Clinical Significance of Antibodies to TS1-RNA in Patients with Mixed Connective Tissue Disease

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ABSTRACT. ***Objective.*** To investigate the clinical significance of anti-TS1-RNA antibodies in patients with mixed connective tissue disease (MCTD).

Methods. Anti-TS1-RNA antibodies were detected by immunoprecipitation using ^{32}P-UTP labeled TS1-RNA as the antigen source. In total, 104 patients with MCTD, 30 with Sjögren's syndrome, 30 with systemic lupus erythematosus (SLE), 25 with systemic sclerosis, 23 with polymyositis or dermatomyositis, and 10 with rheumatoid arthritis were examined. Specificity of anti-TS1-RNA antibodies was analyzed by immunoprecipitation using HeLa cell extracts.

Results. The frequency of anti-TS1-RNA antibodies was 31.7% in patients with MCTD, significantly higher than in SLE ($p < 0.05$). In anti-TS1-RNA positive patients, the incidence of hypertension and proteinuria and the frequency of anti-Sm and anti-dsDNA antibodies associated with SLE were higher than those of anti-TS1-RNA negative patients. Clinical features of SS such as sicca complex, the serum level of IgA, and anti-SSA antibodies were also elevated. The frequency of anti-TS1-RNA antibodies was significantly higher in SLE patients with anti-U1-RNP antibodies ($p < 0.01$); however, anti-TS1-RNA positive sera did not precipitate the specific RNA including U1 RNA in immunoprecipitation using HeLa cell extracts. In longitudinal studies, the level of anti-TS1-RNA antibodies changed in parallel with disease activity.

Conclusion. We found that the level of anti-TS1-RNA antibodies was possibly correlated with the disease activity of lupus-like clinical features in patients with MCTD. (J Rheumatol 2003;30:998–1005)

Key Indexing Terms:
TS1-RNA U1RNP ANTIBODY
MIXED CONNECTIVE TISSUE DISEASE SYSTEMIC LUPUS ERYTHEMATOSUS

Patients with connective tissue diseases produce a variety of autoantibodies directed against normal cellular components such as DNA, RNA, and protein. Recent studies have shown that RNA-protein complexes are the major targets for such immune responses[1]. In addition to antibodies against the protein part, antibodies to the RNA moiety have been found[2-4]. These autoantibodies are known to react with different naked RNA that include stem-loops II and IV of U1 RNA[5-8], hY5 SSA/Ro RNA[9], alanine transfer RNA[10,11], initiator methionine transfer RNA[5], histidine transfer RNA[12], and 28S ribosomal RNA[13]. Although antibodies to DNA have not revealed strong apparent sequence specificities[14], certain anti-RNA antibodies have been shown to be highly sequence-specific and correlate with disease activity[15]. However, the majority of the antigenic determinants of the RNA and the clinical presentations correlated with antibodies have not yet been clarified.

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Tsai, *et al* identified the numbers and locations of RNA epitopes on U1 RNA by using a random RNA selection protocol[16]. This method is unique since it allows the screening of billions of sequences at one time through the use of degenerate nucleic acid pools followed by multiple rounds of amplification and selection, whereas previous methods require identification of potential RNA ligands to analyze the epitopes[5-8]. Using this random RNA selection, we clarified TS1-RNA containing 25-oligonucleotides, a novel RNA epitope recognized in serum from Patient 1 with Sjögren's syndrome (SS)[17], and investigated distribution of antibodies to TS1-RNA in sera from patients with connective tissue diseases. Moreover, we found that the frequency of anti-TS1-RNA antibodies was significantly higher in patients with SS (52.5%) compared to patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (19.5% and 12.0%, respectively), suggesting that anti-TS1-RNA antibodies were novel antibodies against sequence-specific RNA in patients with SS.

Mixed connective tissue disease (MCTD) was first reported by Sharp, *et al* as a distinct clinical entity with overlapping clinical features of SLE, scleroderma, and polymyositis (PM), and high titer of antibodies to U1 ribonucleoprotein (RNP)[18]. Further studies revealed that patients with MCTD had a close association with sicca complex[19], which has been shown to correlate to anti-TS1-RNA antibodies[17]. In addition, we reported the frequency of anti-TS1-RNA antibodies was relatively high in SLE[17].

Therefore, we examined antibodies to TS1-RNA and attempted to clarify its clinical significance in patients with MCTD diagnosed using the Kasukawa criteria[20], which do not have the exclusion item of anti-Sm antibodies.

MATERIALS AND METHODS

Sera. Sera obtained from 104 patients with MCTD, 30 with SS, 30 with SLE who were negative for anti-U1-RNP antibodies, 25 with systemic sclerosis (SSc), 23 with polymyositis or dermatomyositis (PM/DM), 10 with RA, and 25 healthy subjects were used to examine the reactivity to TS1-RNA selected from a random RNA library[17]. In addition, sera from 3 patients with MCTD who were followed up over a 3 year period were tested serially. Moreover, 31 lupus patients with anti-U1-RNP antibodies were also tested to study the relationship between anti-U1-RNP and anti-TS1-RNA antibodies.

All the patients with MCTD were diagnosed following the criteria proposed by the Special Research Committee for MCTD of the Japanese Ministry of Health and Welfare[20], which were the same as the Kasukawa criteria described by Smolen, *et al*[21] and Burdt, *et al*[22] in recent studies. These criteria do not exclude patients who fulfilled the criteria for other connective tissue diseasess and patients with anti-Sm antibodies. Patients with SS were diagnosed according to the criteria proposed by the European Community[23]. All patients with SLE, SSc, and RA met the relevant American College of Rheumatology classification criteria[24-26]. The patients with PM/DM were diagnosed in accord with the criteria of Bohan, *et al* for PM/DM[27].

The serum sample was selected randomly, and clinical and laboratory findings were evaluated at the time the serum sample was taken.

