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Antinucleosome antibodies correlate with the disease severity in children with systemic lupus erythematosus

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Abstract

We compared the serum levels of antinucleosome antibodies (anti-NCS Abs) in thirty pediatric systemic lupus erythematosus (SLE) patients by using enzyme-linked immunosorbent assay (ELISA) to 29 adult SLE patients, 30 healthy controls, 21 juvenile idiopathic arthritis (JIA) and 23 Henoch-Schonlein purpura (HSP) patients as autoimmune disease controls. The mean anti-NCS Ab titer in the pediatric SLE patients was 1552.7 ± 1842.2 U/ml, higher than those of adult SLE patients (194.3 ± 402.7 U/ml), normal controls (9.5 ± 5.7 U/ml) and disease controls (JIA: 7.7 ± 4.0 U/ml, HSP: 5.7 ± 4.4 U/ml) ($p < 0.05$). The prevalence of both anti-NCS Ab (90%) and anti-ds DNA Ab (76.7%) in pediatric SLE patients were higher ($p < 0.05$) than that of adult SLE patients (58.6% and 48.3%). A positive correlation was demonstrated between anti-NCS Ab and anti-dsDNA Ab as well as the SLEDAI scores in pediatric and adult patients ($p < 0.05$). The inverse correlation of anti-NCS Ab levels with C3 was observed in both pediatric and adult SLE patients (pediatrics, $r = -0.61$, $p = 0.0003$; adult, $r = -0.44$, $p = 0.02$). Our data suggested that in pediatric SLE patients, anti-NCS Ab could be as good a marker for SLE diagnosis and disease activity assessment as in adult SLE patients.

Keywords: Anti-double stranded DNA antibodies; Antinucleosome antibodies; Systemic lupus erythematosus

1. Introduction

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by a wide range of clinical manifestations. Nephritis is the most important feature of lupus since kidney damage is a major threat to long-term survival. Renal involvement was found in 40–80% of pediatric lupus patients [1].

The pathogenesis and precise etiology of SLE are still not fully understood. However, SLE is characterized by the production of a variety of auto-antibodies, especially against nuclear components, elevated circulating immune complexes and complement consumption [2–4]. Nucleosomes are generated during cell apoptosis by cleaving the chromatin with endonucleases. In normal conditions, phagocytes engulf these apoptotic cells to prevent the release of cell constituents into the extracellular space. In SLE, programmed cell death might be aggravated and result in the increased release of nucleosomes. Defects in macrophage phagocytosis and complement abnormalities have been suggested in SLE disease pathogenesis [5,6]. In addition, virus infections could trigger autoimmune responses by contributing to the immunogenicity of nucleosomes [7]. The reactivity of SLE serum to nucleosomes involves antinucleosome antibodies (anti-NCS Ab) recognizing only

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quaternary epitopes of the nucleosome as well as the anti-double stranded DNA antibodies (anti-dsDNA Ab) and antihistone antibodies recognizing the individual components of the nucleosome [8–11]. Bruns et al., have demonstrated the cross-reactivity of anti-NCS Ab, both with ssDNA and dsDNA, which is commonly explained by epitope spreading [12]. In murine lupus, nucleosome-specific $CD4^+$T-cells are detected earlier than the pathogenic autoantibodies are produced and nucleosome-specific $CD4^+$T-cells were thought to play a role in the molecular mechanism of the disease [13]. With the identification of critical $CD4^+$T-cell autoepitopes in nucleosomes, Suen et al., successfully performed peptide-based therapy in a lupus animal model [14].

Although many auto-antibodies have been found in SLE patients, none have been a good diagnostic marker nor correlate well with disease activity. Anti-ds DNA Ab and antinuclear antibody (ANA) are the most frequently used markers at present. However, their sensitivity, specificity for SLE diagnosis, and correlation with disease activity are unsatisfactory. More recently, anti-NCS Ab have been investigated as a disease marker for SLE [15,16]. However, due to many controversial aspects, the greater diagnostic ability of anti-NCS Ab versus that of anti-dsDNA Ab still deserves careful investigation.

Additionally, although there are many studies assessing anti-NCS Ab in SLE patients, none have focused on pediatric SLE patients [15,17]. Whether anti-NCS Ab plays a different role in pediatric versus adult SLE patients is unclear. In this study, we compared the serum levels of anti-NCS Ab between pediatric and adult SLE patients. In addition, we investigated the association between anti-NCS Ab and SLE disease activity, renal manifestation, and humoural parameters in pediatric SLE patients.

