Impact of Homozygous C4A Deficiency on Clinical Presentation of Systemic Lupus Erythematosus

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ABSTRACT

Objective: To investigate the association of C4A null allele (C4AQ0) with systemic lupus erythematosus (SLE) and determine the clinical presentation of SLE in relation to C4A null allele.

Study Design: Descriptive study.

Place and Duration of Study: Armed Forces Institute of Pathology (AFIP), Rawalpindi, Immunology Department, from December 2018 to December 2019.

Methodology: Patients referred to AFIP, who fulfilled American College of Rheumatology (ACR) criteria of 1997 for diagnosis of SLE were included in the study. Approval from the Institutional Ethical Review Board was taken. C4A and C4B null alleles were determined in 66 SLE patients and 40 age- and gender-matched healthy controls by polymerase chain reaction (PCR) using sequence-specific primers (PCR-SSP). Various clinical features and laboratory findings in the SLE patients were analysed in relation with C4A null allele.

Results: The mean age of the study population was 30.56 ±10.08 years. C4A null allele was detected in 7 (10.6%) patients; whereas, C4B null allele was detected in only two (3%) patients. SLE patients with C4A null allele had increased incidence of arthritis (100%) and renal damage (85.7%); compared to those with normal C4A allele, 57.6% and 32%, respectively. Fisher’s Exact test revealed strong association of C4A null allele with arthritis and renal damage, (p = 0.039 and 0.01, respectively).

Conclusion: Homozygous absence of C4A alleles was encountered in 10.6% of Pakistani patients of SLE and is closely related with clinical features of arthritis and renal damage. Knowledge of C4A null allele in SLE patients at diagnosis can predict disease course.

Key Words: SLE, C4A null alleles, C4AQ0, Homozygous C4A deficiency.

INTRODUCTION

SLE is a heterogeneous autoimmune disease with a wide spectrum of clinical and serological manifestations resulting from an interaction of genetic, environmental and hormonal factors.¹² It is a systemic autoimmune disorder characterised by multiple auto-antibodies against cell nuclear constituents DNA — histones and ribonucleoprotein.³ Its incidence ranges from 1 to 10 per 10,000 person-year and prevalence from 20 to 70 per 100,000.⁴ Reported prevalence of SLE in South Asia is 3.1 per 100,000.⁵ It affects women of child-bearing age with the highest incidence in African-Americans.⁴

SLE shows great heterogeneity in disease pathology, commonly involving skin, articular joints and kidneys.⁵ Constitutional symptoms like fatigue, fever and weight loss occur in most patients. Arthritis is symmetrical, poly-articular, and non-erosive occurs in 65 to 70% cases. Renal involvement occurs in about 50% cases and is the main cause of morbidity and mortality.⁵⁷

Many complement component deficiencies, both inherited and acquired, are associated with SLE. One of the most common genetic associations in SLE is deficiency of components of classical pathway C1, C4 and C2.⁸ However, the strength and severity of SLE disease is highest for homozygous C1q, relative to homozygous C4 and C2 deficiency. C4 genes are present in class III region of MHC on chromosome 6p21.3.⁹ C4 has two highly polymorphic isotypes, C4A and C4B with at least 13 allelic forms for C4A and 22 alleles for C4B including C4AQ0 and C4BQ0; the null alleles that have no protein expression.¹⁰¹¹ Null allele is associated with SLE and is present in 5-14% of patients with SLE and 1-6% of general population.¹⁰¹² C4A has higher affinity for immunoglobulins and, therefore, more efficient than C4B. It also functions to regulate and maintain B-cell
tolerance.\textsuperscript{13} Defects in C4A cause decreased clearance of autoantibodies, resulting in SLE and other immune complex mediated diseases. C4A plays important role in opsonisation, solubilisation and clearance of immune complex and C4B in mediating immune hemolysis.\textsuperscript{14}

