

CONCISE REPORT

Polymerase chain reaction based C4AQ0 and C4BQ0 genotyping: association with systemic lupus erythematosus in southwest Han Chinese

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Objective: To investigate the association of complement C4 null genes (C4Q0, including C4AQ0 and C4BQ0) and C2 gene with systemic lupus erythematosus (SLE) in southwest Han Chinese; 136 patients with SLE and 174 matched controls were genotyped.

Methods: C4 null genes were determined by a polymerase chain reaction (PCR) procedure with sequence specific primers (PCR-SSP). The 2 bp insertion in exon 29, which was previously identified in non-Chinese populations and caused defective C4A genes, was directly typed by sequencing the whole exon 29 using exon specific primers. The exon 6 of complement C2 was also sequenced in both the patients and controls.

Results: The frequency of homozygous C4AQ0 allele was 12.5% (17/136) in patients with SLE compared with 1.1% (2/174) in controls ($p < 0.001$, odds ratio (OR)=12.286, 95% confidence interval (95% CI) 2.786 to 54.170). There was no significant difference for homozygous C4BQ0 allele between patients with SLE and controls ($p = 0.699$). Patients with the C4AQ0 gene had an increased risk of acquiring renal disorder, serositis, and anti-dsDNA antibodies compared with those without C4AQ0 (for renal disorder, $p = 0.018$, OR=8.951, 95% CI 1.132 to 70.804; for serositis, $p = 0.011$, OR 4.891, 95% CI 1.574 to 15.198; for anti-dsDNA, $p = 0.004$, OR 7.630, 95% CI 1.636 to 35.584). None of the patients or controls had the 2 bp insertion in exon 29 of the C4 gene. The type I C2 deficiency was not detected in the 310 samples.

Conclusion: It is suggested that deficiency of C4A (not due to a 2 bp insertion in exon 29), but not C4B or C2, may be a risk factor for acquiring SLE in south west Han Chinese; this results in increased risk of renal disorder, serositis, and anti-dsDNA antibodies in patients with SLE. Racial differences seem to be relevant in susceptibility to SLE

The association of C4 null genes (C4Q0, including C4AQ0 and C4BQ0) with systemic lupus erythematosus (SLE) has previously been well documented based on electrophoretic mobility of C4 protein in plasma.¹ The C4A null gene was increased in SLE in most populations studied, including Anglo-Irish, North Americans, Australians, Japanese, Koreans, southern Chinese, black Americans, and Mexican Americans.² The present study extensively examined the possible association between homozygous C4Q0 and SLE in a large cohort of Han Chinese patients with SLE from south west China, with the further intention of identifying the molecular basis of C4Q0 and investigating type I C2 deficiency.

PATIENTS AND METHODS

The patient group comprised 136 unrelated patients who fulfilled the American College of Rheumatology classification criteria for SLE. Among them, women represented 89% (121/136) and men 11% (15/136). The median age was 32.9 (SD 11.9) years (range 10–67 years). One hundred and seventy four randomly selected, unrelated healthy people whose sex, age, and home town matched those of the patients were investigated as controls. This study was approved by the patients and was in accordance with the principles of the Declaration of Helsinki. Genomic DNA was extracted from whole blood with a standard phenol/chloroform method.

PCR amplification of C4 null genes

According to Barba *et al*, a polymerase chain reaction (PCR) with sequence specific primers (PCR-SSP) was used to define C4Q0.³ A set of four isotype specific primers has been designated to amplify the two isotypes C4A and C4B. Table 1 shows the primers and length of fragments amplified. Amplification was performed with a touchdown protocol. After a first denaturation step at 94°C for five minutes, the first six cycles were carried out at decreasing annealing temperatures in 1°C steps for each cycle, from 68°C to 63°C, followed by 29 cycles using the following conditions: 30 seconds at 94°C, one minute at 63°C, and one minute at 72°C. Primers C4F and C4R (table 1) were used for specific amplification of exon 29 in complement C4 under the same conditions as those of C4Q0 but with different cycles (95°C for two minutes, 35 cycles at 94°C for 50 seconds, 63°C for one minute, and 72°C for one minute and five minutes). Type I C2 deficiency was defined by using primer pairs C2F and C2R (table 1) as described previously.⁴ The products were then run on a 5% polyacrylamide gel and visualised by ethidium bromide staining.

DNA sequencing

All 310 samples were sequenced for exon 29 in C4. Fifty samples (24 patients and 26 controls) randomly selected from a total of 310 subjects were sequenced for C2 exon 6 after typing by polyacrylamide gel electrophoresis. Products from the PCR, purified on spin columns (Watson BioTechnologies Inc, Shanghai), were directly sequenced for both strands with a BigDye™ Terminator Cycle Sequence Kit (ABI Applied Biosystems).

