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ORIGINAL ARTICLE

Clinical usefulness of anti-cell membrane DNA autoantibodies in serology negative systemic lupus erythematosus

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Abstract

Introduction: Systemic lupus erythematosus (SLE) diagnosis is dependent on the detection of serum autoantibodies. To date, there is no autoantibody highly sensitive and specific enough to be considered as a gold standard. This study aimed to determine the diagnostic usefulness of anti-cmDNA antibodies which found to be associated with SLE. *Materials and Methods:* Serum samples from 83 SLE, 86 other connective tissue diseases (OCTD) and 61 healthy subjects were randomly selected for the study. The OCTD cases included 56 rheumatoid arthritis, 12 scleroderma, 10 Sjogren's syndrome and 8 mixed connected tissue diseases. All samples were assayed for anti-cmDNA by indirect immunofluorescence assay (IFA) using Raji cells as substrate. SLE samples were also tested for anti-dsDNA and anti-Sm antibodies using enzyme-immunoassays. *Results:* Anti-cmDNA positivity was highest in SLE (55.4%) compared to OCTD (9.3%) and healthy subjects (0%). It was 100% specific at differentiating SLE from healthy subjects and 90.7% specific at differentiating SLE from OCTD. There were no significant differences in the sensitivity (55.4%) of anti-cmDNA at differentiating SLE from OCTD and healthy groups. Anti-cmDNA was present in 52.9% of SLE samples negative for standard SLE-specific autoantibodies. It was detected in 7 (36.8%) of anti-dsDNA, 25 (52.1%) of anti-Sm and 5 (31.3%) of both anti-Sm and anti-dsDNA negative samples. Anti-cmDNA positive SLE was significantly associated with arthritis (p=0.019). *Conclusion:* The high specificity of anti-cmDNA detection by IFA makes it an excellent diagnostic test for SLE. Anti-cmDNA is also useful for identifying SLE with negative anti-dsDNA or/and anti-Sm antibodies.

Keywords: anti-cmDNA, Raji cells, immunofluorescence assay, SLE, autoantibodies

INTRODUCTION

Diagnosing SLE remains a challenge mainly due to its broad-spectrum clinical manifestations. SLE is characterised by production of autoantibodies directed against the cellular nuclear components. SLE-specific autoantibodies which are anti-double stranded DNA (dsDNA) and anti-Smith (Sm) antibodies are of clinical importance and were included in the American College of Rheumatology (ACR) criteria for SLE classification.[1,2] Both autoantibodies have different sensitivities and specificities toward SLE and none of them is able to independently confirm the diagnosis. Anti-dsDNA antibodies provide a high specificity (97%) but only present in 60-80% of SLE,[3] while remain persistently negative in some patients. Percentage of SLE with negative anti-dsDNA can be as high as 25%.[4] Anti-Sm antibodies are almost exclusive for SLE but only detectable in about 30% of patients.[1,5] Despite their disadvantages and continuous research for other potential biomarkers, anti-dsDNA and anti-Sm remain the only SLE-specific autoantibodies to date. Therefore, the quest of identifying a biomarker with better diagnostic accuracy for SLE is still relevant.

Cytoplasmic membrane associated-DNA (cmDNA) is a 17-kb polynucleotide chain present on the cell membrane of B lymphocytes and macrophages. It was first described by Lerner and co-workers in 1971. Studies have shown that cmDNA has different physical and

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chemical properties from the chromosomal and mitochondrial DNAs.[6,7] The possibility of cmDNA as contamination of mycoplasma or viral DNA has also been excluded. Examination of purified cytoplasmic membrane fragments under an electron microscope appeared as small linear DNA molecules.[8]

Anti-cell membrane-associated DNA (anti-cmDNA) antibodies were described in the late 90's as a potential specific marker for identification of SLE.[9] Subsequent studies also agreed that anti-cmDNA antibodies provided better diagnostic accuracy as compared to the standard autoantibodies for diagnosing SLE. However, the potential of anti-cmDNA antibodies was not fully explored as the number of studies is scarce. The published studies are limited to several populations particularly of China and Belgium origin.[10,11] The objectives of this study were to evaluate the diagnostic accuracy of anti-cmDNA in a multi-ethnic Malaysian SLE population and to determine the positive rate of anti-cmDNA in patients lacking SLE-specific autoantibodies.