Standard immunologic laboratory tests for diagnosing SLE include detecting antinuclear antibodies (ANA), anti-double stranded DNA antibodies (anti-dsDNA), extractable nuclear antigen antibodies (anti-ENA) and complement proteins C3 and C4. Proteins C3 and C4 play a pivotal role in pathogenesis of immune complex mediated damage.\textsuperscript{15} C4A and C4B are tested by their differences in electrophoresis mobility. Their phenotype is difficult to determine during active disease due to low C4 levels as a result of increased consumption and complement activation.\textsuperscript{12} With molecular typing methods, determination of C4 homozygous and heterozygous deficiency states have become more accurate.\textsuperscript{15}

The presence of C4A null allele (C4AQ0) with SLE is observed in Chinese, Caucasian and Japanese population; whereas, no association was found with C4B null allele.\textsuperscript{11} However, few studies reported no association of C4A null allele with SLE, as in Malaysian, Spanish and French population.\textsuperscript{11,13}

This study was planned with the objective to determine association of C4A null allele (C4AQ0) with systemic lupus erythematosus and its effect on clinical picture in terms of severity, organ involvement and laboratory findings in Pakistani population.

**METHODOLOGY**

This cross-sectional study was conducted from December 2018 to December 2019. Patients presenting at Immunology Department, AFIP who fulfilled 4 or more parameters of the American College of Rheumatology classification criteria (ACR 1997) of SLE, were included in this study.\textsuperscript{14} Patients without a clear diagnosis or those with other comorbid autoimmune diseases like diabetes or thyroid diseases were excluded from the study.

Permission from the Institutional Ethics Committee was sought prior to commencement of study. All the patients signed the informed consent before being enrolled. Sample size was calculated with Raosoft software; keeping margin of error 5%, confidence level 95%, and disease incidence 3.1 per 100,000. Sixty-six diagnosed patients of SLE and 40 age-matched control were enrolled. About 3ml venous blood samples in EDTA tubes were taken from all patients and controls. Genomic DNA was extracted from peripheral mononuclear cells using Flexi Gene DNA purification kit (Qiagen) as per manufacturer’s instructions. Extracted DNA was suspended in ethanol at a final concentration of 100ng/µl. Purity of DNA was kept between 1.65 to 1.95, determined by Epoch microplate spectrophotometer (BioTek Instruments, USA) at 260/280nm. DNA from all samples were saved at -20°C until analysis.

PCR master mix consisted of DNA Taq polymerase (thermo Fisher), dNTPs, MgCl2 (both from Thermo Fisher Scientific), Taq buffer used was 10X with (NH4)2 S04; Thermo Fisher Scientific, USA. Internal control used was HLA-DRB1 locus-specific primers (Alpha DNA). About 15µl of PCR master mix was put in each of the tubes and negative control tubes along with 3 microlitre of patient DNA. Forward and reverse primers (1 µl) of customised C4A and C4B (Alpha DNA, Montreal QC, Canada) were added in 4 mini Eppendorf tubes, according to primers mix combination. PCR was performed on Multigene OptiMax Labnet international thermo-cycler. First denaturation was carried out at 94°C for 5 minutes. The next five cycles were performed at decreasing annealing temperature, one degree for every cycle from 68°C to 63°C. Next 29 cycles were carried out at 30 seconds at 94°C, 1 min at 64°C, and 1 min at 72°C. Agarose gel 2% (w/v) was prepared in 0.1X Tris/borate/EDTA buffer. Next, ethidium bromide and bromphenol dye were added to each PCR tube. The product was run on 2% agarose gel stained with ethidium bromide under ultraviolet light. The gel was finally examined in ultraviolet light and results documented. Primers used are shown in Table I. Other investigation results anti-Sjogren’s syndrome antibody, anti-Smith antibody, anti Jo-1 and anti-Ribonucleoprotein antibody were also taken into account.

Creatinine clearance less than 80ml/min/1.73m² on more than two occasions was taken as diagnostic for renal impairment. Severity of renal damage was graded from 1 to 5 as per KDIGO criteria. Arthritis was diagnosed clinically in the presence of swelling, tenderness or effusion in two or more joints. Clinical characteristics and laboratory investigations of SLE were compared with C4A and C4B null alleles.