2. Materials and methods

2.1. Subject

Thirty pediatric patients with SLE followed-up at the National Taiwan University Hospital (NTUH) were included in this study. Twenty-nine adult SLE patients were also examined as an equivalent group. These subjects met at least four of the revised criteria for SLE of the American College of Rheumatology [18]. All SLE patients were assessed for disease activity using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score [19]. Thirty healthy individuals who were age- and gender-matched to the pediatric patients with SLE were included as the control group. Twenty-one patients with juvenile idiopathic arthritis (JIA) and 23 patients with Henoch-Schonlein purpura (HSP) were also tested as disease controls. JIA and HSP were diagnosed according to the criteria proposed by the International League of Associations for Rheumatology (ILAR) [20] and the criteria proposed by Mills [21], respectively.

2.2. Methods

Anti-NCS Abs were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Anti-Nucleosome ORG 528, Orgentec Diagnostika, Germany), according to the manufacturer's recommendation. Briefly, anti-NCS Ab from patients' sera reacted with purified human nucleosomes coated on the plate during incubation at room temperature for 30 min. After 15 min of incubation with horseradish peroxidase-conjugated anti-human IgG, tetramethylbenzidine substrate was then added and incubated for 15 min at room temperature. The developing color of the substrate is positively correlated with the amount of anti-NCS Ab in the tested samples. A five point calibration curve, a positive control, and a negative control were included in each plate.

Using the same serum sample, each SLE patient was also assayed for anti-ds DNA Abs by commercial enzyme-linked immunosorbent assay (ELISA) kits (Human Anti dsDNA Enzyme Immunoassay Kit MK017, BINDAZYME). Anti-ds DNA Abs from the patients' sera reacted with the calf thymus dsDNA antigen pre-coated on the plate during incubation. After washing, purified peroxidase labeled rabbit anti-human IgG conjugate was added to bind to the captured human auto-antibody. After excess unbound conjugate was removed by a further washing step, the bound conjugate was visualized with tetramethylbenzidine substrate, the intensity of which was proportional to the concentration of auto-antibodies in the sample.

In addition to anti-NCS Abs and anti-dsDNA Abs, blood cell counts, hemoglobin (Hb), C3 and C4 were also measured for each pediatric SLE patient using the same serum sample. Venous blood samples were drawn and centrifuged. Sera were stored at $-20\,^{\circ}C$ prior to use. The study was approved by the ethics committee at the National Taiwan University Hospital.

2.3. Statistical analysis

Continuous variables were expressed as means $\pm$ S.D. Differences among multiple diagnostic groups were assessed by analysis of variance (ANOVA). Pairs of groups were compared using the independent *t*-test. The Fisher's exact probability test was applied to determine significant levels of observed frequencies. The correlations were determined by Spearman's rank correlation. Statistical significance was set at a probability (p) value of $\leqq 0.05$.

3. Results

The demographic features of pediatric and adult SLE patients, healthy controls and other pediatric autoimmune diseases are summarized in Table 1. A total of 30 pediatric and 29 adult SLE patients in NTUH were included in this study; the average age at blood sampling was 13 ± 2.9 years and 31.7 ± 12.3 years; respectively. The mean SLEDAI of the pediatric patients was 7.7 ± 7.2, which was not significantly different to that of adult patients. Active renal involvement,

Table 1
Demographic features of pediatric and adult SLE patients, healthy controls and other pediatric autoimmune diseases

Demographic Features	pSLE ($n = 30$)	CTL ($n = 30$)	aSLE ($n = 29$)	JIA ($n = 21$)	HSP ($n = 23$)
Gender (male/female)	5/25	5/25	3/26	14/7	12/11
Age at blood sampling[a] (years)	13.0 ± 2.9 (5–17)	13.1 ± 2.7 (8–17)	31.7 ± 12.3 (20–59)	10.2 ± 3.5 (4–16)	6.5 ± 3.6 (3–17)
Age at diagnosis[a] (years)	10.5 ± 2.6 (4–16)		23.5 ± 11.6 (10–51)		
SLEDAI[a]	7.7 ± 7.2 (0–28)		6.4 ± 4.4[b] (0–16)		

pSLE: pediatric SLE; CTL: control; aSLE: adult SLE; JIA: juvenile idiopathic arthritis; HSP: Henoch-Schonlein purpura.
[a] Expressed as mean ± S.D. (range).
[b] Data compared with corresponding values of the pSLE group at $p > 0.05$ level with independent t test.

