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Clinical significance of anti-DFS70 antibody in antinuclear antibody-positive samples with a dense fine speckled pattern

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Abstract

Background: When the dense fine speckled (DFS) pattern-antinuclear antibodies (ANA) are detected in the indirect immunofluorescence (IIF) assay, the presence of anti-dense fine speckles 70 (DFS70) antibodies has been suggested to facilitate the exclusion of ANA-associated rheumatic diseases (AARD). We evaluated the potential use of anti-DFS70 antibodies for verifying AARD in patients with a positive ANA result of the DFS pattern.

Methods: A total of 5509 patients who were requested ANA testing were included. The DFS pattern was confirmed using two IIF assays. Semiquantitative DFS70 ELISA (Euroimmun, Germany) was examined in samples with the DFS pattern.

Results: Among 639 ANA-positive patients, 19.6% displayed the DFS pattern. And 17.6% of patients with the DFS pattern were diagnosed with AARD. The low titer of 1:80 was more prevalent in the non-AARD group than in the AARD group (64.1% vs. 4.5%, $p < 0.0001$). Anti-DFS70 antibodies were positive in 60.0% of patients with the DFS pattern. The frequency of anti-DFS70 positivity was higher in the non-rheumatic disease (NRD) group (74.2%) than in the other rheumatic disease group (43.2%, $p = 0.003$) and the AARD group (45.5%, $p = 0.019$).

Conclusions: The DFS pattern is present in both AARD and non-AARD cases. In the DFS pattern, a low titer of 1:80 and isolated anti-DFS70 antibodies without AARD-associated antibodies represent a low likelihood of

AARD. The presence of anti-DFS70 antibodies cannot exclude AARD and should be analyzed in combination with AARD-associated antibodies in the diagnostic algorithm.

Keywords: ANA-associated rheumatic diseases; antinuclear antibody; DFS70; indirect immunofluorescence.

Introduction

The presence of antinuclear antibodies (ANA) against intracellular antigens is a hallmark of ANA-associated rheumatic diseases (AARD). Despite advances in methodologies for the determination of ANA, indirect immunofluorescence (IIF) using human epithelial cell (HEp-2) substrate remains the most prevalent method [1]. However, a positive ANA test alone does not indicate AARD because it was reported that positive ANA results were present in 12.9% of healthy individuals [2].

In subjects with a positive ANA result, the titer and the IIF pattern are proposed to be a relevant parameter that can be used for discriminating between ANA-positive healthy individuals and AARD patients. It was noted that titers $\leq 1:160$ and the dense fine speckled (DFS) pattern are predominantly observed in individuals without AARD [2–5]. But recognition of the DFS pattern can be confused with other chromatin-staining patterns and be affected by evaluator expertise.

The DFS pattern has been described as a staining pattern that shows distribution of the speckles throughout the interphase nucleus and some coarse speckles standing out on metaphase plate. The distinct DFS pattern has been indicated to be associated with antibodies targeting dense fine speckles 70 (DFS70), which was identified as a protein called transcription coactivator p75 and lens epithelium-derived growth factor [6, 7]. It is recommended that a positive ANA result of the DFS pattern need to be followed by an immunoassay for anti-DFS70 antibodies because accurate interpretation of the DFS pattern can be

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challenging in the IIF method although the use of the new substrate can be helpful in resolving this limitation [8, 9].

DFS70 is known to have multiple biologic functions, but clear disease associations have not been confirmed. Based on long-term patient outcomes, none of the 40 healthy individuals with isolated anti-DFS70 reactivity developed AARD within an average of 4 years of follow-up [2]. Anti-DFS70 antibodies have been detected in various non-rheumatic conditions including several inflammatory diseases, and the frequency of anti-DFS70 antibodies was significantly lower in AARD patients compared to patients without AARD [3, 4, 10–12].

