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A study of the genetic basis of C4A protein deficiency. Detection of C4A gene deletion by long-range PCR and its associated haplotypes

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Objective: To study the frequency of a C4A gene deletions as the genetic basis of C4A protein deficiency (C4AQ0) and its associated haplotypes in Icelandic families with systemic lupus erythematosus (SLE).

Methods: Nine multiplex SLE families were genotyped for C4A gene deletions using a long-range polymerase chain reaction (LR-PCR) method, and major histocompatibility complex (MHC) haplotypes were defined.

Results: Of the SLE patients, first-degree and second-degree relatives, 53.8%, 47.9%, and 28.6% had C4AQ0, respectively. A C4A gene deletion was found to be the genetic basis for C4AQ0 in 64.3% of SLE patients, 60.0% of first-degree and 50.0% of second-degree relatives. All individuals carrying haplotype B8-C4AQ0-C4B1-DR3 had a deletion, and the deletion was also found on haplotypes B8-C4AQ0-C4B1-DR7 and B7-C4AQ0-C4B1-DR3.

Conclusion: The study shows that a C4A gene deletion is the most common genetic basis for C4AQ0. It accounts for two-thirds of C4AQ0 and is found on different MHC haplotypes. One-third of C4AQ0 is due to other as yet undefined genetic changes. The results demonstrate a heterogeneous genetic background for C4AQ0, giving further support for the hypothesis that C4AQ0 may be an independent risk factor for SLE.

Epidemiological and genetic data suggest that in systemic lupus erythematosus (SLE) disease expression reflects a complex interplay between genetic, environmental, and hormonal factors (1). Genes within the major histocompatibility complex (MHC) region have been implicated in SLE, in particular MHC class III genes, among them genes encoding complement component C4, as well as MHC class I and II genes encoding molecules, which participate in antigen presentation (2–4). Assessing the risks associated with individual MHC alleles is, however, complicated by the strong linkage disequilibrium among the different alleles within the region. C4A protein deficiency (C4AQ0) has been associated with SLE (5, 7). In Icelandic SLE patients the frequency of C4AQ0 (homo- and heterozygotes combined) is >50% compared to 25% in a population control group (8, 9).

C4 ‘null alleles’ are defined by protein electrophoresis (10), relying on visual inspection of protein band intensities to identify heterozygous ‘null

alleles’. Homozygous ‘null alleles’ are identified by the lack of a protein band. Detection of heterozygous ‘null alleles’ may, however, be difficult when relying only on protein band intensities, resulting in heterozygous ‘null alleles’ being identified as normal.

The molecular basis for C4AQ0 alleles differs between ethnic groups. In Caucasians a large deletion, covering most of the C4A gene and the 21-hydroxylase gene (21-OHA), has been estimated to account for up to two-thirds of C4AQ0 (3). The gene deletion has been associated with the haplotype B8-C4AQ0-C4B1-DR3 (11). Other mutations in the C4A gene, leading to C4AQ0, have been described; a 2 base pair insertion in exon 29 (12) and a 1 base pair deletion in exon 20 (13).

The C4A and C4B genes show 99% homology. All C4A genes analysed to date have been found to be 22 kb long, containing a 6.5 kb retroviral insert (HERV-K(C4) in intron 9, and the C4B gene has the retrovirus present in approximately a 2:1 ratio (C4B long and C4B short) (14). This has made detection of the C4A gene deletion in the heterozygous form difficult using standard polymerase chain reaction (PCR) protocols. The conventional method for detecting C4A gene deletion is restriction fragment length polymorphism (RFLP) analysis with Southern blot (15), which requires large amounts of DNA.

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Recently, we established a long-range PCR (LR-PCR) method to detect the C4A gene deletion in both the heterozygous and homozygous forms, using a specific assay for the C4A deletion and another assay specific for non-deleted C4A genes (16).

In the present study on multiplex SLE families the frequency of a C4A gene deletion was determined by LR-PCR, and haplotypes carrying the deletion were identified.

Materials and methods

Study groups

From nine Icelandic multiplex SLE families, all with at least two SLE patients, a total of 113 family members were studied. Of 32 patients diagnosed with SLE, samples were not available from six patients.