the most common disease manifestation in our pediatric patients, occurred in 40% of pediatric patients, which was not significantly different to that of adults. Arthritis, the most common disease manifestation in adult patients, occurred more frequently in adult patients than in pediatric patients (44.8% vs. 6.7%; $p < 0.05$). Fever occurred in 20% of pediatric patients but not for the adult patients ($p < 0.05$). Malar rash was found in 20% and 24% of our pediatric and adult patients, respectively ($p > 0.05$). Haematological involvement, oral ulcer, alopecia, pleurisy and neurological involvement were found in less than 15% of both adult and pediatric patients.

At blood sampling, there was no significant difference between the medication prescribed in pediatric and adult patients except for azathioprine, which was prescribed marginally more often in pediatric than in adult cases ($p = 0.06$; 50% vs. 24%). Hydroxychloroquine was the most frequently used medication in pediatric and adult SLE patients (77% vs. 69%). Steroids – including pulse-, full- and low-dose therapies – were the second most frequently prescribed medication for both pediatric and adult patients (73% vs. 55%). NSAIDs were prescribed in 23% and 35% of pediatric and adult patients, respectively. Cyclophosphamide, sulfasalazine and cyclosporin A were the least prescribed medication at blood sampling.

Antinucleosome antibody levels in serum from patients with SLE and other pediatric autoimmune diseases and normal controls were assessed by ELISA (Table 2 and Fig. 1). The mean anti-NCS Ab titer in the pediatric SLE patients was 1552.7 ± 1842.2 U/ml, which was higher than that of adult SLE patients (194.3 ± 402.7; $p < 0.05$). The mean titer of anti-NCS Ab in normal controls, JIA, and HSP were 9.5 ± 5.7, 7.7 ± 4.0 and 5.7 ± 4.4 U/ml, respectively, which were significantly lower than that of SLE patients ($p < 0.05$). There was no significant difference between normal controls and disease controls ($p < 0.05$).

For anti-ds DNA Ab, as described in the manufacturer's instructions, the upper limit of normal was expressed as the mean concentration of normal blood donors +5 S.D. (equal to 30 IU/ml, with a mean of 9.2 and S.D. of 4.4 IU/ml). For anti-NCS Ab, comparable to anti-dsDNA Ab, absorbance values greater than the mean + 5 S.D. of the healthy control serum samples were considered positive in this study (38.1 U/ml, with a mean of 9.5 and S.D. of 5.7 U/ml). As shown in Table 3, 27 of the 30 pediatric SLE patients and 17 of the 29 adult SLE patients had anti-NCS Ab levels outside the normal range, while none of the 30 controls had anti-NCS Ab titers beyond the normal range. Twenty-three of the 30 pediatric SLE patients and 14 of the 29 adult SLE patients had positive results of anti-ds DNA Ab. The prevalence of anti-NCS Ab and anti-ds DNA Ab in pediatric SLE patients were both higher than that of adult SLE patients (90% and 76.7% in comparison of 58.6% and 48.3%; $p < 0.05$). There was no significant difference between the prevalence of anti-NCS Ab and anti-dsDNA Ab in SLE patients. Anti-NCS Ab had a sensitivity of 90% and specificity of 100% for SLE diagnosis in pediatric patients.

In 12 pediatric patients with active renal diseases, 11 had positive anti-NCS Ab, while 10 had positive anti-ds DNA Ab. In eight adult patients with active renal diseases, six had positive anti-NCS Ab, while six had positive anti-ds DNA Ab. There was no significant difference between the prevalence of anti-NCS Ab and anti-dsDNA Ab in active renal diseases in both pediatric and adult patients ($p > 0.05$). We also compared the prevalence of anti-NCS Ab between patients with and without active renal diseases (pediatric: 91.7% vs. 88.9%; adult: 75% vs. 52.4%). The prevalence of anti-dsDNA Ab between patients with and without active renal involvement was also compared (pediatric: 83.3% vs. 72.2%; adult: 75% vs. 42.9%). In neither pediatric nor adult patients, no significant difference was found. Moreover, there was no significant difference between the titers of anti-NCS Ab and anti-dsDNA Ab between patients with and without active renal disease, both in pediatric and adult patients ($p > 0.05$).