Specificity of antibodies to U1-RNP, Sm, and SSA was tested by double immunodiffusion using standard sera containing the above antibodies, which were kindly donated by Dr. E.M. Tan (Scripps Research Institute, La Jolla, CA, USA).

Preparation of TS1-RNA. The cDNA encoding TS1-RNA was prepared by subjecting 10 ng of linear oligonucleotide to 25 cycles in a Techne Thermal Cycler (1 min 94°C, 1 min 50°C, 2 min 72°C). The following buffer was employed: 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 µg T7Univ primer, 0.1 µg RevUniv primer, 200 µM dNTP, and 2.5 units Taq DNA polymerase (Takara, Tokyo, Japan). Any tandem products were reduced to monomers by cutting them with BamHI (Life Technologies, Rockville, MD, USA). Amplified cDNA encoding TS1-RNA was transcribed *in vitro* with T7 RNA polymerase (Life Technologies) in the presence of α-^{32}P-UTP (Amersham; 10 mCi/ml). The transcript was purified by gel filtration on a Sephadex G-50 column (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Detection of antibodies to TS1-RNA. ^{32}P labeled TS1-RNA (5'-TGACATTACGCCGATCGGACTTTCG-3': approximately 200,000 cpm) was immunoprecipitated with 2 µl of patient sera as follows: protein A-Sepharose CL-4B beads (Sigma, St. Louis, MO, USA; 4 mg per 100 µl of reaction mixture) were washed in NT2 buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40, pH 7.4), mixed with 2 µl of patient serum, incubated on ice for 20 min, and washed in NT2. The beads were resuspended in 100 µl of RNA binding buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM KCl, 1 mM EGTA, 0.05% Nonidet P-40, 1 mM $MgCl_2$, 2.5% polyvinyl alcohol, RNase inhibitor (Toyobo, Tokyo, Japan) at 40 units/ml, polyA RNA at 50 µg/ml, *Escherichia coli* transfer RNA at 0.5 mg/ml, 1 mM DTT, bovine serum albumin at 100 µg/ml, 0.4 mM vanadyl ribonucleoside complex]. Then 200 to 500 ng of ^{32}P labeled TS1-RNA were added, and the reactions were incubated at room temperature for 5 min. The pellets were washed 5 times with NT2 buffer including 0.5 mM urea. The bound RNA was recovered by phenol extraction and ethanol precipitation. The bound RNA was quantified by scintillation counting. The binding for quantitative measurement of anti-TS1-RNA antibody level was calculated as follows: percentage binding = 100 × (cpm of ^{32}P labeled TS1-RNA precipitated with patient serum)/(cpm of ^{32}P labeled TS1-RNA added in the binding reaction).

Immunoprecipitation of RNA. Immunoprecipitation of RNA was as described[6,28]. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (streptomycin/penicillin G). HeLa cells were resuspended in 1 ml of NET-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Nonidet P40, pH 7.4) and sonicated (cell concentration 6×10^6/ml). Then the lysates were cleaned by centrifugation and supernatant was used as antigen source. Then 2 mg of protein A-Sepharose CL-4B beads were suspended in 500 ml of IPP buffer (10 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P40, pH 8.0) and incubated with 10 µl of patient serum for 12 h at 4°C with rotation. After washing of IgG coated beads, 200 µl of cell supernatant and 400 µl NET-2 buffer were added and incubated 2 h at 4°C. The protein A-Sepharose CL-4B beads were washed 5 times with 500 µl of NET-2 buffer and resuspended in 300 µl of NET-2 buffer. RNA from the immunoprecipitation were extracted with 300 µl of PCA (phenol, chloroform, isoamyl alcohol 50:50:1), 30 µl of 10% sodium dodecyl sulfate and 30 µl of sodium acetate, precipitated with ethanol, and resolved in a 10% polyacrylamide gel containing 7 M urea. They were detected using a silver staining kit (Bio-Rad, Hercules, CA, USA).

In addition, RNA was extracted from HeLa cell supernatant with PCA, and reactivity of patients' sera to the deproteinized RNA in the HeLa cell extract was tested as described above.

Statistical evaluation. Fisher's exact test was utilized to analyze the difference of frequency of anti-TS1-RNA antibodies and the relationship between anti-TS1-RNA antibodies and clinical features in various CTD. When multiple comparisons were made, p values less than 0.05 were regarded as statistically significant. Bonferroni correction was utilized to confirm the results.

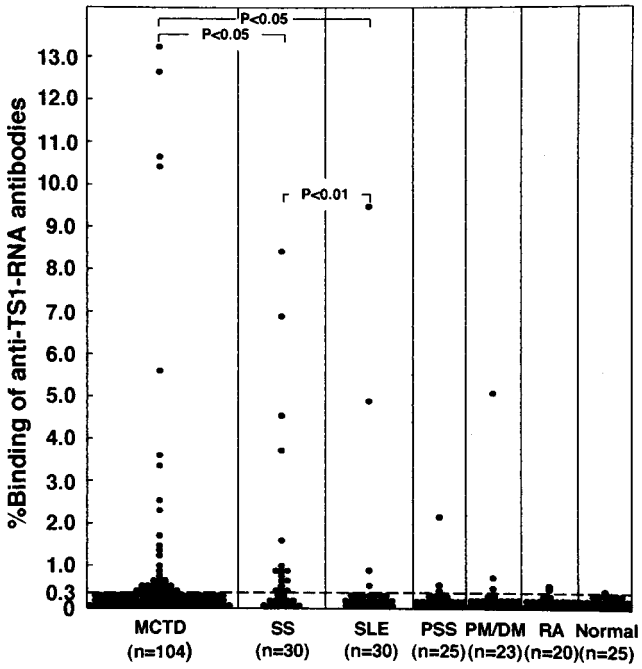

Figure 1. Anti-TS1-RNA antibodies in various connective tissue diseases. Reactivity of sera from patients with MCTD, SS, SLE, PM/DM, and RA to TS1-RNA was tested by immunoprecipitation. Broken line represents the cutoff determined by the upper limit of normal range (0.3, 2 SD above the mean value in 25 healthy controls). The frequency of anti-TS1-RNA antibodies was significantly higher in patients with SS (53.3%) compared to patients with MCTD (31.7%; $p < 0.05$) and SLE (13.3%; $p < 0.01$). In addition, anti-TS1-RNA antibodies were also significantly higher in patients with MCTD than in patients with SLE ($p < 0.05$). PSS: progressive systemic sclerosis.