A few previous studies included the testing for anti-DFS70 antibodies as a component of diagnostic algorithms when the DFS pattern is identified, and the presence of anti-DFS70 antibodies showed a potential as diagnostic determinants between ANA-positive subjects with and without AARD [13, 14]. However, in other studies, the significance of the DFS pattern and anti-DFS70 antibodies in patients with rheumatic symptoms and positive ANA results remains to be established [10, 11].

The present study aimed to assess the frequency of the DFS pattern and anti-DFS70 antibodies in a cohort of patients undergoing routine ANA testing. In addition, we evaluated the usefulness of the DFS pattern and anti-DFS70 autoantibodies in excluding AARD diagnosis and analyzed changes in ANA reactivity during follow-up testing.

Materials and methods

Subjects

ANA testing was referred to 5509 patients for AARD screening from June 2016 to February 2018. Patients showing the DFS pattern were included in our study and were diagnosed according to the established criteria. Clinical diagnoses were classified into AARD and non-AARD, and then non-AARD was subdivided into other autoimmune rheumatic diseases (ORD) and non-rheumatic diseases (NRD). The following were considered as AARD: systemic lupus erythematosus (SLE), systemic sclerosis, Sjögren's syndrome (SjS), mixed connective tissue disease, polymyositis/dermatomyositis (PM/DM) and undifferentiated connective tissue disease (UCTD). Rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA) and spondyloarthropathies (SpAs) were classified as ORD. This study was approved by the Institutional Review Board of our institution.

ANA IIF testing and anti-DFS70 assay

Serum samples were examined using the IIF assay, FLUORO HEPANA TEST (MBL, Nagoya, Japan) for ANA screening. The screening dilution of ANA IIF testing was 1:80, and ANA titer was determined up to 1:5120 by successive twofold dilutions. ANA titers and patterns were determined by two independent evaluators using an Olympus BX 51 fluorescence microscope (Olympus Corporation, Tokyo, Japan). In samples classified as ANA positive, IIF was performed again using Mosaic HEp-2010 (Euroimmun, Lübeck, Germany). The ANA pattern was determined as specific patterns if the same pattern was identified in both substrates. In patients with positive ANA results of DFS pattern, the ANA IIF testing was additionally examined after 6 months.

All serum samples displaying the DFS pattern were tested for anti-DFS70 antibodies by semiquantitative DFS70 ELISA (Euroimmun). A total of 50 sera that were positive for ANA with other IIF patterns (homogenous, $n=15$; speckled, $n=15$; discrete speckled, $n=10$; nucleolar, $n=5$; cytoplasmic, $n=5$) and a titer of 1:160 or 1:320 were used as controls. According to the manufacturer's instructions, 1:201 diluted 100 μ L of serum was transferred to DFS70 antigen-coated microplate wells and incubated for 30 min at room temperature (RT). After washing, the plate was incubated with 100 μ L of enzyme conjugate for 30 min at RT. Afterward, each well was added with 100 μ L of chromogen/substrate solution and incubated for 15 min at RT in the dark. After adding 100 μ L of stop solution, the optical absorbance of each well was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The levels of anti-DFS70 antibodies were determined by calculating the ratio of the extinction value of the sample over the extinction value of the calibrator. Antibody levels higher than 1.0 ratio were considered positive.

Detection of other autoantibodies

Rheumatoid factor (RF) was determined by nephelometry on an automated chemical analyzer TBA-2000 FR (Toshiba, Otawara, Japan). Levels of anti-cyclic citrullinated peptide (CCP) antibodies were measured using a chemiluminescent immunoassay Architect anti-CCP assay (Abbott Laboratories, Abbott Park, IL, USA). AARD-associated antibodies against double stranded DNA (dsDNA) and six subtypes of extractable nuclear antigens (ENA), namely Sjögren's syndrome A (SSA), Sjögren's syndrome B (SSB), ribonucleoprotein (RNP), Sm, Jo-1 and Scl70 were analyzed using automated enzyme immunoassay (EIA) (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed using MedCalc version 14.12.0 (MedCalc software, Ostend, Belgium). Independent t-test or Mann-Whitney test was used for comparison of continuous variables, and Fisher's exact test was used for categorical variables. Receiver-operating characteristic (ROC) curve analysis was used to discriminate between patients with and without AARD. A p-value <0.05 was considered statistically significant.

