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Autoantibody to NA14 is an independent marker primarily for Sjögren's syndrome

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Abstract

Nuclear Autoantigen of 14 kDa (NA14) was originally identified using the serum of a Sjögren's syndrome (SS) patient as probe in screening a human testis cDNA expression library. To date there is no report in the systematic analysis of the prevalence of autoantibodies to NA14. In this study, anti-NA14 was determined in several rheumatic diseases from independent cohorts in the US and Japan. The prevalence of anti-NA14 were 18/132 (13.6%) in primary SS, 0/50 (0%) secondary SS, 2/100 (2%) SLE, 1/43 (2.3%) scleroderma, 0/54 (0%) rheumatoid arthritis, 1/29 (3.4%) polymyositis/dermatomyositis, and 0/58 (0%) normal healthy controls. The frequencies of anti-NA14 positive sera in primary SS are statistically greater than normal healthy controls ($p=0.006$), secondary SS ($p=0.044$), and other rheumatic diseases. Furthermore, among 11 anti-NA14 positive primary SS sera, 4/11 (36.3%) sera were negative for both anti-SS-A/Ro and SS-B/La antibodies. Thus anti-NA14 autoantibodies may be useful for the discrimination of primary versus secondary SS and serve as a diagnostic marker for primary SS especially in seronegative (anti-SS-A/Ro and anti-SS-B/La antibodies negative) patients with SS.

Keywords

NA14; Sjögren's syndrome; Autoantigen; Autoantibody; Coiled-Coil Protein; Review

2. INTRODUCTION

Sjögren's syndrome (SS) is a member of the family of systemic autoimmune diseases, in which the immune response is activated to recognize a limited subset of ubiquitously expressed self antigens (1,2). Although SS is characterized primarily by chronic sialadenitis and dacryoadenitis, a variety of systemic manifestations have been described. The disease may be isolated (primary SS) or it may occur in association with other rheumatic diseases (secondary SS) such as rheumatoid arthritis (RA), scleroderma (SSc), and systemic lupus erythematosus (SLE). Although the pathogenesis of SS remains unclear, one of the central clues comes from the observation that the immune system in SS targets a restricted and highly specific group of intracellular autoantigens (3). These cellular antigens that are often targeted by high-titer autoantibody response are a diverse group of macromolecules that are ubiquitously expressed. In systemic autoimmune diseases, many of these cellular antigens are components of protein-nucleic acid complexes but otherwise share no obvious features in terms of subcellular distribution, protein structure, or function (4). In spite of the diversity of self antigens, the specificity of the autoimmune response is remarkably predictive of disease phenotype, such that specific autoantibodies have become useful in clinical diagnosis and prognosis (5–8). The predominant autoantigen targets in SS are the ribonucleoprotein autoantigens SS-A/Ro and SS-B/La (9). There have been many reports of other less prevalent autoantibodies such as coiled-coil-rich proteins including nuclear mitotic apparatus protein (NuMA) (10,11), members of golgin family (12), alpha-fodrin (13) and M3 muscarinic receptors (14,15). Other autoantigens described in SS include poly (ADP) ribose polymerase (PARP) (16), 90-KDa nucleolar organizer region protein (NOR90) (17), p80-coilin (18), and many others that do not appear to be restricted to SS. Although autoantibodies to SS-A/Ro and SS-B/La are indicative of SS, their presence is not specific since they are found with some frequency in patients with other systemic autoimmune diseases such as SLE (5,9).

Nuclear autoantigen 14 kDa (NA14) was originally identified as a novel autoantigen recognized by an autoimmune serum from a patient with SS (19). Ramos-Morales *et al* reported that neither the autoimmune serum nor a polyclonal antibodies raised against recombinant NA14 recognized endogenous NA14 by conventional IIF using HeLa cells (19). For this reason, subcellular localization was achieved by expression of an HA-tagged version of NA14. Under these conditions, transfected NA14 localized to the nucleus and thus NA14 was reported as a nuclear autoantigen (19). Although physiological role of NA14 remains unknown so far, several reports recently have suggested NA14 protein localizes at not only nucleus but also centrosomes and play an important role in cell division and proliferation (20–22). Although expression of NA14 in salivary and lacrimal glands has not been determined, it is reasonable to expect that it is expressed in these tissues since NA14 seems to be widely expressed.