RESULTS

Anti-TS1-RNA antibodies in patients with CTD. The results of immunoprecipitation for anti-TS1-RNA antibodies in patients with various CTD are shown in Figure 1. The percentage of sera containing anti-TS1-RNA antibodies was calculated using a cutoff defined as mean percentage binding in the sera of normal controls + 2 SD (0.30).

Anti-TS1-RNA antibodies were detected in 33 of 104 sera from patients with MCTD (31.7%). Similarly, anti-TS1-RNA antibodies were detected in 16 of 30 sera from patients with SS (53.3%), in 4 of 30 SLE sera (13.3%), 3 of 25 SSc sera (12.0%), 3 of 23 PM/DM sera (13.0%), and one of 10 RA sera (10.0%). The frequency of anti-TS1-RNA antibodies was significantly higher in patients with SS compared to patients with MCTD ($p < 0.05$), SLE ($p < 0.01$), SSc ($p < 0.01$), PM/DM ($p < 0.01$), and RA ($p < 0.01$). In addition, anti-TS1-RNA antibodies were also significantly higher in patients with MCTD than in patients with SLE ($p < 0.05$), SSc ($p < 0.05$), PM/DM ($p < 0.05$), and RA ($p < 0.05$).

Clinical features and laboratory findings in MCTD patients with and without anti-TS1-RNA antibodies. Because anti-TS1-RNA antibodies were highly detected in patients with MCTD, we analyzed the clinical characteristics of patients with and without anti-TS1-RNA antibodies. As shown in Table 1, the incidences of hypertension (24.2%) and sicca complex (63.6%) were more predominant among patients with anti-TS1-RNA antibodies. On the other hand, the incidences of swollen fingers or hands (80.3%), myalgia (49.3%), and muscle weakness (46.5%) were higher in patients without anti-TS1-RNA antibodies.

The comparison of laboratory findings in patients with and without anti-TS1-RNA antibodies is shown in Table 2. The incidence of proteinuria (60.6%); the frequency of anti-Sm antibodies (36.4%), anti-SSA antibodies (54.5%), and anti-dsDNA antibodies (54.5%); and increased serum levels of IgA (45.5%) were more predominant among patients with anti-TS1-RNA antibodies. In contrast, the frequency of rheumatoid factor (RF), 36.6%, was lower than that of patients without anti-TS1-RNA antibodies.

These data suggest that anti-TS1-RNA antibodies can associate with particular clinical and laboratory features, although these findings were not statistically significant after analysis using Bonferroni correction.

Anti-TS1-RNA antibodies in SLE patients with and without anti-U1-RNP antibodies. When we studied the frequency of anti-TS1-RNA antibodies in CTD, we tested sera from SLE patients without anti-U1-RNP antibodies in order to show the difference from MCTD. However, we found that clinical

Table 1. Comparison of clinical features in MCTD patients with and without anti-TS1-RNA antibodies.

Clinical Features	Anti-TS1-RNA Antibodies (n = 104)	
	With (n = 33), %	Without (n = 71), %
Raynaud's phenomenon	87.9	95.8
Swollen fingers or hands	51.5	80.3
Polyarthritis	81.8	77.5
Lymphadenopathy	24.2	16.9
Malar rash	21.2	23.9
Eruption	27.3	23.9
Photosensitivity	18.2	12.7
Oral ulcer	6.1	15.5
Alopecia	9.0	5.6
Pericarditis	6.1	4.2
Pleuritis	6.1	1.4
Hypertension	24.2	8.5
CNS involvement	3.0	2.8
Sclerodactyly	42.4	36.6
Skin ulcer	12.1	7.0
Telangiectasia	3.0	5.6
Interstitial pneumonitis	39.4	28.2
Abnormality of pulmonary function tests	45.5	39.4
Pulmonary hypertention	3.0	4.2
Hypomotility or dilation of esophagus	9.0	9.9
Myalgia	27.3	49.3
Muscle weakness	30.3	46.5
Sicca complex	63.6	38.0
Thyroiditis	18.2	21.1

CNS: central nervous system.

Table 2. Comparison of laboratory findings in MCTD patients with and without anti-TS1-RNA antibodies.

Laboratory Findings	Anti-TS1-RNA Antibodies (n = 104)	
	With (n = 33), %	Without (n = 71), %
Leukocytopenia (< 3000/μl)	18.2	23.9
Lymphocytopenia (< 1000/μl)	63.6	60.6
Anemia (< 11.2 g/dl)	27.3	26.8
Thrombocytopenia (< 100,000/μl)	9.1	11.3
Increased ESR (> 30 mm/h)	75.8	63.4
Proteinuria (> 0.5 g/day)	60.6	35.2
Increased CPK (> 93 IU/l)	21.2	29.6
Increased amylase (> 400 IU/l)	27.3	26.8
Increased serum CRP (> 0.3 mg/dl)	36.4	32.4
Increased serum IgG (> 1700 mg/dl)	87.9	83.1
Increased serum IgA (> 410 mg/dl)	45.5	25.4
Increased serum IgM (> 260 mg/dl)	6.1	4.2
Hypocomplementemia (< 25 IU)	27.3	15.5
Rheumatoid factor	18.2	36.6
Platelet associated IgG	6.1	5.6
IgG-rheumatoid factor	6.1	8.4
Circulating immune complexes	27.3	26.8
Anticardiolipin antibodies	21.2	22.5
Lupus anticoagulant	9.1	5.6
Anti-Ul-RNP antibodies	100.0	100.0
Anti-Sm antibodies	36.4	16.9
Anti-SSA antibodies	54.5	31.0
Anti-SSB antibodies	3.0	7.0
Anti-topoisomerase I antibodies	0.0	0.0
Anti-Jo-l antibodies	0.0	0.0
Anti-dsDNA antibodies (RIA)	54.5	38.0

RIA: radioimmunoassay, dsDNA: double-stranded DNA.

features associated with SLE were significantly higher in MCTD patients with anti-TS1-RNA antibodies, as shown above. Thus we further tested anti-TS1-RNA antibodies in 31 sera obtained from SLE patients with anti-U1-RNP antibodies to determine whether anti-TS1-RNA antibodies were associated with anti-U1-RNP antibodies from patients with SLE.