Results

Patient characteristics

Positive ANA test results were detected in 639 (11.6%) out of 5509 patients, and 125 (2.3%) patients showed the DFS pattern. Out of the 125 patients, 96 (76.8%) were female. The mean age of patients showing the DFS pattern was 46.2 ± 20.8 years (range, 4–76 years). The prevalence of the DFS pattern was 19.6% of positive ANA test results.

Table 1 shows the demographic characteristics of individuals with the DFS pattern. Among the 125 patients showing the DFS pattern, 66 (52.8%) were classified as the NRD group without evidence of autoimmune disease and 22 (17.6%) were diagnosed with AARD. The AARD group comprised 14 with SLE, five with SjS, two with UCTD and one with DM/PM. In the NRD group, inflammatory diseases, renal disorders and dermatologic diseases were the most common disease entities. The remaining was 37 with ORD (29.6%) and included 34 with RA, two with JIA and one with SpAs.

The 125 patients with the DFS pattern showed a broad range of ANA titers (1:80 to >1:1280). The most common titer was 1:80, which was observed in 67 (53.6%) patients. The remaining 58 patients had ANA titers of 1:160 or higher. The number of patients at each titer was 44 (35.2%) at 1:160, nine (7.2%) at 1:320, two (1.6%) at 1:640, two (1.6%) at 1:1280, and one (0.8%) at >1:1280. The ANA titers of 1:80 or 1:160 were more prevalent in the ORD and NRD groups than in the AARD group ($p < 0.0001$, $p < 0.0001$).

In the follow-up ANA testing after 6 months, 88.0% of patients showed the same or elevated ANA titers, but 15 patients (10 with NRD and five with ORD) with the initial

Table 1: Clinical characteristics and distribution of titer in 125 ANA-positive patients with the DFS pattern.

Diseases	Titer				
	1:80 (n=67)	1:160 (n=44)	1:320 (n=9)	1:640 (n=2)	≥1:1280 (n=3)
AARD (n=22)	1 (1.5%)	9 (20.4%)	7 (77.8%)	2 (100.0%)	3 (100.0%)
SLE (n=14)	1 (1.5%)	5 (11.4%)	5 (55.6%)	1 (50.0%)	2 (66.7%)
SjS (n=5)		1 (2.3%)	2 (22.2%)	1 (50.0%)	1 (33.3%)
UCTD (n=2)		2 (4.5%)			
PM/DM (n=1)		1 (2.3%)			
ORD (n=37)	23 (34.3%)	13 (29.6%)	1 (11.1%)		
RA (n=34)	21 (31.3%)	12 (27.3%)	1 (11.1%)		
JIA (n=2)	2 (3.0%)				
SpAs (n=1)		1 (2.3%)			
NRD (n=66)	43 (64.2%)	22 (50.0%)	1 (11.1%)		
Inflammatory diseases (n=18)	8 (11.9%)	10 (22.7%)			
Renal diseases (n=12)	10 (14.9%)	2 (4.5%)			
Dermatologic diseases (n=10)	6 (8.9%)	3 (6.8%)	1 (11.1%)		
Infections (n=6)	5 (7.5%)	1 (2.3%)			
Liver diseases (n=5)	4 (6.0%)	1 (2.3%)			
Neurologic diseases (n=4)	4 (6.0%)				
Hematologic diseases (n=2)	2 (3.0%)				
Pulmonary diseases (n=1)		1 (2.3%)			
Other diseases (n=8)	4 (6.0%)	4 (9.1%)			

ANA, antinuclear antibodies; DFS, dense fine speckled; AARD, ANA-associated rheumatic diseases; ORD, other autoimmune rheumatic diseases; NRD, non-rheumatic diseases; SLE, systemic lupus erythematosus; SjS, Sjögren's syndrome; UCTD, undifferentiated connective tissue disease; PM/DM, polymyositis/dermatomyositis; RA, rheumatoid arthritis; JIA, juvenile idiopathic arthritis; SpAs, spondyloarthropathies.

titer of 1:80 showed negative conversion of ANA results (<1:80).