NA14 was identified as an autoantigen recognized by a human SS serum. However, there is no report regarding the prevalence of autoantibodies to NA14 in systemic autoimmune diseases to date and correlation with clinical manifestation is completely absent. In this study, anti-NA14 autoantibodies were determined in patients with various rheumatic diseases from cohorts in both US and Japan.

3. MATERIALS AND METHODS

3.1. Human sera

Human rheumatic diseases sera were obtained from laboratory serum bank of Juntendo University Hospital and the University of Florida Autoimmune Disease Center with institutional ethics approval. Patient sera with primary SS (1'SS, n=132), secondary SS (2'SS, n=50), SLE (n=100), RA (n=54), SSc (n=43), PM/DM (n=29) and normal healthy controls (NHS, n=58) were assessed. Patients with SS fulfilled the American–European Consensus

Criteria (23). Patients with RA, SLE, and SSc fulfilled the criteria of American College of Rheumatology (24–26). Patients with PM/DM fulfilled the Bohan's criteria (27). Patients sera with secondary SS was derived from patients with SS in association with other rheumatic diseases, including SLE (n=27), RA (n=15), PM/DM (n=3), SSc (n=2), primary APS (n=2), and polyarteritis nodosa (PN, n=1).

3.2. Recombinant NA14 protein

NA14 full length cDNA were cloned into pET28 expression vector and introduced into *Escherichia coli* BL21 (DE3; Novagen, Madison, WI, USA) as previously described (28). The expression construct was confirmed by direct DNA sequencing in both strands. Bacterial pellets were suspended in 6M guanidium hydrochloride containing buffer, and the recombinant proteins were purified by nickel column chromatography according to manufacturer's instructions (Qiagen, Valencia, CA, USA). The concentration of the purified recombinant protein was measured by a Protein DC Kit (Bio-Rad, Hercules, CA, USA) and the samples were stored at -80°C .

3.3. Enzyme-linked immunosorbent assay

The ELISA protocol described by Rubin (29) was used with some modifications. In brief, nickel column affinity purified recombinant protein was diluted in phosphate-buffered saline to a final concentration of $1\mu\text{g/ml}$ and then coated on Immunolon2 microtiter plates (Dynatech Laboratories, Alexandria, VA, USA) overnight at 4°C . Human sera were diluted at 1:1000 and then incubated in the antigen-coated wells. Horseradish peroxidase-conjugated goat anti human IgG (CALTAG Laboratories, San Francisco, CA, USA) was used at 1:5000 dilution and the substrate 2,2'-azinobis (3-ethylbenzthiazoline) sulfonic acid was added as the detection reagent. Samples were analyzed in duplicate and the average optical density (OD) at 405 nm with a substrate development of 15 min was used for data analysis. The cut off value designating a positive reaction was the mean OD of normal healthy controls +5 standard deviation (SD). In some anti-NA14 antibody positive sera with primary SS, antibodies to SS-A/Ro and SS-B/La were measured by ELISA system (MASCAP test) according to manufacturer's instructions (MBL, Tokyo, Japan). Statistical analysis was performed by Chi-square test.

3.4. Immunoblotting

Affinity purified recombinant proteins were loaded on 15% SDS-PAGE gels ($1\mu\text{g/lane}$), separated by electrophoresis, and transferred to nitrocellulose membranes using a Semi-Dry Tran-Blot apparatus (Bio-Rad) as described previously (28,30). Human sera containing anti-NA14 antibodies were used at dilutions of 1:100. Detection of bound antibodies was achieved using horseradish peroxidase-conjugated goat anti-human IgG antibody (CALTAG), used at 1:5000 dilution, and developed with Supersignal enhanced chemiluminescence (Pierce Products, Rockford, IL, USA).