Thus, 26 SLE patients were studied along with 73 first- and 14 second-degree relatives (Figure 1). The multiplex SLE families have previously been studied for C4AQ0 by protein electrophoresis and analysed for MHC haplotypes. All SLE patients fulfilled at least four of the American Rheumatism Association (ARA) 1982 criteria for the classification of SLE.

C4A deletion genotyping by long-range PCR

LR-PCR was carried out on 100 ng of genomic DNA as described by Grant et al (16), using G11 universal forward primer (5'-TCTAGCTTCAG-TACTTCCAGCCTGT-3'), C4A gene deletion reverse primer (5'-GATGACACAAAATACCAG-GATGTGA-3'), and C4A non-deletion reverse primer (5'-TGGTCCCCAACATGTCTGTGCATGCTG-3').

Figure 1. Pedigrees of Icelandic multiplex SLE families. ■ SLE, □ Normal (non-SLE), ⊞ SLE patient not tested, ⊠ Deceased, □ Not tested. Descriptors below symbol: MHC haplotypes carrying C4A deletion: *a: B8-C4AQ0-C4B1-DR3, *d: B8-C4AQ0-C4B1-DR7, *m: B7-C4AQ0-C4B1-DR3.

Screening for mutations in exons 20 and 29

PCR methods were applied according to protocol to screen for a point mutation in exon 20 (12) and exon 29 (13) of the C4A gene.

C4 protein analysis and MHC typing

C4A and B proteins were analysed by high-voltage agarose electrophoresis on carboxypeptidase- (Sigma Type I) and neuraminidase- (Sigma Type VIII) treated serum samples (17). Typing of HLA-B allotypes was performed on serum samples using the lymphocytotoxicity test (18) and typing of HLA-DR alleles by PCR with sequence specific primers (PCR-SSP) (Dynal) (19).

Results

Frequency of C4A protein deficiency (C4AQ0)

The frequency of C4AQ0 in the families was studied by protein electrophoresis (Table 1A). The overall frequency of C4AQ0 in all family members (n=113) was 46.9% (10.6% homozygous and 36.3% heterozygous). The population frequency of C4AQ0 (heterozygous and homozygous combined) in Iceland is 25%. A high background of C4AQ0 is seen in seven of the nine families. Only families 3 and 6 had frequency of C4AQ0 less 25%.

SLE patients, first-degree and second-degree relatives of the patients were analysed as separate groups. Of the 26 SLE patients studied, 14 (53.8%) (11.5% homozygous and 42.3% heterozygous) had C4AQ0; of the 73 first-degree relatives 35 (47.9%) had C4AQ0 (12.3% homozygous and 35.6% heterozygous); and of the 14 second-degree relatives, four (28.6%) had C4AQ0 (all were heterozygous) (Table 1A).

Frequency of C4A gene deletion in family members with C4A protein deficiency

All family members (n=113) were genotyped for C4A gene deletion using LR-PCR, but to confirm the frequency of C4A deletions as the genetic basis for C4AQ0, calculations were performed only on family members with C4AQ0 (n=53) (Table 1B). Of all family members with C4AQ0 (n=53), 32 (60.4%) had a C4A deletion (5.7% homozygous and 54.7% heterozygous). Of 14 SLE patients with C4AQ0, nine (64.3%) carried a C4A gene deletion. No SLE patient was homozygous for a gene deletion. Of the 35 first-degree relatives with C4AQ0, 21 (60.0%) carried a C4A gene deletion, three (8.6%) were homozygous, and 18 (51.4%) heterozygous. Of the four second-degree relatives with C4AQ0, two (50%) carried a C4A gene deletion (both were heterozygous).

Homozygosity for C4AQ0 and/or C4A gene deletions

A total of 12 family members were homozygous for C4A protein deficiency (C4AQ0), three (11.5%) SLE patients and nine first-degree relatives (Table 1A). Only three family members, all second-degree relatives, were homozygous for C4A gene deletion (Table 1B).

Comparison of C4A protein data and LR-PCR gene deletion data

The LR-PCR revealed discrepancies in C4A protein analysis. Two family members previously analysed as having normal C4A protein status by protein analysis were, by LR-PCR, found to be heterozygous for C4A gene deletion.

Table 1. (A) C4A protein analysis. Frequency of C4A protein deficiency (C4AQ0) among family members of the multiplex SLE families. (B) Results of C4A gene deletion genotyping with LR-PCR. Frequency of C4A gene deletion among family members with C4AQ0.