We then examined the correlation between anti-NCS Ab titers and other activity markers (Table 4). Levels of anti-NCS

Table 2
Anti-NCS Ab titers in pediatric and adult SLE patients, healthy controls and other pediatric autoimmune diseases

	pSLE ($n = 30$)	CTL ($n = 30$)	aSLE ($n = 29$)	JIA ($n = 21$)	HSP ($n = 23$)
Anti-NCS Ab[a] (U/ml)	1552.7 ± 1842.2 (13.5–6363.2)	9.5 ± 5.7 (2.7–28)	194.3 ± 402.7 (7.4–1939.7)	7.7 ± 4.0 (2.1–16.8)	5.7 ± 4.4 (1.9–11.7)

pSLE: pediatric SLE; CTL: control; aSLE: adult SLE; JIA: juvenile idiopathic arthritis; HSP: Henoch-Schonlein purpura.
[a] Expressed as mean ± S.D. (range).

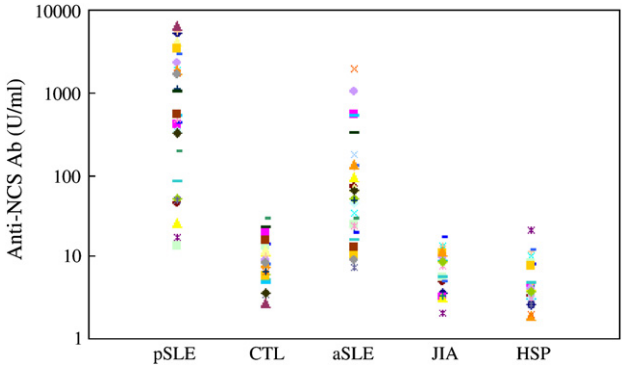

Fig. 1. Levels of antinucleosome antibody in pediatric and adult SLE patients, healthy controls and other pediatric autoimmune diseases. Antinucleosome antibody titers were examined by ELISA. The cut-off value, 38.1 U/ml, was set for mean + 5 S.D. of the control samples.

Ab correlated to anti-dsDNA Ab levels better in adult patients than in pediatric patients (adult, $r = 0.6$, $p = 0.0009$; pediatrics, $r = 0.37$, $p = 0.004$), but correlated to SLEDAI better in pediatric patients than in adult patients (pediatrics, $r = 0.85$, $p < 0.0001$; adult, $r = 0.37$, $p = 0.047$). The inverse correlation of anti-NCS Ab levels to C3 was observed in both pediatric and adult SLE patients (pediatrics, $r = -0.61$, $p = 0.0003$; adult, $r = -0.44$, $p = 0.02$). However, the inverse correlation of anti-NCS Ab levels to C4 was observed only in pediatric SLE patients ($r = -0.56$, $p = 0.0015$).

4. Discussion

The titers of anti-NCS Ab in our adult patients were comparable to those reported in adult patients elsewhere [15,17]. In Simon's study [15], the reported mean anti-NCS Ab in SLE patients was 244 ± 88 U/ml (57–481) (mean age 25.6 years, mean disease duration 5.5 months, modified SLEDAI 5.3). In Ghirardello's study [17], the mean anti-NCS Ab in SLE patients was 53.9 ± 41.9 U/ml (mean age 29.8 years, mean disease duration 80.2 months, ECLAM 2.1). Our study is interesting in demonstrating mean anti-NCS Ab titers in pediatric SLE patients to be much higher than in adult patients (1552.7 ± 1842.2 U/ml v.s. 194.3 ± 402.7 U/ml; $p < 0.05$). Many factors contribute to the circulating level of anti-NCS Ab, including the production and clearance of nucleosomes and anti-NCS Ab, and the formation of nucleosome-autoantibody complexes. The effect of age in the shaping of the immune repertoire through influencing the rate of apoptosis or the removal of apoptotic cells, the function of macrophages, the activation of polyclonal B-cells, and the rate of Fc receptor-mediated clearance of immune complexes may need further study. Furthermore, the balance among the levels of circulating nucleosomes, deoxyribonuclease (DNase), the enzyme responsible for nucleosome degradation, and anti-NCS Ab has been reported to likely be dynamic [22]. In Amoura's study [23], nucleosome levels were shown to be negatively correlated with anti-NCS Ab and anti-dsDNA Ab, which suggest that the clearance of circulating nucleosomes involves both anti-NCS Ab and anti-dsDNA Ab. Therefore, in a cross-sectional study, we faced the challenge of proving a causative role for decreased DNase levels, and consequently, elevated nucleosome antigen levels, followed by elevated anti-NCS Ab and anti-dsDNA Ab levels. To find a possible association, it was necessary to simultaneously investigate DNase activity, circulating nucleosomes, anti-NCS Ab and anti-dsDNA Ab levels, and conduct follow-up for a certain period of time.