4. RESULTS

As NA14 was originally reported as autoantigen recognized by patient sera with SS, we initiated investigation in the prevalence of autoantibodies to NA14 using affinity purified recombinant protein in ELISA format. To avoid experimental deviation caused by individual center study, ELISA was independently performed using the same protocol at Juntendo University and the University of Florida testing patients sera with various rheumatic diseases including primary SS, secondary SS, SLE, SSc, RA, PM/DM, and normal healthy controls. Figure 1 shows independent data derived from Japan (upper panel) and USA (lower panel) respectively. The combined prevalence of anti-NA14 were 18/132 (13.6%) primary SS, 0/50 (0%) secondary SS, 2/100 (2%) SLE, 1/43 (2.3%) SSc, 0/54 RA (0%), and 1/29 (3.4%) PM/DM. As expected, anti-NA14 antibody positive sera were observed most frequently in primary

SS. Interestingly, none of the secondary SS patients had autoantibodies to NA14. There were no obvious differences observed between patient cohorts in Japan and USA. Although a few anti-NA14 positive sera were observed in other rheumatic diseases like SLE, SSc, and PM/DM, anti-NA14 autoantibodies were almost exclusively observed in only primary SS compared to other rheumatic diseases.

To compare the prevalence of anti-NA14 autoantibodies in primary SS to those of other rheumatic diseases more precisely, we combined Japanese and USA data and performed further statistical analysis (Table 1). Although p value was not significant comparing to PM/DM due to small number of the sera, the prevalence of anti-NA14 autoantibodies in primary SS was statistically greater than in other rheumatic diseases and normal healthy controls. Interestingly, none of sera from patients with secondary SS reacted with NA14 and the frequency of anti-NA14 positive sera in primary SS are statistically greater than normal healthy controls ($p=0.002$) and secondary SS ($p=0.0041$) and other rheumatic diseases. The reactivity against NA14 recombinant protein in primary SS was also confirmed by immunoblotting (Figure 2).

To investigate the clinical association between primary SS and anti-NA14 autoantibodies further, we measured anti-SS-A/Ro and SS-B/La antibodies in anti-NA14 positive sera with primary SS. Among 11 available anti-NA14 positive sera, 4/11 (36.3%) sera were negative for both anti-SS-A/Ro and SS-B/La antibodies (Table 2). These data suggest anti-NA14 antibodies not only can discriminate primary and secondary SS serologically, but also it can be used as a potential diagnostic marker for primary SS even in the case when both anti-SS-A/Ro and SS-B/La antibodies are negative.

5. DISCUSSION

5.1. Diseases specificity of anti NA-14 antibodies

In our current study, anti-NA14 autoantibodies were observed primarily in primary SS compared to other rheumatic diseases including secondary SS. Our data showed anti-NA14 antibodies appeared independent of anti-SS-A/Ro anti-SS-B/La antibodies and 36.4% (4/11) of anti-NA14 positive sera was negative for anti-SS-A/Ro and anti-SS-B/La antibodies. It has been proposed that primary SS negative for anti-SS-A/Ro and SS-B/La antibodies represent immunologically distinct populations compared to anti-SS-A/Ro and SS-B/La antibodies positive population. Kelly *et al.* reported that the presence of anti-SS-A/Ro antibodies identified patients with more severe systemic diseases (31). Subsequently, Davidson *et al.* described that parotid swelling and lymphadenopathy were more common in anti-SS-A/Ro and SS-B/La antibodies positive patients and the risk of developing non-Hodgkin's lymphoma was higher in this group compared to anti SS-A/Ro and SS-B/La antibodies seronegative patients (32). Therefore, primary SS may be divided into clinically distinct groups based on the presence of specific autoantibodies such as anti-SS-A/Ro antibodies. Although we have not been able to analyze the characteristics of anti-NA14 antibodies positive patients due to the lack of clinical information, the detection of anti-NA14 antibodies may provide useful information with primary SS and future studies will be needed to examine correlations with clinical activities.

5.2. Coiled-coil proteins elicit autoantibodies production

The unique characteristic of NA14 is that it is predicted to contain coiled-coil secondary structure in the N-terminal half. This is interesting since we and others reported a class of coiled-coil proteins that are recognized as intracellular autoantigens in systemic autoimmune diseases (12,33). Coiled-coil rich autoantigens have been noted in several cytoplasmic organelles, including a family of Golgi complex antigens known as golgins (12,33), endosomal proteins EEA1 (34) and CLIP-170 (35), centrosomal proteins pericentrin (36), ninein (37), and

Cep250 and Cep110 (38). The mitotic organelles are also known to be associated with coiled-coil rich autoantigens, including NuMA (10,11,39), and centromere associated protein CENP-E (38,40,41) and CENP-F (42,43). It is not clear why coiled-coil rich protein can selectively elicit autoimmune response; however, one possible explanation is that this protein structure may be more stable and can promote the induction and production of antibodies in certain diseases states such as SS. It is important to note that the immune response is not merely directed at crossreactive coiled-coil structure, because the human autoimmune response to coiled-coil proteins appears to be highly specific in our study (28,30).