Statistical analysis

Statistical analyses were performed using SPSS 10.0 for Windows. Categorical variables were compared by Fisher's exact test. Continuous variables were compared by Student's *t* test.

Abbreviations: C4 null genes, C4Q0, including C4AQ0 and C4BQ0; 95% CI, 95% confidence interval; OR, odds ratio; PCR-SSP polymerase chain reaction with sequence specific primers; SLE, systemic lupus erythematosus

Table 1 Genotyping primers used in current study

Gene	Primer	Sequence (5'-3')	Length of PCR Products (bp)	Reference
C4A/C4AQ0	Aup	GCATGCTCCTGTCTAACACTGGAC	377/null	Barba <i>et al</i> ³
	L3	TGCGGATCCAGCAGTTTCGGAAG		
	Ado	AGGACCCCTGTCCAGTGTAGAC	578/null	
	L4	ATAGGATCCTAAGGTCCCCTGGGCCT		
C4B/C4BQ0	Bup	TGCTCCTATGATCACTGGAGAGA	377/null	Barba <i>et al</i> ³
	L3	TGCGGATCCAGCAGTTTCGGAAG		
	Bdo	AGGACCTCTCCAGTGATACAT	578/null	
	L4	ATAGGATCCTAAGGTCCCCTGGGCCT		
C4 exon 29	C4F	CTTCTGTTTACTTGTGGTCTCC	478	Current study
	C4R	AGCTGCAGCGCTGGGACTTGA		
C2 exon 6	C2F	AAAGCTTGGGCCGTAATAATCCAGCG	180	Johnson <i>et al</i> ⁴
	C2R	GAGCACAGGAAGGCCTCTCTGCAGG		

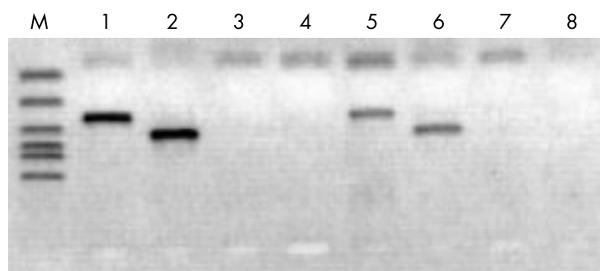


Figure 1 Specific PCR products of the C4Q0 homozygotes. Lanes 1 (Aup/L3) and 2 (Ado/L4) are the products of a sample without C4AQ0; lanes 3 (Aup/L3) and 4 (Ado/L4) are the products of a sample with C4AQ0; lanes 5 (Bup/L3) and 6 (Bdo/L4) are the products of a sample without C4BQ0; lanes 7 (Bup/L3) and 8 (Bdo/L4) are the products of a sample with C4BQ0, respectively. M, DNA Marker DL2000 (TaKaRa, code No D501 CA). A C2 gene specific PCR was used as positive control.

The possible associations between C4Q0 alleles and a range of clinical features were estimated by χ^2 analyses. The odds ratio (OR) and 95% confidence interval (95% CI) for each association were determined.

RESULTS

PCR amplification of the C4Q0 genes

Two fragments, 377 bp and 578 bp, were clearly discerned on 2% agarose gel (fig 1). Samples without C4A genes at both C4A loci or those without C4B genes at both C4B loci could not be amplified by the two pairs of primers.

Homozygous C4Q0 gene frequencies in patients with SLE and controls

There were seventeen patients (12.5%) with the C4AQ0 gene compared with only two controls (1.1%) ($p < 0.01$, OR=12.286; 95% CI 2.786 to 54.170). Table 2 shows the association between phenotypes of SLE and C4AQ0. There was no correlation between anti-dsDNA antibodies and serositis ($p = 0.169$), or between anti-dsDNA antibodies and renal disorder ($p = 0.171$) in the patient group.

Two of 136 (1.5%) patients and four of 174 controls were C4BQ0 positive (2.3%) ($p = 0.699$). The prevalence of C4AQ0 compared with C4BQ0 was significantly different in patients ($p = 0.015$), but not in controls ($p = 1.00$).