As shown in Figure 2, the frequency of anti-TS1-RNA antibodies in SLE patients with anti-U1-RNP antibodies was significantly higher than that of patients without anti-U1-RNP antibodies — 48.4% and 13.3%, respectively ($p < 0.01$).

Immunoprecipitation of HeLa cell extracts by anti-TS1-RNA antibodies. Since a significant relationship between anti-TS1-RNA antibodies and anti-U1-RNP antibodies was observed in SLE patients, we further analyzed the reactivity of anti-TS1-RNA antibodies to somatic RNA in immunoprecipitation using whole cell and deproteinized extracts from HeLa cells (Figures 3A, 3B), and compared the reactivity of anti-U1-RNP and anti-SSA antibodies to those antigens.

Using whole cell extracts as antigen (Figure 3A), we observed anti-TS1-RNA serum from Patient 1 (lane 2) that was also positive for anti-U1-RNP and anti-SSA precipitated Y small cytoplasmic (sc) RNA and U1 RNA that in the precipitates of anti-SSA serum of Patient 2 (lane 6) and anti-U1-RNP serum of Patient 3 (lane 7), respectively, in addition to unspecified RNA. Anti-TS1-RNA serum of Patient 4 was also positive for anti-SSA precipitated Y scRNA (lane 3). Anti-TS1-RNA serum of Patient 5 positive for anti-Sm and anti-U1-RNP precipitated U small nuclear (sn) RNA (lane 4), and anti-TS1-RNA serum of Patient 6 also positive for anti-U1 RNP precipitated U1 and U2 RNA (lane 5). These data revealed that there were no common RNA precipitated by anti-TS1-RNA positive sera, although the sera precipitated RNA that were associated with other autoantibodies accompanied with anti-TS1-RNA antibodies.

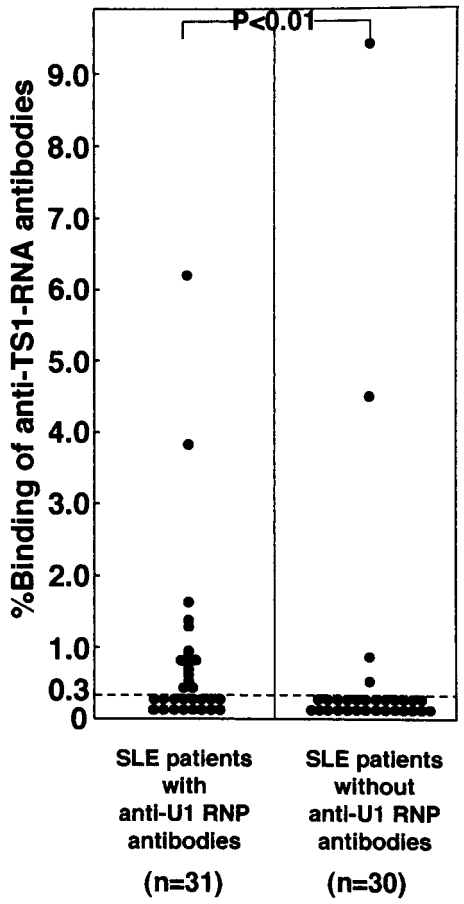

Figure 2. Anti-TS1-RNA antibodies in anti-U1 RNP positive and negative patients with SLE. The frequency of anti-TS1-RNA antibodies was significantly higher in SLE patients with anti-U1-RNP than patients negative for anti-U1-RNP ($p < 0.01$).

In contrast, only anti-TS1-RNA serum of Patient 1 precipitated U1 RNA (Figure 3B, lane 2), and serum from Patient 5 precipitated some RNA reacting with the deproteinized cell extracts (Figure 3B, lane 4).

These data suggest that anti-TS1-RNA sera did not react with the particular somatic RNA in HeLa cells, and were not cross-reactive with U1-RNP and SSA antigens, although they tended to associate with anti-SSA in MCTD and were significantly associated with anti-U1-RNP in SLE.

Relationship between clinical courses and anti-TS1-RNA antibodies in 3 patients with MCTD. To analyze the clinical significance of anti-TS1-RNA antibodies in patients with MCTD further, we performed longitudinal studies using sera from 3 patients with MCTD who were followed for over 3 years.

Case 1, a 33-year-old woman, had only Raynaud's phenomenon when she first visited our hospital at the beginning of 1997 (Figure 4). Since August 1997, she developed swollen hands, polyarthralgia, sclerodactyly, and leukocytopenia, with anti-U1-RNP antibodies and increased anti-TS1-RNA antibodies, and was diagnosed as having MCTD. In parallel with increased level of anti-U1-RNP antibodies and anti-TS1-RNA antibodies, all symptoms worsened, and the level of anti-TS1-RNA antibodies increased maximally when she developed pulmonary thrombosis in 1999. Then she was urgently admitted to hospital and was treated with methylprednisolone pulse therapy, and the level of anti-TS1-RNA antibodies decreased in parallel with improvement of the clinical features.