METHODOLOGY

Serum Sample Collection

This was a cross-sectional study using serum samples sent to a reference autoimmune laboratory in the Allergy and Immunology Research Centre (AIRC), Institute of Medical Research (IMR), Malaysia. This laboratory receives samples from various hospitals in Malaysia for specialised autoimmune tests. The SLE and OCTD samples were randomly selected from leftover sera received by the AIRC from January 2017 to December 2018. There were 83 SLE and 86 other connective tissue diseases (OCTD) serum samples included for this study. OCTD samples consisted of 56 rheumatoid arthritis, 12 scleroderma, 10 Sjogren's syndrome and 8 mixed connective tissue disease (MCTD). All diagnoses were established by treating physicians and fulfilled the American College of Rheumatology classification criteria. The 61 healthy serum samples were obtained from consented donors recruited during Malaysian Stem Cell Registry (MSCR) campaigns. The serum samples were aliquoted and stored at -80°C until respective analysis was carried out. Approval of this study was obtained from the Medical Review & Ethics Committee (MREC), Ministry of Health Malaysia [NMRR-15-2461-28371 (IIR)].

Raji Cell Culture

Raji cells (ATC.CCL-86) from American Type Culture Collection (ATCC, USA) were maintained in RPMI-1640 (NacalaiTesque, Japan) culture medium supplemented with 10% fetal bovine serum (Tico Europe Ltd, Netherland) and 100 U/ml antibiotic. The cells were incubated at 37°C in 5% CO_2 and the respective suspension cells achieving exponential growth phase of 1×10^6/ml were harvested through centrifugation at 1800 RPM for 10 minutes. By using the trypan blue method, cell viability was consistently assessed during sub-culturing.

Indirect Immunofluorescence Assay (IFA)

The procedure of IFA using Raji cells as substrate was carried out according to the protocol described by Chen *et al*., 2008.[10] In summary, Raji cell culture that has reached a cell density of $1x10^6$/ ml were harvested and washed with RPMI-1640 culture medium. Fifty µl of the cell suspension at a cell density of $0.5x10^6$/ml was transferred onto each well of chambered glass slide and incubated for 30 minutes at room temperature. The cell fixation was performed using 4% formaldehyde at room temperature for 15 minutes. The Raji cells-fixed slide was then incubated with 1:40 diluted serum samples (diluted with PBS 10 mM, 0.05% Tween-20, pH 7.4) for 30 minutes at room temperature. Serum samples (1:40) with the highest level of anti-cmDNA measured using ELISA kit for human anti-cmDNA (Sunlong Biotech, China) were used as positive control. Healthy serum samples (1:40) with undetectable level of anti-cmDNA were used as negative control. After washing, the slide was incubated with 50 µl of FITC-conjugated goat anti-human IgG (ThermoFisher Scientific, USA) at 1:60 dilution for 30 minutes at room temperature in a dark environment. The slide was then washed and mounted with Flouromount Aqueous mounting media (Sigma Aldrich, USA) prior to visualisation under a fluorescence microscope. The immunofluorescence patterns of the Raji cell membrane were classified as follows: (i) absence of cell membrane fluorescence indicated a negative result; (ii) punctuate or continuous ring fluorescence of the cell membrane indicated a positive result (Figs. 1 and 2).