Statistical analysis was performed by using the SPSS 23.0. Frequency and percentages were calculated for the qualitative variable while mean and standard deviation were calculated for the quantitative variables. Fisher’s Exact test was used to determine the association of C4A null allele with various clinical and laboratory parameters with p-value less than or equal to 0.05 as statistically significant.

**RESULTS**

Sixty-six patients of SLE were recruited in the study. Mean age of the study participants was 30.56 (±10.08) years. Sixty-four patients were females while only two patients were males, as shown in Table II. C4A was deficient in 7 (10.6%) out of total 66 patients; and in 1 (2.5%) out of 40 in the control group (Table II). Six (85.7%) patients with C4A null allele had renal damage and all 7 patients (100%) with C4A null allele had arthritis. Fisher’s Exact test revealed significant association of C4A null allele with arthritis and renal damage (P=0.039 and 0.01, respectively) as shown in Table III. Anti-Sjögren’s-syndrome-related antigen A antibody (anti-SSA) was positive among 5 (71.4%) patients with C4AQ0 (P=0.001). C4B allele was deficient in 2 (3%) out of 66 SLE patients and in none of the control group. No association of C4B null allele was found with clinical or laboratory parameters of SLE. Male patients had severe disease in terms of clinical manifestations and severity of renal impairment.
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Table I: Genotyping primers used. Sequence specific primers for PCR used in current study for detection of C4A & C4B null allele.

| Gene       | Primer   | Sequence (5-3)                  | Length of PCR | Reference |
|------------|----------|---------------------------------|---------------|-----------|
| C4A/C4AQ0  | Aup      | GCATGCTCTGCTTACACCTGGAC         | 377/null      | Barba et al. |
|            | L3       | TGGCAATCCAGCGATTTCGGAA          | 578/null      |           |
|            | Ado      | AAGAACCCTCTGCAAGTCTTACAC       | 377/null      |           |
|            | L4       | ATAGGATCCCTAGTGCCCCCGGCT        | 578/null      |           |
| C4B/C4BQ0  | Bup      | TGTCTCTATGTATCTACTGGAGAGA      | 377/null      | Barba et al. |
|            | L3       | TGGCAATCCAGCGATTTCGGAA          | 578/null      |           |
|            | Bdo      | AAGAACCCTCTGCAAGTCTTACAC       | 377/null      |           |
|            | L4       | ATAGGATCCCTAGTGCCCCCGGCT        | 578/null      |           |

Table II: Demographic data and clinical presentation of patients included in the study.

| Age (years): Mean ± SD Range (min-max) | Patients (n=66) | Controls (n=40) |
|---------------------------------------|-----------------|-----------------|
| 10 years - 60 years                   | 30.56 (±10.08)  | 30.75 (±9.22)   |
| Gender: Male                          | 64 (97%)        | 39 (97.5%)      |
|                                          | 02 (3%)         | 01 (2.5%)       |
| C4 A Null Allele: Present             | 07 (10.6%)      | 1 (2.5%)        |
|                                          | 59 (89.4%)      | 39 (97.5%)      |
| C4 B Null Allele: Present             | 02 (3%)         | 0               |
|                                          | 64 (97%)        | 40              |
| anti-dsDNA:                            | 64 (97%)        |                 |
| anti ENA antibodies                    |                 |                 |
| anti-SSA                               | 07 (10.6%)      |                 |
| anti-SBB                               | 01 (1.5%)       |                 |
| anti-Smith                             | 02 (3.0%)       |                 |
| anti-RNP                               | 02 (3.0%)       |                 |
| anti Jo-1                              | 00 (0%)         |                 |
| Clinical features:                     |                 |                 |
| Rash                                  | 36 (54.5%)      |                 |
| Photosensitivity                       | 22 (33.3%)      |                 |
| Oral ulcer                            | 20 (30.3%)      |                 |
| Arthritis                             | 41 (62.1%)      |                 |
| Renal damage                          | 25 (37.9%)      |                 |
| Serositis                             | 11 (16.7%)      |                 |
| Hematologic involvement               | 14 (21.2%)      |                 |

DISCUSSION

This study was planned with the objective to determine association of C4 null alleles (C4A0 and C4B0) with systemic lupus erythematosus in the local population and its impact on clinical presentation and laboratory findings. An increased frequency of C4A null allele was found in SLE compared to the control group (10.6% vs 02.5%) and increased risk of arthritis and renal damage in these patients.