Case 2, a 29-year-old woman, had suspected MCTD at first because of having only 4 findings, Raynaud's phenomenon, swollen hands, sclerodactyly, and anti-U1-RNP antibodies in 1997, and was diagnosed as having MCTD when she developed proteinuria, leukocytopenia, and positive anti-Sm antibodies in 1998 (Figure 5). Soon after that, pyrexia, proteinuria, and leukocytopenia were aggravated in parallel with the increased level of anti-TS1-RNA antibodies. Then she was treated with methylprednisolone pulse therapy, and proteinuria improved, with decreased level of

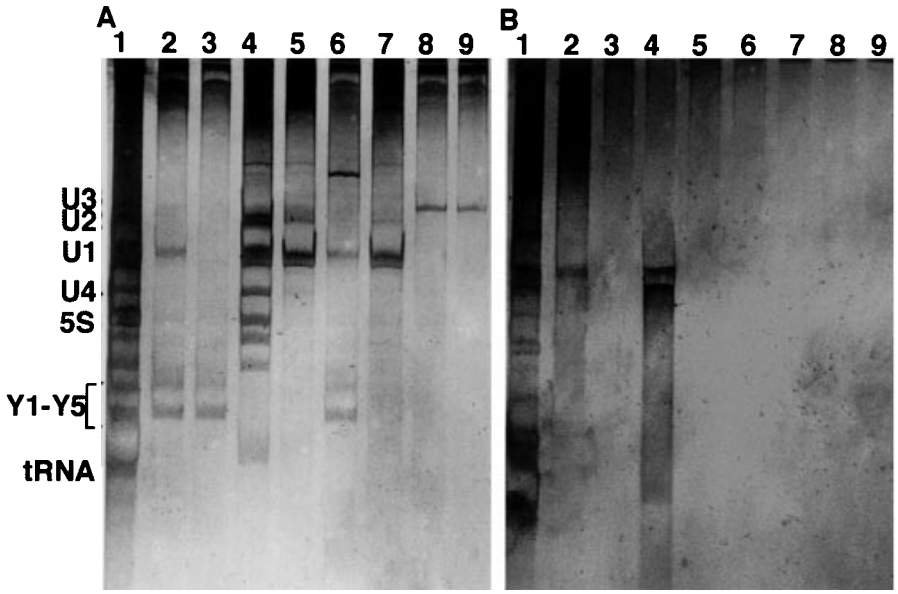

Figure 3. RNA immunoprecipitation using HeLa cell extracts with anti-TS1-RNA sera. A. Patient serum samples were incubated with whole cell extracts. Precipitated RNA were extracted with phenol, fractionated on polyacrylamide gels, and detected by silver staining. Anti-TS1-RNA serum of Patient 1 (lane 2) that was also positive for anti-U1-RNP and SSA precipitated Y small cytoplasmic (sc) RNA and U1 RNA that were observed in the precipitates of anti-SSA serum of Patient 2 (lane 6) and anti-U1-RNP serum of Patient 3 (lane 7), in addition to unspecified RNA. Anti-TS1-RNA serum of Patient 4 positive for anti-SSA precipitated Y scRNA (lane 3). Anti-TS1-RNA serum of Patient 5 positive for anti-Sm and U1-RNP precipitated U small nuclear (sn) RNA (lane 4), and anti-TS1-RNA serum of Patient 6 also positive for anti-U1-RNP precipitated U1 and U2 RNA (lane 5). Lane 1: RNA in whole HeLa cell extract; lanes 8 and 9: anti-U3-RNP positive sera used as controls. B. Patient serum samples were incubated with deproteinized RNA. Only anti-TS1-RNA serum from Patient 1 precipitated U1 RNA (lane 2), and serum from Patient 5 precipitated some RNA reacting with the deproteinized cell extract (lane 4). Lane 1, both panels: total RNA extracted from HeLa cells. These findings suggest that anti-TS1-RNA sera does not react with the particular somatic RNA in HeLa cells, and was not cross-reactive with U1-RNP and SSA.

anti-TS1-RNA antibodies. In this patient, the titer of anti-U1-RNP antibodies did not change during the clinical course, but the level of anti-TS1-RNA antibodies increased in parallel with disease activities.

Case 3, a 30-year-old woman, presented with suspected SLE because of arthralgia and positive anti-dsDNA antibodies in 1997 (Figure 6). Before polyarthralgia and myalgia worsened in 1997, she was positive for anti-TS1-RNA antibodies, but negative for anti-U1-RNP antibodies. After the level of anti-TS1-RNA antibodies maximally increased, she developed Raynaud's phenomenon and swollen fingers in addition to worsened arthralgia and myalgia, and she was also positive for anti-U1-RNP antibodies. The clinical features improved after administration of increased prednisolone combined with sarpogrelate hydrochloride. However, symptoms worsened again since the beginning of 2000, after the level of anti-TS1-RNA antibodies increased in December 1999. In this case, the level of anti-TS1-RNA antibodies increased prior to the disease flare and the elevation of anti-U1-RNP antibodies and anti-dsDNA antibodies.

DISCUSSION

When we first studied the frequency of anti-TS1-RNA antibodies in patients with various connective tissue diseases, anti-TS1-RNA antibodies were detected in 52.5% of patients with SS, significantly higher than in patients with other diseases including SLE[17]. Our results also revealed that the frequency of anti-TS1-RNA antibodies was the highest, 53%, in patients with SS, among the various connective tissue diseases. But we found that anti-TS1-RNA antibodies could be detected at high levels in patients with MCTD (31.7%), a frequency that was significantly higher than in patients with SLE (13.3%). It was possible that anti-TS1-RNA antibodies in patients with MCTD were detected in association with the sicca complex because patients with MCTD frequently had SS in addition to clinical features associated with SLE, SSc, and PM[18,19]. Indeed, the incidence of sicca complex was higher in MCTD patients with anti-TS1-RNA antibodies than in MCTD patients without anti-TS1-RNA. But 27 out of 48 MCTD patients with SS (56.3%) were negative for anti-TS1-RNA antibodies, and anti-TS1-RNA antibodies were detected in