Evaluation of cmDNA Expression with DNase Pre-Treatment

Briefly, the Raji cells-fixed slides were separately pre-treated with RNase-free DNase, RNaseand

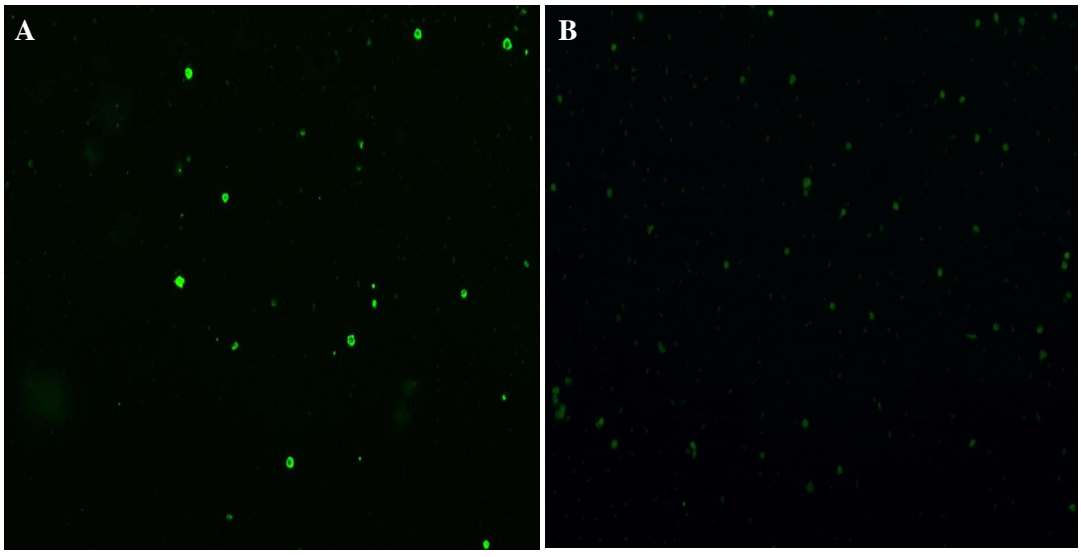

FIG. 1: Immunofluorescent staining of Raji cells for detection of anti-cmDNA (A) Positive = presence of ring fluorescence of the cell membrane; (B) Negative = absence of ring fluorescence of the cell membrane (magnification x10).

trypsin to evaluate the expression and localisation of cmDNA. The slides were subsequently incubated with anti-cmDNA positive serum samples as previously described. The cmDNA expression and specificity were confirmed by the extinction of cell membrane fluorescence pattern on slides pre-treated with RNase-free DNase, but not RNase and trypsin.

DNase Serum Inhibition Assay

SLE serum samples that produced the strongest cmDNA immunofluorescence staining were selected. A 50 µl volume of serum (1:40 dilution) was added with 5 µl DNase and incubated for 60 minutes at room temperature. Enzyme-free buffer was used as control. Five µl of ethylenediamine tetraacetic acid (EDTA; 0.5 M) was then added to stop the DNase activity. The serum samples