5.3. Pathogenesis of NA14 in primary SS

Although it has been reported that NA14 play an important role for cell division and proliferation (20–22), the exact physiological roles of NA14 remain unknown. Recently, Aki T *et al* reported that the orphan receptor Transmembrane Protein Regulated in Adipocytes of 40kDa (TPRA40/GPR175) could bind to NA14 and TPRA40 regulated cell division in mouse embryocytes through the interaction with NA14 (44). TPRA40 was originally cloned and its level found to be significantly increased in diabetic mice (45). Recently, a full-length cDNA encoding a human seven transmembrane domain orphan receptor (GenBank accession number AB037108) was isolated from human fetal brain. The deduced amino acid sequence shows 85.8% homology to rat TPRA40 and 83.3% homology to mouse TPRA40 (46). The regulation of cell division by TPRA40 was also observed in human HeLa cells (44). Although the report showed the interaction of TPRA40 and NA14 can regulate cell division, the precise mechanism of interaction of NA14 and TPRA40 has not been elucidated (44). TPRA40 is considered to be a membrane receptor, therefore, its ligand should exist in soluble form like chemokines or as cell surface membrane protein which can function in promoting cell-cell interaction. Since NA14 is found to be an intracellular protein to date, it is surprising that NA14 interacts with the cell surface TPRA40. One wild speculation is that NA14, being a relatively short polypeptide, may exist not only as an intracellular protein but also a secreted protein like chemokines. As it was reported that NA14 is widely expressed in variety of tissues, NA14 may be overexpressed in the salivary and lacrimal glands tissues in primary SS and this may induce autoantibody production by an antigen-driven mechanism described in SS (47). It is also possible that NA14 has chemokine-like activity and play a role in lymphocytes infiltration in salivary and lacrimal tissues in primary SS patients and the infiltration leads the tissue destruction. If NA14 is overexpressed in salivary and lacrimal tissues in primary SS, it might serve as a chemokine and attract lymphocytes from blood vessel to the tissues. We are now conducting experiments to examine the expression of NA14 in salivary tissues with primary SS patients and assay NA14 for chemokine-like activities.

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Kazuhisa Nozawa and Keigo Ikeda contributed equally to this study.

Abbreviations

APS	anti phospholipid syndrome
NA14	nuclear autoantigen 14 kDa
IIF	indirect immunofluorescence
PM/DM	polymyositis/dermatomyositis
SSc	Scleroderma
SLE	systemic lupus erythematosus