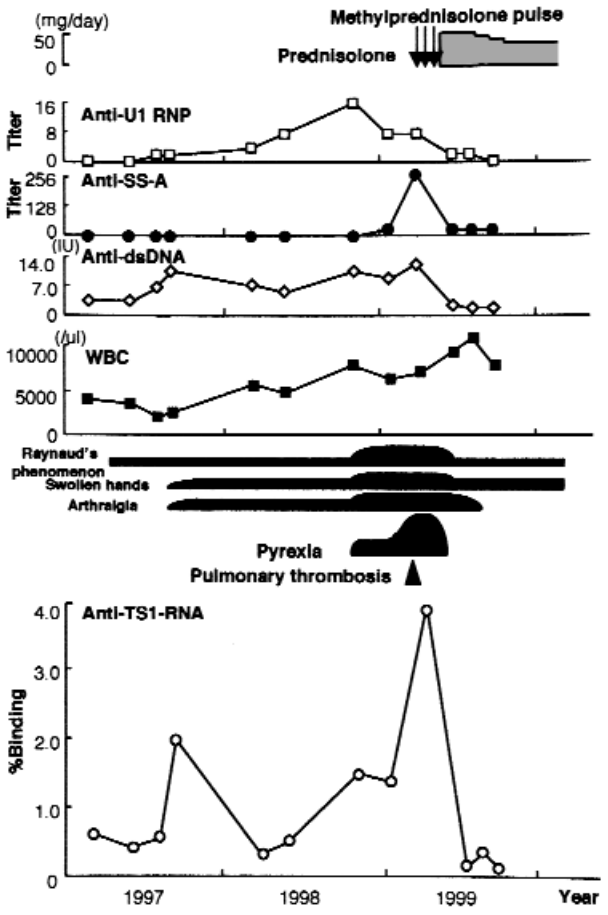

Figure 4. Case 1 (33-year-old woman): the relationship between clinical features and the titer of anti-TS1-RNA antibodies was compared longitudinally. MCTD was diagnosed in August 1997. Symptoms and laboratory data had worsened in parallel with increased level of anti-TS1-RNA antibodies. Anti-TS1-RNA antibodies had been positive before anti-U1-RNP and dsDNA antibodies were recognized.

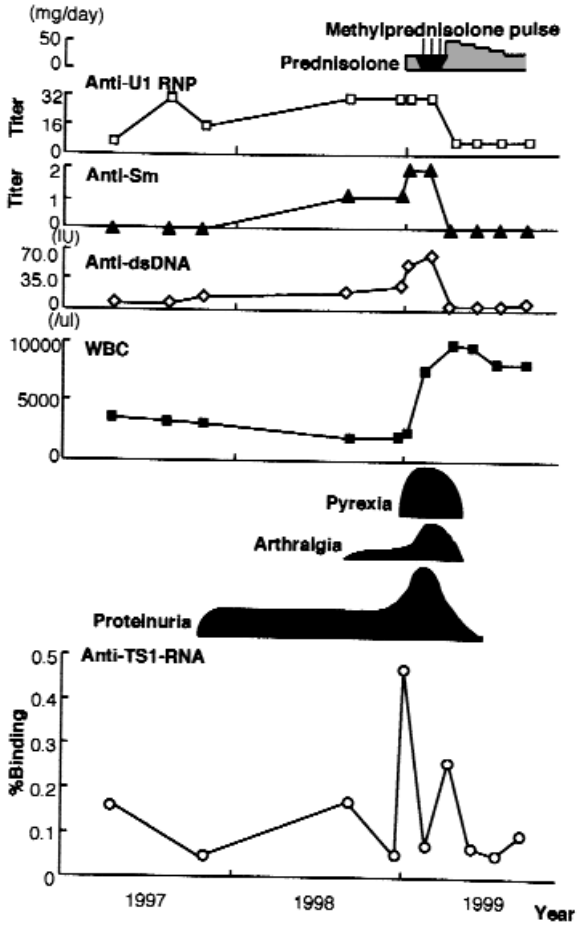

Figure 5. Case 2 (29-year-old woman): MCTD had been diagnosed in October 1997. Proteinuria and leukocytopenia had worsened in parallel with increased level of anti-TS1-RNA antibodies. Titer of anti-U1-RNP antibodies did not change during the clinical course, but the level of anti-TS1-RNA antibodies increased in parallel with disease activity.

21.4% (12 of 56) of MCTD patients without sicca complex. These results revealed that the frequency of anti-TS1-RNA antibodies were not always associated with SS in MCTD patients. And the interesting observation was that patients with anti-TS1-RNA antibodies tended to show a higher frequency of proteinuria, anti-Sm antibodies, and anti-dsDNA antibodies. Indeed, these are known to be the characteristic clinical features of SLE, and these patients also fulfilled the ACR criteria for SLE. When Sharp, *et al* proposed the criteria for MCTD[29], patients with anti-Sm were excluded. But there are no particular diagnostic criteria that have been widely accepted[21], and the criteria proposed by Alarcon-Segovia, *et al*[30] and Kahn, *et al*[31] do not exclude patients with anti-Sm, the same as the Kasukawa criteria[32] that were used in this study. Burdt, *et al* also used the Kasukawa criteria, and reported that anti-Sm and anti-dsDNA antibodies occurred in 22% and 19%, respectively, of patients during active MCTD[22], the same as our results. Although it is still controversial whether patients with anti-Sm must be excluded from the MCTD, our observations showed the possible association of anti-TS1-RNA antibodies with the clinical features in patients with MCTD diagnosed by the particular criteria. However, we must be aware that the results shown here would not apply if the Sharp criteria were used.