C4 exon 29 and C2 exon 6 typing

None of the 136 patients (including 17 C4AQ0 positive) or the 174 controls (including two C4AQ0 positive) sequenced for the exon 29 in C4 gene had the 2 bp insertion. No patients or

Table 2 Association of C4AQ0 with SLE phenotypes

SLE phenotype	C4AQ0 positive	C4AQ0 negative	p Value	OR (95% CI)
Malar rash	12	80	0.745	0.796 (0.231 to 2.748)
Discoid erythema	5	27	0.753	1.186 (0.345 to 4.081)
Photosensitivity	5	43	0.402	0.550 (0.163 to 1.852)
Oral ulcers	4	29	0.759	0.730 (0.190 to 2.800)
Arthritis	11	86	0.150	0.390 (0.118 to 1.295)
Serositis	10	27	0.011*	4.891 (1.574 to 15.198)
Renal disorder	16	72	0.018*	8.951 (1.132 to 70.804)
Neurological	6	17	0.129	3.000 (0.891 to 10.096)
Haematological	12	64	0.409	1.742 (0.517 to 5.866)
Anti-dsDNA	15	54	0.004**	7.630 (1.636 to 35.584)
ANA	14	70	0.223	0.333 (0.071 to 15.71)
Anti-Sm	5	31	1.000	0.907 (0.250 to 3.293)
Anti-U1RNP	6	26	0.508	1.656 (0.474 to 5.790)
Anti-Ro	9	32	0.063	3.448 (0.954 to 12.458)
Anti-La	5	20	0.262	2.179 (0.567 to 8.372)
24 h urinary protein (SD) (g/24 h)	3.49 (4.55)	1.38 (2.60)	0.045*†	

*Difference is significant at the 0.05 level (two tailed).
 **Difference is significant at the 0.01 level (two tailed).
 †† test.
 p Value from Fisher's exact test (two tailed).

controls had the 28 bp deletion in exon 6 of the C2 gene. Further sequencing of the region in a randomly selected 50 samples confirmed the absence of the deletion in these samples.

DISCUSSION

Previous studies have shown that homozygous and heterozygous C4 deficiency was more prevalent in patients with SLE than in controls.^{5,6} Our data here also suggested a strong association between homozygous C4AQ0 and SLE (12.5% *v* 1.1%, OR=12.286, *p*<0.01), but no association between the C4BQ0 distribution and SLE, which was in general agreement with other studies which found no association between C4BQ0 and SLE based on serum phenotype and restriction fragment length polymorphism,⁷ although the C4B null allele played an important part in SLE susceptibility in the Spanish population.⁸ The prevalence of C4AQ0 increased compared with C4BQ0 in the SLE group suggesting a specific role for C4A deficiency as a predisposing factor in south west Han Chinese with SLE.

Most of the previous studies concluded that C4AQ0 did not correlate with clinical or laboratory subsets of SLE. However, in our study, we found that the frequencies of renal disorder, serositis, and anti-dsDNA antibodies were higher in patients with C4AQ0 than in those without (although the different clinical subgroups were relatively small). The ORs for them were 8.951, 4.891, and 7.630, respectively. Combined with an increase in 24 hour protein in urine in patients with C4AQ0, these results suggested that C4AQ0 might be a highly predisposing factor for lupus nephritis, and this could explain why the kidneys were not involved in some patients, whereas in others there was rapidly progressive destruction of the renal parenchyma. Our suggestions are in accordance with the results of Clemenceau *et al*, who reported that renal involvement was more frequent in patients with C4AQ0 or C4AQ0/C4AQ0 than in the patients without null C4 (9/11 *v* 3/12).⁹

Anti-dsDNA antibody is thought to play an important part in the pathogenesis of the autoimmune diseases and to be a specific autoantibody for SLE. We found that anti-dsDNA was correlated with C4AQ0, but anti-dsDNA antibody had no association with renal disorder and serositis in our study. Thus, C4AQ0 was probably one cause, but not the only one, in producing anti-dsDNA antibody.

There was an obvious difference in the relative prevalence of C4A and C4B deficiencies in our samples from patients with SLE. C4A is more efficient at processing immune complexes and C4B has greater haemolytic activity. Deficiency of C4A results in impairment in the clearance of immune complexes and hence their overproduction. These complexes will deposit at blood abundant sites such as the kidney, synovium, skin, and lung parenchyma and incite inflammation, giving rise to, for example, nephritis and serositis.¹⁰

Previous studies have shown that one of the causes of a C4A null allele is a 2 bp (CT) insertion in exon 29 that leads to a premature stop codon;¹¹ the 2 bp insertion was found more often in patients with SLE compared with controls and was more common in white patients (3.4%) than in Afro-American patients with SLE (1.8%).¹² However, in 136 patients and 174 controls in this study, we did not detect the 2 bp insertion. It seems that the 2 bp insertion in exon 29 was not a main cause for C4A null alleles in south west Han Chinese.

