 1.77 (1.28-2.45) |
| C4T=5-8, OR: | 0.55 (0.45-0.68) | 0.53 (0.30-0.94) |
| ***C4A*** | | |
| SLE - GCI: | 2.09 ± 0.79 | 1.80 ± 0.90 |
| Controls - GCI: | 2.25 ± 0.82 | 2.09 ± 0.75 |
| difference | −0.16 | −0.30 |
| p | $3.4\times10^{-6}$ | $3.4\times10^{-6}$ |
| C4A=0, OR: | 12.4 (1.57-97.9) | 8.57 (2.81-26.1) |
| C4A=1, OR: | 1.28 (1.00-1.65) | 1.97 (1.36-2.85) |
| C4A≥3, OR: | 0.58 (0.47-0.71) | 0.55 (0.37-0.83) |
| ***C4B*** | | |
| SLE - GCI: | 1.85 ± 0.68 | 1.78 ± 0.59 |
| Controls - GCI: | 1.88 ± 0.71 | 1.73 ± 0.63 |
| difference | −0.03 | −0.04 |
| p | ns | ns |
| ***Long C4*** | | |
| SLE - GCI: | 2.88 ± 0.79 | 2.63 ± 1.16 |
| Controls - GCI: | 2.94 ± 0.82 | 2.95 ± 0.98 |
| difference | −0.06 | −0.31 |
| p | ns | 0.0002 |
| C4L≤2, OR: | na | 1.66 (1.26-2.18) |
| ***Short C4*** | | |
| SLE - GCI: | 1.05 ± 0.97 | 0.98 ± 0.85 |
| Controls - GCI: | 1.17 ± 0.94 | 0.89 ± 0.79 |
| difference | −0.12 | 0.09 |
| p | 0.005 | ns |
| C4S=0, OR: | 1.48 (1.22-1.80) | na |

na, not applicable; ns, not significant. GCI: gene copy-index - the mean of gene copy-number (± standard deviation).

[¶]See also Supporting Information Figure S5.

[*]European-American data derived from White Ohio healthy subjects (N=500) and 232 White Ohio SLE patients (25).