These data suggest that anti-TS1-RNA antibodies are related to anti-U1-RNP antibody itself and the clinical features associated with SLE. When we then studied the association of anti-TS1-RNA antibodies to anti-U1-RNP antibodies in SLE patients, we found that anti-TS1-RNA antibodies were significantly higher in SLE patients with anti-U1-RNP than in patients without anti-U1-RNP. Although these results suggest a strong association of anti-TS1-RNA antibodies with anti-U1-RNP antibodies, it is known that the sequence of nucleotides in TS1-RNA is not identical to that of U1 RNA[17], and TS1-RNA does not have

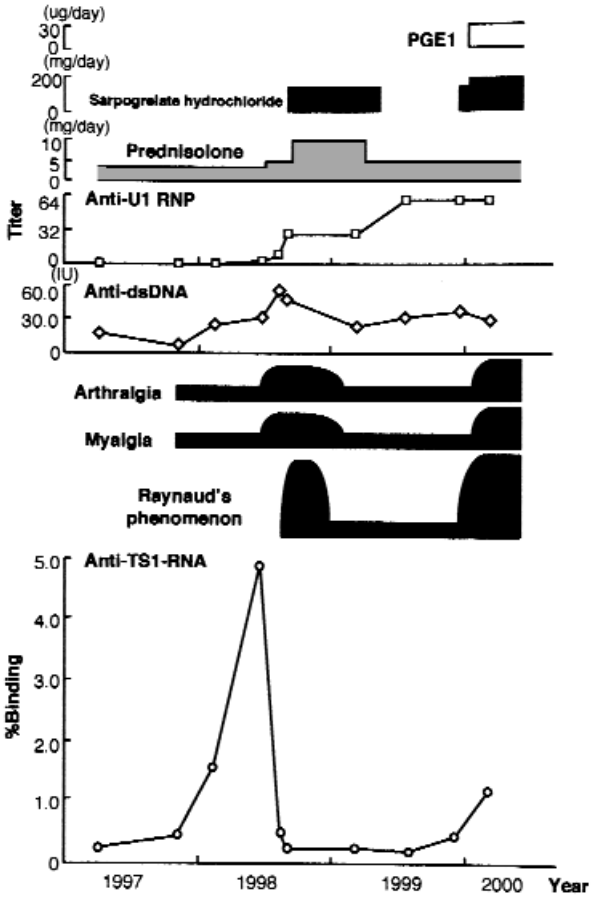

Figure 6. Case 3 (30-year-old woman): SLE diagnosis was suspected at first. After the emergence of anti-TS1-RNA antibodies, anti-U1-RNP antibodies and Raynaud's phenomenon were recognized, and MCTD was diagnosed. The level of anti-TS1-RNA antibodies increased prior to the disease flare and elevation of anti-U1-RNP and dsDNA antibodies.

the stem-loop structure through the RNA epitopes. In addition, the inhibition study using purified U1-RNP showed that anti-TS1-RNA antibodies were not cross-reactive with U1-RNP[17]. Our data from immunoprecipitation analysis using whole cell and deproteinized extracts of HeLa cells also strongly suggested that anti-TS1-RNA antibodies do not cross-react with U snRNA and Y scRNA, or associated proteins, although anti-TS1-RNA may be associated with anti-U1-RNP and anti-SSA antibodies.

We could not define the somatic RNA reactive with anti-TS1-RNA antibodies, but it is interesting that the reactivity to TS1-RNA is significantly associated with the diseases in which autoantibodies to somatic RNA such as U1 RNA[7] and hY RNA[9] can be detected. A number of models, including antigen driven viral infection[33], idiotype networks[6], and immune cross-reactivity with RNA recognition motif on proteins[34], have been proposed to explain the existence of anti-RNA antibodies, and it is possible that the mechanisms to induce antibodies to these RNA are related to each other in patients with SS, MCTD, and SLE.

The relationship between anti-TS1-RNA antibodies and disease activity in MCTD was another important observation in this study. It has been reported that antibodies to the 70 kDa protein, which are detectable in immunoblots and immunoprecipitation, occur in 75 to 95% of patients with MCTD[35]. These antibodies may be less frequent in comparable groups of patients with SLE, in which anti-70 kDa antibodies detectable by immunoblotting appear to occur in one-fifth to one-half of individuals[35]. Snowden, *et al* reported that anti-70 kDa antibodies may also be markers for the former diagnosis when present in high titers, and that the titers may vary during the course of the disease[36]. By contrast, longitudinal studies indicate that anti-70 kDa antibody titers vary over time; however, there is no clear evidence whether the titers reflect underlying disease activity[37].

In contrast, Hoet, *et al* have shown that antibodies to U1-RNP in patients with MCTD often recognize the stem-loops II and/or IV of U1 RNA as epitopes, and the titer of these antibodies correlates with disease activity in patients with MCTD[8]. Our results were interesting because anti-TS1-RNA antibodies that recognized the sequence-specificity of RNA were strongly associated with the disease activity of MCTD in patients in the longitudinal study, the same as antibodies to U1 RNA, as described[8]. In 3 different patients with MCTD, it was shown that the level of anti-TS1-RNA antibodies increased in parallel with worsening of clinical or laboratory findings, and anti-TS1-RNA antibodies increased before deterioration of symptoms observed in cases 2 and 3 above. Further, the markedly increased level of anti-TS1-RNA antibodies was seen prior to the elevation of other autoantibodies such as anti-U1-RNP, anti-Sm, and anti-dsDNA, although it is possible that this phenomenon was affected by the higher sensitivity of the immunoprecipitation test used to detect anti-TS1-RNA compared to double immunodiffusion. These data suggest the association of anti-TS1-RNA antibodies to characteristic clinical features of SLE in patients with MCTD, and the possibility that detection of anti-TS1-RNA antibodies would be useful to predict flare in MCTD.

We found that anti-TS1-RNA antibodies were frequently detected in patients with MCTD diagnosed by the Kasukawa criteria and tended to be associated with the clinical features of SLE. We found that the level of anti-TS1-RNA antibodies was possibly correlated with disease activity in patients with MCTD.

REFERENCES

1. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 1989;44:93-152.
2. Eilat D, Lotan C. The reaction of human SLE antibodies with native, single stranded RNA: analysis of nucleotide sequence specificities and correlation with Sm and nRNP activities. Clin Exp Immunol 1982;49:283-9.