SS Sjögren's syndrome

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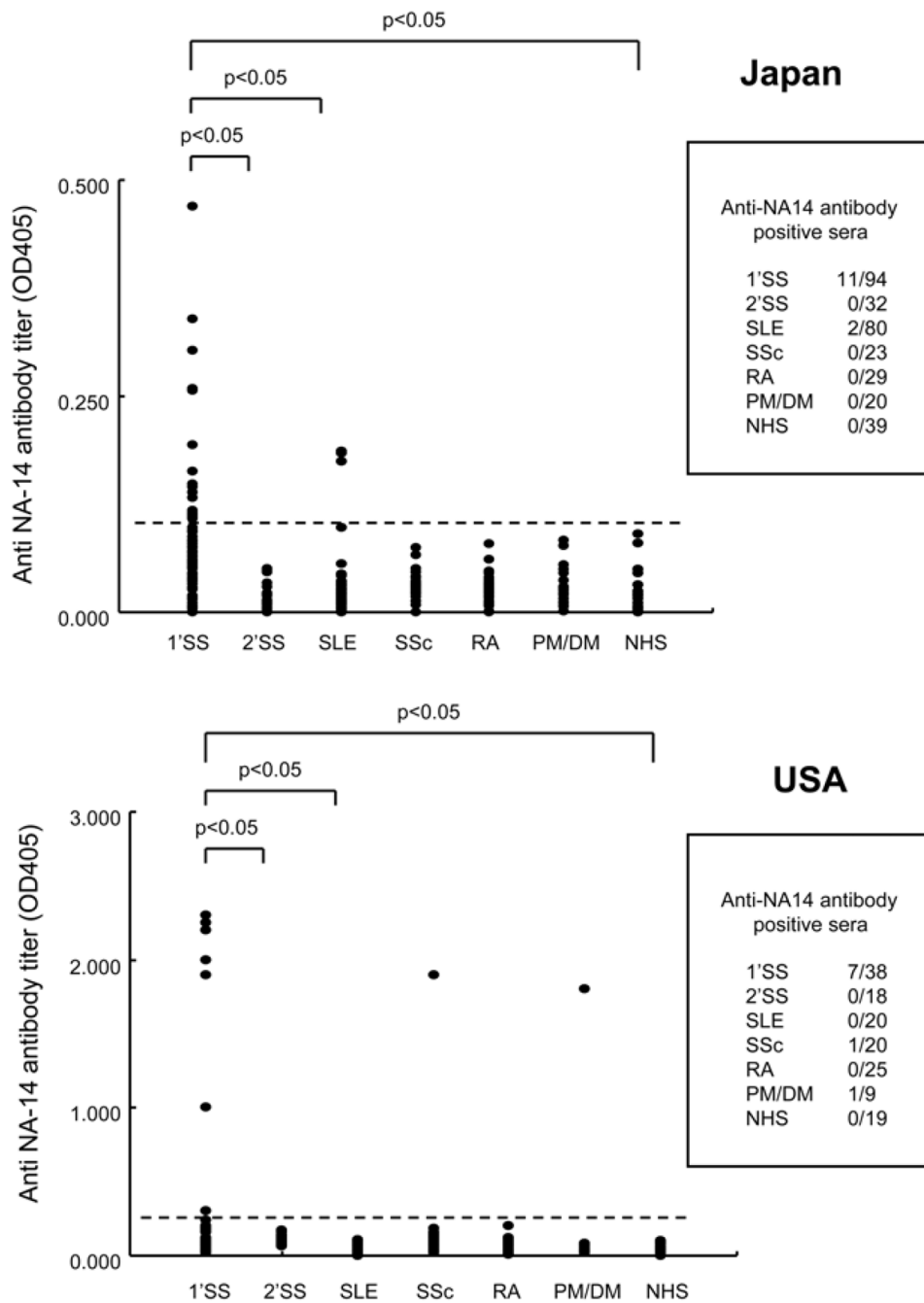


Figure 1. Serum antibodies to NA14 in rheumatic disease cohorts from Juntendo University (Japan) and University of Florida (USA). ELISA using recombinant NA14 antigens were used and the cut off value designating a positive reaction was the mean OD (405 nm) of normal healthy controls +5 standard deviations. The preferential positive reaction was observed in sera from primary SS (1'SS). Statistical analysis was performed by Chi-square test.