Anti-NCS Ab has been successively detected in different connective tissue diseases such as SLE, mixed connective tissues disease (MCTD) [12,15,24] and scleroderma [16,17,24–26]. Anti-NCS Ab level in SLE was largely higher than in other connective tissue diseases [17]. In anti-NCS Ab-positive scleroderma patients, antibody levels seemed to be located within borderline values [25,27]. Furthermore, there tend to be anti-NCS Ab in the absence of anti-dsDNA Ab [25]. The specificity of anti-NCS Ab for SLE diagnosis related to the definition of the cut-off value and medium/high levels is highly specific for SLE [17]. Although we collected data from patients with JIA (the most common rheumatic disease in childhood) and HSP (the most common small vessel vasculitis in childhood) as disease controls, we were unable to collect serum from pediatric patients of MCTD and scleroderma. This could limit our findings of the specificity of anti-NCS Ab for pediatric SLE diagnosis.

The sensitivity of an assay depends not only on its cut-off value or different solid-phase antigen preparations [17,27], but also on the clinical characteristics of the SLE patients, such as disease activity, disease duration and medical treatment [15,28]. The reported sensitivity of anti-NCS Ab for SLE diagnosis varied widely [12,16,17,24,29]. Pinpointing where the discrepancy came from was not easy, because different studies used different antigen preparations and activity indices. In addition, only a few studies recorded medical treatment and clearly defined their cut-offs. In our study, we demonstrated that the sensitivity of anti-NCS Ab in pediatric SLE patients was higher than that of adult patients. We eliminated the influence of disease activity, medical treatment, antigen preparation and the definition of cut-off values because the same ELISA kit and cut-off values were applied and the

Table 3
Prevalence of anti-NCS Ab and anti-dsDNA Ab in pediatric and adult SLE patients, healthy controls and other pediatric autoimmune diseases

Prevalence	pSLE ($n = 30$)	CTL ($n = 30$)	aSLE ($n = 29$)	JIA ($n = 21$)	HSP ($n = 23$)
Anti-dsDNA Ab[a]	23/30 (76.7%)		14/29 (48.3%)		
Anti-NCS Ab[a]	27/30 (90.0%)	0/30 (0%)	17/29 (58.6%)	0/21 (0%)	0/23 (0%)

pSLE: pediatric SLE; CTL: control; aSLE: adult SLE; JIA: juvenile idiopathic arthritis; HSP: Henoch-Schonlein purpura.
[a] Expressed as the number of patients with positive findings over total patients checked (percentage).

Table 4
Correlation between anti-NCS Ab titer and other activity markers

	pSLE		aSLE	
	r^a	p^a	r^a	p^a
Anti-dsDNA	0.37	0.004	0.60	0.0009
C3	−0.61	0.0003	−0.44	0.02
C4	−0.56	0.002	−0.30	N.S.
SLEDAI	0.85	<0.0001	0.37	0.047
Modified SLEDAI	0.43	0.02	0.29	N.S.

pSLE: pediatric SLE; aSLE: adult SLE; N.S.: not significant.
[a] Calculated by Spearman's rank correlation.

SLEDAI and medical therapy were equivalent in two groups. However, the influence of differences in disease duration could not be excluded in our study.