In this study, seven out of the 66 patients (10.6%) had homozygous C4A deficient alleles. This is in accordance with the international data which reports 10-15% SLE patients being homozygous deficient.\(^5\) Petri et al. observed significant increase in C4A null alleles among SLE patients versus controls in African-American (61% vs 13%) and European-American population (43% vs 25%).\(^17\) In a study of French-Canadian and non-French-Canadian lupus patients, a significant association of C4AQ0 in SLE patients in non-French-Canadian group was seen.\(^18\) In the Asian population, incidence of C4AQ0 is less relative to western data and more comparable to the results of this study. Man et al. reported 12.5% incidence of C4AQ0 in SLE patients and 1.1% in the control group in South-West Chinese population.\(^19\) However, Spanish, Greek and Malaysian studies failed to detect a significant association of lupus patients with C4A null alleles.\(^13,20,21\)

A significant association of C4AQ0 with arthritis and renal damage was found. All patients with C4A deficiency had arthritis and 85.7% (6 out of 7) had renal impairment; \(p=0.039\) and 0.01, respectively (Table III). Similar results are reported by Clemenceau et al. who found increased risk of renal involvement, in 9 out of 11 patients with C4AQ0.\(^22\) However, no such association was found by Schur et al. in USA and Kramer et al. in Hungry.\(^23,24\) This can be due to clinical heterogeneity and ethnic differences in populations.

Arthritis was significantly more among the SLE patients with C4AQ0 compared to the patients without C4A deficiency, 100% vs 57.6% (Table III). Tsang et al. also reported increased severity of arthritis in SLE patients with C4A null allele.\(^25\) Juptner et al. observed early onset and severe disease in SLE patients with C4AQ0 in German population, though no such association with age was observed in this study.\(^10\) This early disease onset and increased severity is due to C4A deficiency, that results in impaired clearance of immune complexes which get deposited at various locations like kidney, synovium and serosa leading to inflammation and damage.\(^16,17\)

In this study, 5 out of 7 patients (71.4%) with C4A null allele showed antibody to SSA (Sjögren’s-syndrome-related antigen A); \(p<0.001\), which is in agreement with Naves et al. and Revellive et al. who also demonstrated this association.\(^20,21\)
Table III: Fisher’s Exact test for relationship of various clinical and laboratory findings with the presence of C4A null alleles among the SLE patients. This table shows association of C4A null allele with clinical features & diagnostic tests in SLE patients.

| Characteristics | Status | Present n=59 | Absent n=7 | Total | p-value |
|-----------------|--------|--------------|------------|-------|---------|
| Age             | <25 years | 21 (35.6%)  | 2 (28.6%)  | 23    | >0.999  |
|                 | >25 years | 38 (64.4%)  | 5 (71.4%)  | 43    | >0.999  |
| Malar rash      | Present  | 32 (54.2%)  | 4 (57.1%)  | 36    | 0.425   |
|                 | Absent   | 27 (45.8%)  | 3 (42.9%)  | 30    |         |
| Oral ulcer      | Present  | 17 (28.8%)  | 3 (42.9%)  | 20    |         |
|                 | Absent   | 42 (71.2%)  | 4 (57.1%)  | 46    |         |
| Arthritis       | Present  | 34 (57.6%)  | 7 (100%)   | 41    | 0.039   |
|                 | Absent   | 25 (42.4%)  | 0          | 25    |         |
| Renal damage    | Present  | 19 (32.2%)  | 6 (85.7%)  | 25    | 0.01    |
|                 | Absent   | 40 (67.8%)  | 1 (14.3%)  | 41    |         |
| Anti-dsDNA      | Present  | 58 (98.3%)  | 6 (85.7%)  | 64    | 0.202   |
|                 | Absent   | 1 (1.7%)    | 1 (14.3%)  | 2     |         |
| Anti SSA        | Present  | 2 (3.4%)    | 5 (71.4%)  | 7     | <0.001  |
|                 | Absent   | 57 (96.6%)  | 2 (28.6%)  | 59    |         |
| Anti Smith      | Present  | 1 (1.7%)    | 1 (14.3%)  | 2     | 0.202   |
|                 | Absent   | 58 (98.3%)  | 6 (85.7%)  | 64    |         |
| Anti Jo-1       | Present  | 0           | 0          | 0     | **      |
|                 | Absent   | 59          | 7          | 66    |         |
| Anti RNP        | Present  | 2 (3.4%)    | 0 (0%)     | 2     | >0.999  |
|                 | Absent   | 57 (96.6%)  | 7 (100%)   | 64    |         |