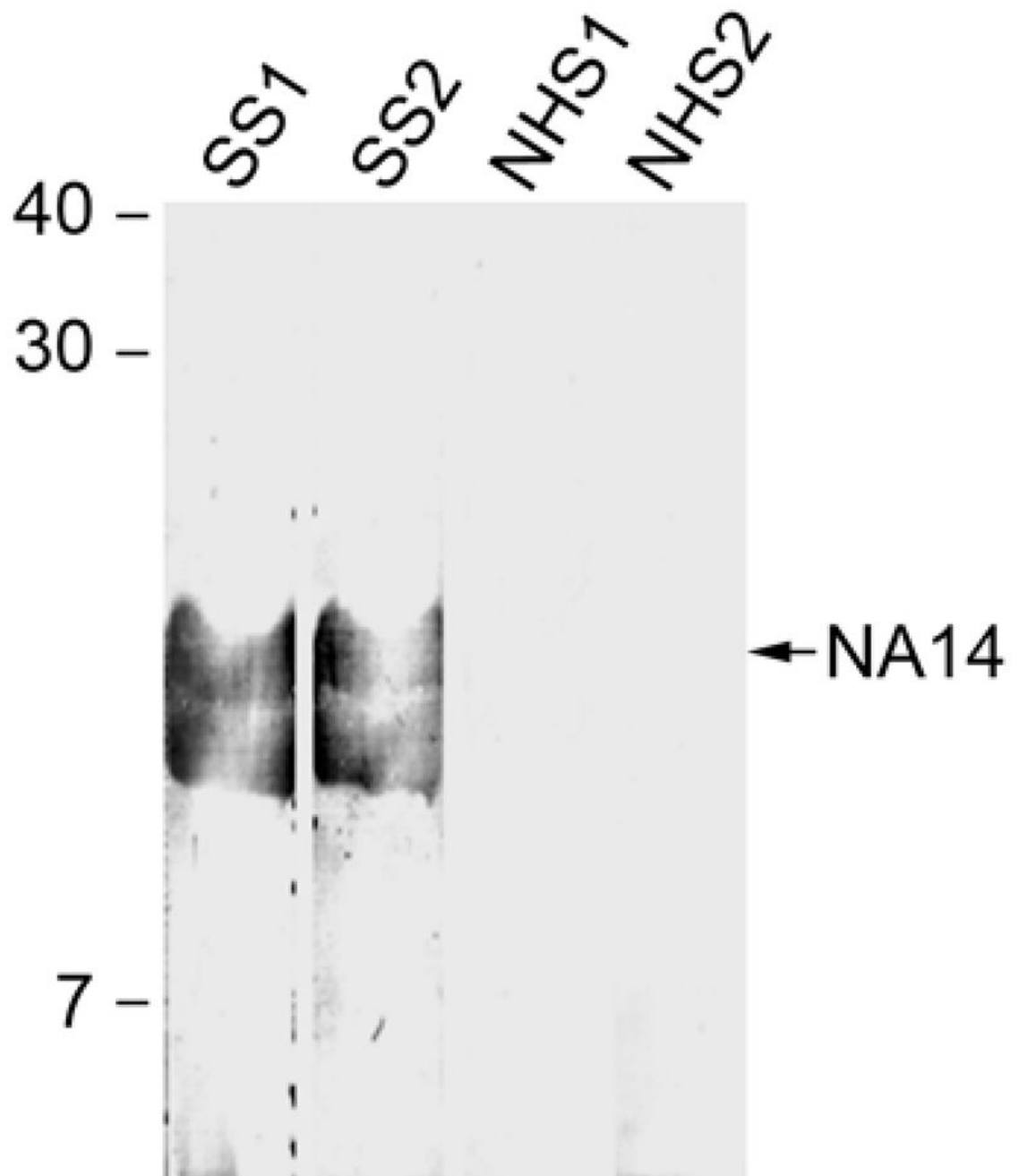


Figure 2. Confirmation of ELISA positive sera with Western blot against recombinant NA14 protein. The results of two representative SS sera and two normal human sera each are shown.

Table 1

Prevalence of anti-NA14 autoantibodies in patients with rheumatic diseases

	1'SS	2'SS	SLE	SSc	RA	PM/DM	NHS
Number of patients	132	50	100	43	54	29	58
Number of anti-NA14 antibody positive patients	18	0	2	1	0	1	0
Prevalence of anti-NA14 antibody	13.6%	0%	2%	2.3%	0%	3.4%	0%
Statistical analysis (vs 1'SS)		P=0.0041	P=0.001	P=0.0281	P=0.0029	P=0.11	P=0.002

Patient sera from rheumatic diseases were analyzed for reactivity against NA14 recombinant protein. The cut off value designating a positive reaction was the mean OD of NHC sera +5 standard deviation. 2'SS consisted of SS in association with other rheumatic diseases including SLE (n=27), RA (n=15), SSc (n=2), PM/DM (n=3), PN (n=1), and APS (n=2). Statistical analysis was performed by chi square test.

Table 2

Correlation of SS-A/Ro and SS-B/La reactivity in anti-NA14 positive sera

Anti-SS-A/Ro	Anti-SS-B/La	n	percent
+	+	3	28%
+	-	4	36%
-	+	0	0%
-	-	4	36%
		11	100%

11 anti-NA14 positive sera from 1'SS were analyzed for reactivity against SS-A/Ro and SS-B/La