The involvement of C2 deficiencies as a predisposing factor for the occurrence of SLE remains in dispute. Type I C2 deficiency in white people was significantly more common in patients with SLE than in controls, but the 28 bp deletion in C2 was not found in either Afro-American controls or Afro-American patients.¹³ We did not detect the type I C2 deficiency in any of the 136 patients and 174 controls; moreover, in the 210 Taiwanese Han reported on by Lu *et al*, none had the deletion.¹⁴ It thus seemed that the type I C2 deficiency was either very rare or absent in Chinese people, and was not a

factor associated with SLE in Chinese patients. The above data supported the suggestion of an ethnic difference in genetic associations with SLE.¹⁵

In summary, C4AQ0, but not C4BQ0, might be a predisposing factor for SLE in south west Han Chinese patients with SLE. C4AQ0 was also found to be associated with renal disorder, serositis, and anti-dsDNA antibodies in our patients. A 2 bp insertion in exon 29 was not a cause for C4AQ0 in our samples, and type I C2 deficiency was absent in all the 310 subjects. Our results give support to the suggestion that there are appreciable racial and ethnic differences in susceptibility to SLE.¹⁵

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REFERENCES

- Howard PF, Hochberg MC, Bias WB, Arnett FCJ, McLean RH. Relationship between C4 null genes, HLA-D region antigens, and genetic susceptibility to systemic lupus erythematosus in Caucasian and black Americans. *Am J Med* 1986;81:187-93.
- Doherty DG, Ireland R, Demaine AG, Wang F, Veerapan K, Welsh KI, *et al*. Major histocompatibility complex genes and susceptibility to systemic lupus erythematosus in southern Chinese. *Arthritis Rheum* 1992;35:641-6.
- Barba GMR, Braun-Heimer L, Rittner C, Schneider PM. A new PCR-based typing of the Rodgers and Chido antigenic determinants of the fourth component of human complement. *Eur J Immunogenet* 1994;21:325-39.
- Johnson CA, Densen P, Hurford RK, Colten HR, Wetsel RA. Type I human complement C2 deficiency. *J Biol Chem* 1992;267:9347-53.
- Hartung K, Baur MP, Coldevey R, Fricke M, Kalden JR, Lakomek HJ, *et al*. Major histocompatibility complex haplotypes and complement C4 alleles in systemic lupus erythematosus. Results of multicenter study. *J Clin Invest* 1992;90:1346-51.
- Steinsson K, Arnason A, Erendsson K, Fossdal R, Skafstadottir I, Jonsdottir S, *et al*. A study of the major histocompatibility complex in a Caucasian family with multiple cases of systemic lupus erythematosus: association with the C4AQ0 phenotype. *J Rheumatol* 1995;22:1862-6.
- Petri M, Watson R, Winkelstein JA, McLean RH. Clinical expression of systemic lupus erythematosus in patients with C4A deficiency. *Medicine* 1993;72:236-44.
- Naves M, Hajeer AH, Teh LS, Davies EJ, Ordi-Ros J, Perez-Pemén P, *et al*. Complement C4B null allele status confers risk for systemic lupus erythematosus in a Spanish population. *Eur J Immunogenet* 1998;25:317-20.
- Clemenceau S, Castellano F, Montes de OM, Kaplan C, Danon F, Levy M. C4 null alleles in childhood onset systemic lupus erythematosus. Is there any relationship with renal disease? *Pediatr Nephrol* 1990;4:207-12.
- Walport MJ. Complement. Second of two parts. *N Engl J Med*. 2001;344:1140-4.
- Barba G, Rittner C, Schneider PM. Genetic basis of human complement C4A deficiency. *J Clin Invest* 1993;91:1681-6.
- Sullivan K, Kim NA, Goldman D, Petri MA. C4A deficiency due to a 2bp insertion is increased in patients with systemic lupus erythematosus. *J Rheumatol* 1999;26:2144-7.
- Sullivan KE, Petri MA, Schmeckpeper BJ, McLean RH, Winkelstein JA. Prevalence of a mutation causing C2 deficiency in systemic lupus erythematosus. *J Rheumatol* 1994;21:1128-33.
- Lu LY, Ding WZ, Fici D, Deulofeut R, Cheng HH, Cheu CC, *et al*. Molecular analysis of major histocompatibility complex allelic associations with systemic lupus erythematosus in Taiwan. *Arthritis Rheum* 1997;40:1138-45.
- Kristjansdottir H, Bjarnadottir K, Hjalmarsson IB, Grondal G, Arnason A, Steinsson K. A study of C4AQ0 and MHC haplotypes in Icelandic multigene families with systemic lupus erythematosus. *J Rheumatol* 2000;27:2590-6.