Many studies showed a higher prevalence of anti-NCS Ab than anti-dsDNA Ab [10,15,17,24,27,30]. However, with longer disease durations, anti-dsDNA Ab might have a higher prevalence than anti-NCS Ab. In Bruns' study, the mean disease duration was 8 years and a higher prevalence of anti-dsDNA Ab than anti-NCS Ab was noted [12]. Amoura et al. [8], demonstrated that in murine models of lupus, the development of anti-NCS Ab occurred before any other anti-chromatin antibodies. The presence of anti-NCS Ab in SLE patients who were anti-dsDNA Ab-negative [15,16,24] could provide a new reliable diagnostic test. In our pediatric patients, equivalent prevalence of anti-NCS Ab and anti-dsDNA Ab was demonstrated. However, a trend of higher prevalence of anti-NCS Ab than anti-dsDNA Ab was revealed if the sample size was increased.

A positive association of anti-NCS Ab with renal damage has been shown, in both murine models of lupus and in SLE patients [12,13,15,16,28,30–33]. In Ghirardello's study [17], no correlation to renal involvement during follow-up was found, which might raise questions about using anti-nucleosome testing as a marker or predictor of nephritis. In our study, we found no significant difference in the prevalence and titers of anti-NCS Ab between patients with and without active renal involvement. Van Bruggen et al. [34], found that nucelosomes and anti-NCS Ab binded to the glomerular basal membrane (GBM). It was assumed that nucleosomes and anti-NCS Ab binds to the GBM, form the nucleosome-containing immune complexes and cause glomerulonephritis. The cross-reactivity of nucleosome-specific antibodies might also play a role by attacking the glomeruli and causing inflammation. It may be further speculated that the higher levels of anti-NCS Ab in pediatric SLE patients may cause higher prevalence or an exacerbation of lupus glomerulonephritis. However, no significant difference was demonstrated in our study, but this may be due to the limitations of sample size.

Although anti-NCS Ab have been detected both in active and inactive SLEs [12,24], a close relationship between anti-NCS Ab and SLE disease activity evaluated by SLEDAI score [15,23,24,35] or the European Community Lupus Activity Measure (ECLAM) score [16,30] has been summarized. Benucci et al. [16], also demonstrated the significantly lower levels of C3 and C4 in patients with positive anti-NCS Ab. In our pediatric SLE patients, positive correlations between anti-NCS Ab and anti-dsDNA Ab, as well as SLEDAI, were illustrated. We also demonstrated a negative correlation between anti-NCS Ab and C3 as well as C4. Because anti-dsDNA Ab and complement are important components of SLEDAI, the association of anti-NCS Ab with SLEDAI might be a consequence of the strong correlation between anti-NCS Ab, anti-dsDNA Ab and complement. We, therefore, used a modified SLEDAI, in which anti-dsDNA Ab and complement were excluded to avoid overestimation of the correlation. The Spearman's rank correlation coefficient between anti-NCS Ab and modified SLEDAI score was smaller than that between anti-NCS Ab and SLEDAI in pediatric patients. In adult patients, although a correlation between anti-NCS Ab and SLEDAI was demonstrated, there was no significant correlation between anti-NCS Ab with modified SLEDAI. This illustrated that in pediatric patients, but not in adult patients, the correlation of anti-NCS Ab and SLEDAI was also affected by factors other than anti-dsDNA Ab and complement. The clinical relevance of anti-NCS Ab during disease evolution remains highly controversial and further longitudinal studies are needed to clarify this matter.

Our study had some limitations. Cross-reaction among anti-chromatin antibodies was not tested. However, in Simon's study [15], which used the same commercial ELISA kit that ours used, a group of SLE patients was demonstrated to be positive only for anti-NCS Ab and negative for other anti-chromatin antibodies. This was clear evidence of the existence of a group of antibodies that recognized only the quaternary structure of the nucleosomes [36]. Two previous reports demonstrated that this cross-reaction did not affect the interpretation of the test [12,24]. Some studies demonstrated that anti-dsDNA Ab accounted for a minor part ($\leq$30%) of the serum anti-nucleosome reactivity in SLE patients [9,10,24,37].

In conclusion, high sensitivity and specificity of anti-NCS Ab for SLE diagnosis were demonstrated in pediatric patients. Anti-NCS Ab titers were positively correlated to the SLE disease activity in these patients. Therefore, circulating anti-NCS Ab could be a useful parameter for the diagnosis and assessment of disease activity in pediatric SLE patients.

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