Clinical association of anti-DFS70 antibodies

As shown in Table 2, ELISA results showed that 75 (60.0%) out of the 125 samples with the DFS pattern were anti-DFS70 positive. Anti-DFS70 antibodies were detected in 49 NRD patients, 16 ORD patients (13 RA, two JIA and one SpAs) and 10 AARD patients (eight SLE and two SjS). By contrast, anti-DFS70 antibodies were not detected in all 50 controls with non-DFS IIF patterns. The prevalence of anti-DFS70 antibodies was significantly higher in the NRD group (74.2%) than in the ORD (43.2%, $p=0.003$) and AARD groups (45.5%, $p=0.019$).

In specimens of a titer of 1:80, anti-DFS70 antibodies were more frequently detected in the NRD group (67.4%) than in the ORD group (30.4%) ($p=0.005$). In specimens of a titer of $\geq 1:160$, positivity of anti-DFS70 antibodies was significantly higher in the NRD group (87.0%) than in the AARD group (47.6%) ($p=0.009$). On the other hand, the prevalence and median ratio of anti-DFS70 antibodies were not different between NRD patients with a titer of 1:80 and NRD patients with a titer of $\geq 1:160$ ($p=0.145$, $p=0.380$). Out of 15 patients with the negative conversion in the follow-up ANA testing, anti-DFS70 antibodies were present in only four patients. The negative conversion rate of ANA was higher in patients without anti-DFS70 antibodies than in those with anti-DFS70 antibodies (22.0% vs. 5.3%, $p=0.012$).

ROC curve analysis demonstrated that the anti-DFS70 test discriminated between non-AARD patients and AARD patients with an area under the ROC curve (AUC) of 0.642 (95% confidence interval [CI] 0.552–0.726; $p=0.032$). At a cut-off ratio of 1.5, the sensitivity was 58.3% and the specificity was 68.2% in classifying as non-AARD. At a cut-off ratio of 1.0 based on the manufacturer's recommendation, the anti-DFS70 test had a sensitivity of 62.1% and a specificity of 54.6%.

Anti-dsDNA or ENA antibodies were detected in all patients with AARD. In 14 SLE patients with the DFS-ANA pattern, anti-dsDNA antibodies were detected in 12 patients. The frequency of anti-DFS70 antibodies was not significantly different between patients with anti-dsDNA antibodies (6/10, 60%) and patients without anti-dsDNA antibodies (2/2, 100%) ($p=0.515$).

In 34 RA patients with the DFS-ANA pattern, RF or anti-CCP antibodies were detected in all patients. The anti-DFS70-positive and -negative groups showed no significant differences in the mean levels of RF (78.2 ± 67.7 vs. 100.4 ± 68.9 , $p=0.366$) and anti-CCP antibodies (340.3 ± 189.6 vs. 384.3 ± 138.0 , $p=0.202$).

Application of diagnostic algorithm

We analyzed the usefulness of diagnostic algorithm using the presence of anti-DFS70 antibodies and AARD-associated antibodies for the prediction of AARD. When we discriminated the patients by the presence of anti-DFS70 antibodies and AARD-associated antibodies, 95.5% of AARD patients were interpreted as AARD likely and 98.1% of non-AARD patients were interpreted as AARD unlikely. In the case of patients interpreted as AARD unlikely, additional tests for RF and anti-CCP antibodies subdivided 31.4% of non-AARD patients as the ORD group. However, the algorithm displayed a wrong classification in 2.4% of patients (two with RA as AARD likely, one with UCTD as AARD unlikely) (Figure 1).

Discussion

ANA testing is currently referred to the laboratory from a broad range of clinicians besides rheumatologists, and positive results are observed in a sizable proportion of the healthy population. Therefore, accurate interpretation of test results is important to prevent unnecessary further

Table 2: Prevalence of anti-DFS70 antibodies in the AARD, ORD and NRD groups.