A	All family members		SLE patients		1° relatives		2° relatives	
	n=113		n=26		n=73		n=14	
	%	no.	%	no.	%	no.	%	no.
C4AQ0	46.9	53	53.8	14	47.9	35	28.6	4
Homozygous	10.6	12	11.5	3	12.3	9	0.0	0
C4A normal	53.1	60	46.2	12	52.1	38	71.4	10
B	All family members with C4AQ0		SLE patients with C4AQ0		1° relatives with C4AQ0		2° relatives with C4AQ0	
	n=53		n=14		n=35		n=4	
	%	no.	%	no.	%	no.	%	no.
C4A gene deletion (LR-PCR)	60.4	32	64.3	9	60.0	21	50.0	2
Homozygous	5.7	3	0.0	0	8.6	3	0.0	0

Analysis of haplotypes carrying a C4A gene deletion

MHC haplotype analysis was used to identify haplotypes carrying a C4A gene deletion. Three haplotypes were found to carry a deletion: B8-C4AQ0-C4B1-DR3 (*a) and two variant haplotypes, B8-C4AQ0-C4B1-DR7 (*d) and B7-C4AQ0-C4B1-DR3 (*m) (Table 2A).

The frequency of the C4A deletion haplotypes among family members with confirmed C4A gene deletion is as follows. The classical C4A deletion haplotype B8-C4AQ0-C4B1-DR3 (*a) was found in 28 of 32 (87.5%) family members carrying a deletion; that is in eight of the nine (88.9%) SLE patients, 19 of the 21 (90.5%) first-degree relatives, and one of the two (50.0%) second-degree relatives (Table 2A). The haplotype B8-C4AQ0-C4B1-DR3 (*a) was found in six of the nine families (Figure 1).

The variant haplotype B8-C4AQ0-C4B1-DR7 (*d) was found in four of the 32 (12.5%) individuals carrying a deletion; one of the nine (11.1%) SLE patients and three of the 21 (14.3%) first-degree relatives (Table 2A). All four individuals are members of family 8 and the haplotype was traced back three generations in the pedigree (Figure 1).

The variant haplotype B7-C4AQ0-C4B1-DR3 (*m) was found in one second-degree relative (Table 2A) in family 6. The haplotype was found to have penetrated the family through marriage (Figure 1).

Homozygosity for C4A deletion and associated haplotypes was as follows. No SLE patient or second-degree relative was homozygous for C4A gene deletion. Three first-degree relatives were homozygous for C4A gene deletion. Two of them were homozygous for the classical deletion haplotype B8-C4AQ0-C4B1-DR3 (*a) and one carried C4A

gene deletions on haplotypes B8-C4AQ0-C4B1-DR3 (*a) and B8-C4AQ0-C4B1-DR7 (*d) (Table 2B).

Screening for other genetic changes underlying C4AQ0

Family members were screened for mutations in exons 20 and 29 of the C4A gene. These genetic changes were not found in the cohort.

Discussion

Inherited complete deficiency of the early components of the classical complement pathway (C1, C2, and C4) has been associated with increased susceptibility to SLE. The hypothesized underlying mechanisms differ for the different deficiency states (20–24). The importance of an intact complement system in immune complex (IC) handling has been demonstrated in a patient with complete C2 protein deficiency and SLE (20, 25, 26). It may be speculated that complete deficiency of the C4 protein, which is rare, would predispose to disease in a similar manner. Whether the same is true for partial deficiency of C4, or specifically for deficiency of the C4A component (C4AQ0), which is common in SLE, has yet to be demonstrated.

We have previously studied, in multiplex SLE families, the frequency of C4AQ0 and C4AQ0-carrying haplotypes. An increased frequency of C4AQ0 was shown on different MHC haplotypes, with the haplotype B8-C4AQ0-C4B1-DR3 being most common (8). This haplotype has been associated with a deletion of the C4A gene (11, 15).

In the present study, using a novel LR-PCR method, we confirm a C4A gene deletion as the underlying genetic mechanism in up to two-thirds of C4AQ0 in Icelandic multiplex SLE families. Other,

Table 2. (A) MHC haplotypes carrying CA gene deletion. Frequency of the three C4A deletion carrying MHC haplotypes found the multiplex SLE families. (B) Homozygosity for C4A gene deletion. MHC haplotypes found in the three family members homozygous for C4A deletion.