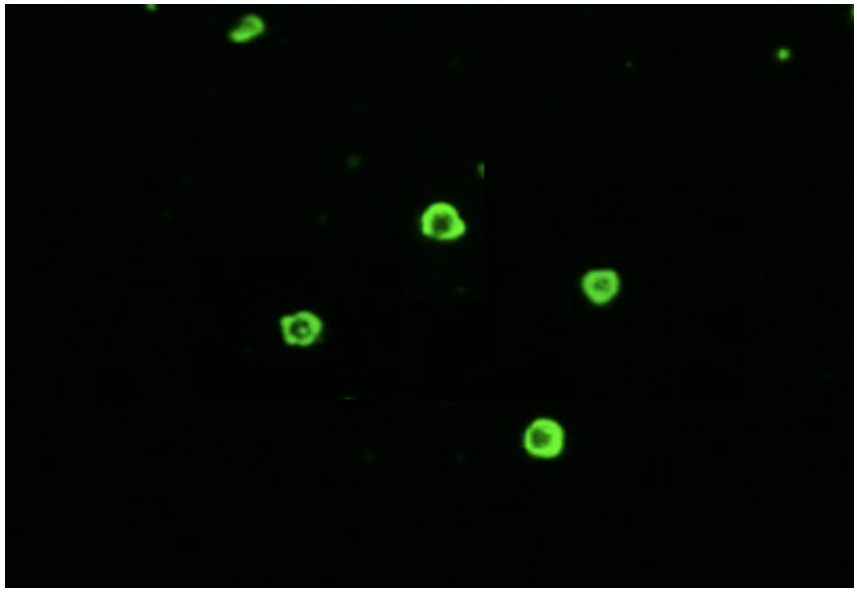

FIG. 2: Immunofluorescent ring of Raji cell membrane in a positive anti-cmDNA sample (magnification x40).

were subsequently assayed for IIF as previously described. This inhibition assay was aimed to eliminate any cross reactivity from DNA that may be present in the serum. The pre-treatment did not change the immunofluorescence pattern.

Enzyme-Immunoassay of anti-dsDNA and anti-Sm

Anti-dsDNA antibodies (INOVA QUANTA Lite dsDNA, Inova Diagnostics, USA) were measured through custom-designed ELISA kit according to the manufacturer's protocol. Anti-Sm antibodies were analysed using fluoroenzyme immunoassay (Phadia 250, Thermo Fisher Scientific, Sweden).

Statistical Analysis

All statistical analysis was performed using Statistical Package for the Social Sciences (SPPS) version 23. Any stated data value is projected as a mean ± standard deviation, with the confidence interval (CI) set at 95 % for mean value estimation. Associations between clinical manifestations and laboratory parameters with anti-cmDNA in SLE were analysed using Chi-square test. The acquired p-value is considered significant when it is less than 0.05.

RESULTS

Characteristics of Study Samples

The majority of 83 SLE cases were between 20 to 40 years old (66.3%), female (89.2%) and Malay ethnicity (57.8%) (Table 1). The 86 OCTD cases comprised of 56 rheumatoid arthritis, 12 scleroderma, 10 Sjogren's syndrome and 8 mixed connected tissue diseases. Most OCTD cases were between 41-60 years old (41.9%), female (96.5%) and Malay ethnicity (40.7%).

Frequency of anti-cmDNA in SLE, OCTD and Healthy Subjects

Anti-cmDNA was detected in 46 (55.4%) SLE and 8 (9.3%) OCTD. The 8 OCTD cases were rheumatoid arthritis (3 cases), Sjogren's syndrome (2 cases), MCTD (2 cases) and scleroderma (1 case). There was a significant difference between the positivity of anti-cmDNA between SLE and OCTD patients ($p<0.001$). Anti-cmDNA was not detected in healthy subjects.

Frequency of anti-dsDNA and anti-Sm in SLE

Anti-dsDNA and anti-Sm were positive in 64 (77.1%) and 35 (50.85%) of SLE patients, respectively.

Diagnostic Accuracy of anti-cmDNA in SLE Diagnosis

Anti-cmDNA was 100% specific at differentiating SLE from healthy subjects and 90.7% specific at differentiating SLE from OCTD. The sensitivity of anti-cmDNA at differentiating SLE from both groups was the same, which was 55.4%. The

TABLE 1: Demographic characteristics of SLE, other connective tissues diseases and healthy subjects

Characteristics	SLE *n* = 83 n (%)	OCTD *n* = 86 n (%)	Healthy Subjects *n* = 61 n (%)
Age (years)	36.42 (13.6)[a]	48.56 (14.2)[a]	25.05 (56.0)[a]
< 20	2 (2.4)	2 (2.3)	0 (0.0)
20-40	55 (66.3)	27 (31.4)	59 (96.7)
41-60	23 (27.7)	36 (41.9)	2 (3.3)
> 60	3 (3.6)	21 (24.4)	0 (0.0)
Gender			
Female	74 (89.2)	83 (96.5)	46 (75.4)
Male	9 (10.8)	3 (3.5)	15 (24.6)
Ethnicity			
Malay	48 (57.8)	35 (40.7)	30 (49.2)
Chinese	24 (28.9)	28 (32.6)	22 (36.1)
Indian	9 (10.8)	21 (24.4)	9 (14.8)
Others	2 (2.4)	2 (2.3)	0 (0.0)