	AARD (n=22)			ORD (n=37)			NRD (n=66)		
	1:80 (n=1)	$\geq 1:160$ (n=21)	Total	1:80 (n=23)	$\geq 1:160$ (n=14)	Total	1:80 (n=43)	$\geq 1:160$ (n=23)	Total
Anti-DFS70 (–) (n=50)	1 (100.0%)	11 (52.4%)	12 (54.5%)	16 (69.6%)	5 (35.7%)	21 (56.8%)	14 (32.6%)	3 (13.0%)	17 (25.8%)
Anti-DFS70 (+) (n=75)	0 (0.0%)	10 (47.6%)	10 (45.5%)	7 (30.4%)	9 (64.3%)	16 (43.2%)	29 (67.4%)	20 (87.0%)	49 (74.2%)
p-Value	0.341	0.009 ^a	0.019 ^a	0.005 ^a	0.215	0.003 ^a			

AARD, ANA-associated rheumatic diseases; ORD, other autoimmune rheumatic diseases; NRD, non-rheumatic diseases; DFS70, dense fine speckles 70. ^aStatistically significant when compared with the NRD group.

Figure 1: The application of diagnostic algorithm in individuals with positive anti-nuclear antibody (ANA) results of the dense fine speckled (DFS) pattern in indirect immunofluorescence (IIF) testing.

AARD, ANA-associated rheumatic diseases; NRD, non-rheumatic diseases; ORD, other rheumatic diseases.

evaluation and undue concern in patients. Recently, the International Consensus on ANA Patterns (ICAP) defined the nomenclature and classification of IIF patterns [15]. Among several competency level patterns, the DFS pattern has to be differentiated from the classical homogenous nuclear pattern because the clinical relevance of these two patterns is different.

The DFS pattern has been reported in a variable range from 0.8% to 12.3% of routine ANA cohorts [4, 5, 10, 16–18]. The wide range of prevalence could be explained by the heterogeneity of the study population (age, gender, ethnic groups, reasons for requesting ANA testing), differences in the method (HEp-2 cell lines, screening dilutions) and subjective identification of the DFS pattern. So, we identified the DFS pattern using two evaluators and two IIF assays to minimize the possibility of misinterpretation.

In the present study, the DFS pattern was observed in 2.3% of clinical samples submitted for ANA screening when a screening dilution of 1:80 was adopted. The prevalence of the DFS pattern was similar to the reported values of 1.7% at 1:100 [10] and 3.8% at 1:40 [5] in Korean studies. And the DFS pattern comprised 19.6% of ANA-positive cases. Previous studies demonstrated that the DFS pattern

was recognized in 24.5%–37.0% of ANA-positive cases [2, 5, 17, 18]. Our finding supports that the DFS pattern is one of the commonly observed IIF patterns in ANA-positive cases although the prevalence of the DFS pattern is relatively low as about 2% in the Korean population.

In assessing the clinical significance of a positive ANA result, it was reported that a low ANA titer (1:80) or the DFS pattern in IIF assay increases the discriminative performance between ANA-positive healthy individuals and AARD patients [2]. And a few studies demonstrated that the DFS pattern tends to occur mostly in patients with non-AARD [11, 17]. In this study, 82.4% of patients with the DFS pattern were associated with non-AARD, and especially, 98.5% of patients with the DFS pattern of a titer of 1:80 were confirmed to have non-AARD. On the contrary, most patients with AARD (95.5%) had titers $\geq 1:160$. Although the positive predictive value of a positive ANA result was poor for the diagnosis of AARD [19], we assured that the analysis of an ANA titer is helpful in excluding AARD in cases with the DFS pattern.

In our population, anti-DFS70 antibodies were detected in 60.0% of patients with the DFS pattern, and the prevalence was similar with 60.2% in the Korean

population [10]. Previous studies reported that the positive rates of anti-DFS70 in individuals with the DFS pattern ranged from 11% to 98.8% [4, 5, 10, 16–18]. The difference in concordance rates between the DFS pattern and anti-DFS70 antibodies could be attributed to interlaboratory variability in detecting the DFS pattern and the presence of autoantibodies that produce a DFS-like pattern [12]. We thought that the recognition of typical DFS pattern is necessary to be confirmed by the presence of anti-DFS70 antibodies to exclude a DFS-like pattern.