A Family members with C4AQ0 and C4A gene deletion underlying†	All family members		SLE patients		1° relatives		2° relatives	
	n=32		n=9		n=21		n=2	
	%	no.	%	no.	%	no.	%	no.
B8-C4AQ0-C4B1-DR3 (*a)	87.5	28	88.9	8	90.5	19	50.0	1
B8-C4AQ0-C4B1-DR7 (*d)	12.5	4	11.1	1	14.3	3	0.0	0
B7-C4AQ0-C4B1-DR3 (*m)	3.13	1	0.0	0	0.0	0	50.0	1
B Family members homozygous for C4A gene deletion	All family members		SLE patients		1° relatives		2° relatives	
	n=32		n=9		n=21		n=2	
	%	no.	%	no.	%	no.	%	no.
B8-C4AQ0-C4B1-DR3/B8-C4AQ0-C4B1-DR3 (*a/*a)	6.25	2	0.0	0	9.5	2	0.0	0
B8-C4AQ0-C4B1-DR3/B8-C4AQ0-C4B1-DR7 (*a/*d)	3.13	1	0.0	0	4.8	1	0.0	0

†One 1° relative is homozygous for a deletion on haplotypes *a and *d and are counted twice.

as yet undefined, genetic changes account for one-third of C4AQ0. Mutations in exons 20 or 29 of the C4A gene were not found to account for C4AQ0 in our SLE family cohort. In addition to the classical deletion haplotype, B8-C4AQ0-C4B1-DR3, two other haplotypes carry the C4A deletion; B8-C4AQ0-C4B1-DR7 and B7-C4AQ0-C4B1-DR3.

The LR-PCR method used to genotype for a C4A gene deletion revealed discrepancies in the C4 protein electrophoresis analysis, where heterozygous C4A 'null alleles' had not been detected. This demonstrates the advantage of this novel LR-PCR method compared to protein analysis. By this method individuals can be reliably analysed for a C4A gene deletion even without family data. Compared to RFLP analysis, smaller amounts of DNA are required and this method is less time-consuming.

The present study, therefore, further confirms the heterogeneous genetic background of C4AQ0 indicated in our previous study on C4AQ0 and MHC class I and II alleles in Icelandic multicase SLE families (8).

In summary, the data from our previous (8) and present studies show that C4AQ0 is found on different MHC haplotypes with varying HLA class I and II alleles. This is despite a strong disequilibrium between class I, II, and III alleles. In our study cohort C4AQ0 shows a stronger association with SLE than HLA-B8 and DR3 (8). A C4A gene deletion accounts for two-thirds of C4AQ0 alleles and the deletion is found on haplotypes carrying B8, B7, DR3, and DR2.

As for the role of C4AQ0 in SLE, an interplay with other genes outside the MHC region is expected and other studies by our group support such a relationship. A previous study on one of the Icelandic families (family 8, see Figure 1) looked at the relationship between C4AQ0 and IC levels. Finding that increased IC levels along with C4AQ0 was seen more often in SLE patients than in their relatives, it was proposed that C4AQ0 is a superimposed primary defect in individuals with an underlying second defect leading to elevated IC levels and increased susceptibility to SLE (27).

In a genome scan on Icelandic multiplex SLE families, C4AQ0 was at one point used as a stratification marker for analysing the data, and families not having C4AQ0 were excluded (i.e. families 3 and 6, see Figure 1). A susceptibility locus, *SLEB2* on chromosome 2q37, was identified with a possible epistatic effect with C4AQ0 (28). Our group has recently reported on a candidate gene, PD-1, within the *SLEB2* region (29). The PD-1 gene is an immunoreceptor-coding gene and participates in T-cell development and peripheral self-tolerance.

Taken together, our results give further support for the hypothesis, put forward in our previous study, that C4AQ0 may be an independent susceptibility factor

for SLE, irrespective of the genetic basis for C4AQ0. C4A protein deficiency may be one of several genetic factors contributing, in unison with other genetic factors such as a PD-1 gene mutation or elevated IC levels, to the pathogenesis of SLE.

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