[a] Mean (SD), OCTD: other connective tissue diseases

TABLE 2: Anti-cmDNA diagnostic profile in SLE and in differentiating SLE from healthy subjects and other connective tissue diseases

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	LR+	LR-
SLE	55.4	94.6	85.2	79.0	80.4	10.18	0.47
SLE vs Healthy Subjects	55.4	100	100	62.2	74.3	-	0.46
SLE vs OCTD	55.4	90.7	85.2	67.8	73.4	5.96	0.49

No calculation for LR+ for SLE vs Health Subjects as specificity is 100%
PPV: positive predictive value; NPV: negative predictive value; LR+: positive likelihood ratio; LR-: negative likelihood ratio; vs: versus

accuracy of anti-cmDNA in diagnosing SLE among 144 SLE and healthy subjects, as well as among 169 SLE and OCTD were 74.3% and 73.4%, respectively. The diagnostic accuracy and discriminating ability of anti-cmDNA are summarised in Table 2. The above values were calculated using Bayes' theorem formula.

Frequency of anti-cmDNA in SLE with Negative Standard SLE-Specific Autoantibodies

Anti-cmDNA was present in 27/51 (52.9%) SLE cases with serology negative of either or both standard SLE-specific autoantibodies (anti-dsDNA and anti-Sm). It was detected in 7 (36.8%) of anti-dsDNA, 25 (52.1%) of anti-Sm and 5 (31.3%) of both anti-Sm and anti-dsDNA negative samples (Table 3).

Association of anti-cmDNA with SLE Clinical and Laboratory Characteristics

SLE patients with positive anti-cmDNA showed to be significantly associated with arthritis (p=0.019). However, no significant associations were found between anti-cmDNA with other clinical manifestations such as mucocutaneous, lupus nephritis, hematological, neurological and serositis in SLE. There was also no significant difference in SLE with positive anti-cmDNA and other studied laboratory parameters (Table 4).

DISCUSSION

Anti-cmDNA antibodies were found to be useful in SLE diagnosis. Several studies have shown that anti-cmDNA can be detected using several human cell lines as substrate by IFA.[10-13] Several types of B cell lines including Wil2 NS, Priess, Daudi and Raji cells, T lymphoma cell line (Jurkat) and human promyelocytic leukaemia (HL60) cell line were identified to be able to demonstrate cell membrane fluorescence. However, Raji cell line was shown to provide the strongest, most clear and reproducible immunofluorescence staining as substrate,[10,12,13] thus was selected to be used in this study.

At serum dilution of 1:40, anti-cmDNA prevalence in SLE was 55.4%. From this study, anti-cmDNA prevalence was lower than anti-dsDNA (84.0%) but higher than anti-Sm (45.7%). In terms of specificity, anti-cmDNA was shown to be highly specific for SLE, at par with anti-Sm reported specificity but with higher sensitivity. Previous studies have reported that anti-cmDNA sensitivity for SLE ranges between 65-85%.[11–14] These differences might be due to the use of different cell lines as substrate for IFA. Depending on the substrate, the specificity of anti-cmDNA was reported to be as high as 98% [9] to as low as 87%.[15] Wil2 NS[11,13] and HL60[15] cell lines were used in those studies,

TABLE 3: Frequency of anti-cmDNA positivity in SLE lacking standard SLE-specific autoantibodies

Antibodies	Total (*n*)	anti-cmDNA positivity	
		n	%
anti-dsDNA (-)	19	7	36.8
anti-Sm (-)	48	25	52.1
anti-Sm& anti-dsDNA (-)	16	5	31.3