The presence of anti-DFS70 antibodies has been proposed as a biomarker for the exclusion of AARD [4, 11, 20]. However, this clinical association remains indistinct because anti-DFS70 antibodies are detected in AARD [10, 14]. A recent study reported that UCTD patients showed the high prevalence of anti-DFS70 antibodies (13.2%) and its presence can be related to an evolution to CTD [21]. In our study, the prevalence of anti-DFS70 antibodies was significantly lower in the AARD group compared to the NRD group, but anti-DFS70 antibodies were also present in AARD patients. And ROC analysis of anti-DFS70 antibodies showed low sensitivity and specificity in discriminating between AARD and non-AARD patients. Therefore, isolated anti-DFS70 antibodies without concomitant AARD-associated antibodies may be an exclusive marker of AARD.

Besides, the positive rate of DFS70 antibodies was significantly lower in the ORD group than in the NRD group. Among ORD patients with the DFS pattern, RA patients were the majority and anti-DFS70 antibodies were detected in 41.2% of RA patients. A previous study reported that the DFS pattern is frequently observed in RA [5]. These results suggested that tests for RA-associated autoantibodies should be considered in patients with the DFS pattern and an isolated detection of anti-DFS70 antibodies in the absence of AARD and RA-associated autoantibodies reflects a likelihood of NRD.

We observed that ANA positivity of the DFS pattern persists like other specific patterns during follow-up testing although some patients with a low titer of 1:80 showed negative conversion. In addition, negative conversion of a positive ANA test result was more frequently observed in patients without anti-DFS70 antibodies, and cases showing negative conversion were classified under the non-AARD group. It was reported that a low titer of 1:80 in healthy individuals had the probability of future negative conversion [2]. We thought that the DFS pattern at a low titer tends to be weakened and the reduction of titer over a follow-up period may happen in patients with non-AARD.

Schmeling et al. [22] showed that anti-DFS70 antibodies were present in children with JIA-associated uveitis.

A study in Japanese children [23] revealed that five out of 10 JIA patients with iridocyclitis showed the presence of anti-DFS70 antibodies. In the present study, anti-DFS70 antibodies were detected in all JIA cases, but concomitant eye diseases were not confirmed. Considering the small number of patients, the association between anti-DFS70 antibodies and JIA complicated with eye diseases remains inconclusive and thus requires further studies.

Patients with AARD showed the presence of at least one AARD-associated antibody. A recent study on an SLE cohort indicated that patients with anti-dsDNA were less likely to have anti-DFS70 antibodies [24]. In this study, the frequency of anti-DFS70 antibodies was not related to the presence of anti-dsDNA antibodies. And, in RA, the prevalence of anti-DFS70 antibodies was not associated with the intensity of RA-associated antibodies. These findings suggest that the presence of rheumatic disease-associated antibodies has no influence on the development of anti-DFS70 antibodies.

Several surveys [10, 13, 25] noted that the DFS pattern should be confirmed by conducting a specific anti-DFS70 test and that the combined use of ENA panel is necessary to verify the diagnostic relevance of the test. In the present study, the diagnostic algorithm incorporating anti-DFS70 and AARD-associated antibodies increased the diagnostic performance between non-AARD and AARD patients with the DFS pattern. The workup test for RA in the absence of AARD-associated antibodies could provide the additional diagnostic value.

Conclusions

The DFS pattern is commonly observed in IIF-ANA-positive cases and is present in both AARD and non-AARD cases. A low titer of 1:80 and isolated anti-DFS70 without AARD-associated antibodies represent the low likelihood of AARD. Anti-DFS70 antibodies should be integrated with AARD-associated antibodies in cases with the DFS pattern to exclude AARD.

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