dsDNA: double stranded DNA, ENA: extractable nuclear antigens, SSA: Anti-Sjögren’s-syndrome-related antigen A, RNP: Ribonucleoprotein. *Presence of C4A null allele has strong, statistically significant association with the clinical feature of arthritis and renal impairment and detection of anti SSA antibodies. **No statistical significance could be calculated due to absence of the variables in both groups.

In our study, no association was found between C4BQ0 and SLE which was in general agreement with other studies. Although C4BQ0 played an important part in SLE susceptibility in Spanish population (OR 6.0, \( p < 0.001 \)). This could be due to difference in ethnicity and haplotype tested. Indeed, majority of studies showed no increase in C4BQ0 allele in Caucasoid, Asian and African-American population.

Most of the patients in this study were females, only two males. This is consistent with the historical data. It was also observed that manifestations of SLE were more severe in male patients as compared to female patients.

In the control group, only one patient had C4A null allele and none had C4B null allele (Table II). Different studies have shown a variable incidence of C4A null allele in control groups that ranged from 13% in African-American, 25% in European-American; whereas, 1.1% in Chinese population. This difference in expression can be due to difference in ethnicity and genetic make-up.

There are various limitations of this present study. Firstly, the sample size is small, as it is often difficult to obtain a large number of SLE patients in a single hospital. Secondly, SLE is a complex disease. Due to the dynamic nature of disease, there may be many different sets of genetic and biologic players causing pathogenesis of SLE and resultant clinical manifestations, which may not be addressed in this study. Thirdly, there is genetic heterogeneity and various ethnic factors that have impact upon development and progression of SLE disease. Pakistani population is diverse and has many different ethnic groups distributed all over the country. The study centre is located in the northern part of the country; therefore, the studied population is not representative of all Pakistani population. A large multicenter prospective study can generate generalised results for our population and we could set our own guidelines for the complement testing among SLE patients.

This study gives an insight into the pattern of C4A deficiency among SLE patients and its impact on clinical presentation in Pakistani population. Knowledge of C4A status at diagnosis in SLE patients will help the physician predict disease course. Close monitoring of risk organs and early intervention will reduce morbidity and improve long term outcome.

CONCLUSION

Homzygous absence of C4A alleles is seen in 10.6% of SLE patients in Pakistani population. It marks increased risk of arthritis, renal impairment and detection of anti SSA antibodies. Testing for C4A null allele should be included in initial testing of SLE to predict disease course and better long-term management.

ETHICAL APPROVAL:
Approval from Institutional Review Board of Armed Forces Institute of Pathology was taken prior to commencement of this study.
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PATIENTS' CONSENT:
Informed consents for participation in the study and publication of results were taken from all the patients at the time of enrollment.

CONFLICT OF INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
AAM: Design of the study, acquisition, analysis, and interpretation of data, preparation of manuscript and final draft.
HNT: Conception and design of study, monitoring, correction and final approval of draft.
DA: Conception and design of study, final approval of draft.
MF: Analysis and interpretation of data, preparation of draft.

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