(-): antibody negative

TABLE 4: Association of anti-cmDNA antibodies with clinical and laboratory characteristics in SLE

Clinical and Laboratory Characteristics	anti-cmDNA		χ^2	*p*-value
	Positive (*n* = 46)	Negative (*n* = 37)		
Clinical manifestations				
Mucocutaneous	23 (50.0)	14 (37.8)	1.316	0.518
Arthritis	26 (56.5)	10 (27.0)	7.918	**0.019** [a]
Serositis	2 (4.4)	4 (10.8)	5.518	0.063
Lupus nephritis	11 (23.9)	15 (40.5)	3.675	0.159
Neurological	3 (6.5)	3 (8.1)	4.917	0.086
Hematological	10 (21.7)	8 (21.6)	0.503	0.778
Laboratory parameters				
ANA	41 (89.1)	32 (86.5)	0.135	0.935
anti-dsDNA	39 (84.0)	25 (67.6)	3.443	0.064
anti-Smith	21 (45.7)	14 (37.8)	0.513	0.510

Categorical variables were expressed by counts and percentages and compared between different groups by the χ^2 test.

[a] Significant at with $p< 0.05$.

apart from Raji cells.[10,12] In this study using Raji cells as substrate, anti-cmDNA specificity was 94.6%. It was 100% specific in differentiating SLE from healthy control and 90.7% specific in differentiating SLE from OCTD. At 1:40 dilution, the specificity and accuracy of anti-cmDNA detection using Raji cells for SLE in this study were consistent as reported by the previous above studies. Anti-cmDNA was also demonstrated to be a useful test for establishing the diagnosis of SLE. SLE patients were shown to be 10 times more likely to have a positive anti-cmDNA (LR+ 10.18) and about 0.5 times as likely to have a negative anti-cmDNA (LR- 0.47) than those without the disease.

Anti-cmDNA was shown to be positive in more than half of SLE patients with negative anti-dsDNA or anti-Sm.[10,12,14] From this study cohort, anti-cmDNA was able to detect an additional 31.3% of SLE that were negative for both anti-dsDNA and anti-Sm. This property is very useful especially in cases with vague clinical presentations in addition to negative SLE-specific autoantibodies as stated in the classification criteria. Having anti-cmDNA as an additional biomarker will increase the detection rate of SLE and its combination will improve the serological diagnostic accuracy as suggested by several previous studies.[12,15]

In our study, anti-cmDNA was found to be significantly associated with arthritis in SLE. Mucocutaneous and haematological involvements as well as SLE-associated autoantibodies were also more frequently seen in anti-cmDNA positive cases but were not statistically significant. Chen *et al*.[10] and Ru *et al*.[12] also reported that there was no significant difference in clinical features and laboratory parameters among SLE with or without anti-cmDNA. The role of anti-cmDNA as disease activity marker has not been fully explored. From the literature review, only one study reported on this and they found no significant correlation between anti-cmDNA and SLE Disease Activity Index (SLEDAI).[12] More studies should look at this aspect and determine the other potential roles of anti-cmDNA. The disease activity status of SLE was not included in our objectives. The significant association might be due to more SLE samples were taken during disease flare among SLE with musculoskeletal involvement. Future studies are warranted to confirm this finding.

CONCLUSION

The high specificity of anti-cmDNA detection using IFA method makes it an excellent diagnostic tool for SLE. Anti-cmDNA is potentially a very useful biomarker for SLE with negative anti-dsDNA or/and anti-Sm antibodies.

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Authors' contribution: Faten Nurul Amira: data acquisition, data analysis and drafting the manuscript; Maha Abdullah: study design and critical revision; Masita Arip: data acquisition and critical revision; Masriana Hassan: data acquisition and critical revision; Hasni Mahayidin: study design, data analysis, drafting the manuscript and final approval.

Conflict of interest: The authors declare no conflict of interest.

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