3. Eilat D, Hochberg M, Fischel R, Laskov R. Antibodies to RNA from autoimmune NZB/NZW mice recognize a similar antigenic determinant and show large idiotypic diversity. Proc Natl Acad Sci USA 1982;79:3818-22.
4. Lamon EW, Bennett JC. Antibodies to homologous RNA in the rabbit following stimulation by exogenous RNA. Proc Soc Exp Biol Med 1970;134:968-70.
5. Wilusz J, Keene JD. Autoantibodies specific for U1 RNA and initiator methionine tRNA. J Biol Chem 1986;261:5467-72.
6. Deutscher SL, Keene JD. A sequence-specific conformational epitope on U1 RNA is recognized by a unique autoantibody. Proc Natl Acad Sci USA 1988;85:3299-303.
7. van Venrooij WJ, Hoet R, Castrop R, et al. Anti-(U1) small nuclear RNA antibodies in anti-small nuclear ribonucleoprotein sera from patients with connective tissue diseases. J Clin Invest 1990;86:2154-60.
8. Hoet RM, De Weerd P, Gunnewiek JK, et al. Epitope regions on U1 small nuclear RNA recognized by anti-U1RNA-specific autoantibodies. J Clin Invest 1992;90:1753-62.
9. Boulanger C, Chabot B, Menard HA, et al. Autoantibodies in human anti-Ro sera specifically recognize deproteinized hY5 Ro RNA. Clin Exp Immunol 1995;99:29-36.
10. Bunn CC, Mathews MB. Autoreactive epitope defined as the anti-codon region of alanine transfer RNA. Science 1987;238:1169-9.
11. Becker HF, Corda Y, Mathews MB, et al. Iosine and N1-methylinosine within a synthetic oligomer mimicking the anticodon loop of human tRNA (Ala) are major epitopes for anti-PL-12 myositis autoantibodies. RNA 1999;5:865-75.
12. Brouwer R, Vree Egberts W, Jongen PH, et al. Frequent occurrence of anti-tRNA (His) autoantibodies that recognize a conformational epitope in sera of patients with myositis. Arthritis Rheum 1998;41:2059-67.
13. Uchiumi T, Traut RR, Elkon K, Kominami R. A human autoantibody specific for a unique conserved region of 28S ribosomal RNA inhibits the interaction of elongation factors 1a and 2 with ribosomes. J Biol Chem 1991;266:2054-62.
14. Sun Y, Fong KY, Chung MC, Yao ZJ. Peptide mimicking antigenic and immunogenic epitope of double-stranded DNA in systemic lupus erythematosus. Int Immunol 2001;13:223-32.
15. Hoet RM, Koornneef I, de Rooij DJ, et al. Changes in anti-U1 RNA antibody levels correlate with disease activity in patients with systemic lupus erythematosus overlap syndrome. Arthritis Rheum 1992;35:1202-10.
16. Tsai DE, Kenan DJ, Keene JD. In vitro selection of RNA epitopes using autoimmune patients serum. J Immunol 1993;150:1137-45.
17. Hirokawa K, Takasaki Y, Takeuchi K, et al. Anti-TS1-RNA: Characterization of a novel antibody against the sequence-specific RNA by a random RNA selection in patients with Sjögren's syndrome. J Rheumatol 2002;29:931-7.
18. Sharp GC, Irvin WS, Tan EM, et al. Mixed connective tissue disease syndrome associated with a specific antibody to an extractable nuclear antigen. Am J Med 1972;52:148-59.
19. Ohtsuka E, Nonaka S, Shingu M, et al. Sjögren's syndrome and mixed connective tissue disease. Clin Exp Rheumatol 1992; 10:339-44.
20. Kasukawa R, Tojo T, Miyawaki S. Mixed connective tissue disease — preliminary diagnostic criteria. Jpn J Rheumatol 1988;1:263-7.
21. Smolen JS, Steiner G. Mixed connective tissue disease: to be or not to be? Arthritis Rheum 1998;41:768-77.
22. Burdt MA, Hoffman RW, Deutscher SL, et al. Long-term outcome in mixed connective tissue disease: longitudinal clinical and serological findings. Arthritis Rheum 1999;42:899-909.
23. Vitali C, Bombardieri S, Moutsopoulos HM, et al. Preliminary criteria for the classification of Sjögren's syndrome. Results of a prospective concerted action supported by the European Community. Arthritis Rheum 1993;36:340-7.
24. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
25. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteira for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581-90.
26. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
27. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of 2 parts). N Engl J Med 1975;292:344-7.
28. Ohosone Y, Ishida M, Takahashi Y, et al. Spectrum and clinical significance of autoantibodies against transfer RNA. Arthritis Rheum 1998;41:1625-31.
29. Sharp GC. Diagnostic criteria for classification of MCTD. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue diseases and antinuclear antibodies. Amsterdam: Elsevier; 1987:23-32.
30. Alarcon-Segovia D, Villareal M. Classification and diagnostic criteria for mixed connective tissue disease. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue diseases and antinuclear antibodies. Amsterdam: Elsevier; 1987:33-40.
31. Kahn MF, Appelboom T. Syndrome de Sharp. In: Kahn MF, Peltier AP, Meyer O, Piette JC, editors. Les maladies systemiques. 3rd ed. Paris: Flammarion; 1991:545-56.
32. Kasukawa R, Tojo T, Miyawaki S. Preliminary diagnostic criteria for classification of mixed connective tissue disease. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue diseases and antinuclear antibodies. Amsterdam: Elsevier; 1987:41-7.
33. Chang YN, Kenan DJ, Keene JD, et al. Direct interactions between autoantigen La and human immunodeficiency virus leader RNA. J Virol 1994;68:7008-20.
34. Keene JD, Query CC. Nuclear RNA-binding proteins. Prog Nucl Acids Res Mol Biol 1991;41:179-202.
35. Pettersson I, Wang G, Smith EI, et al. The use of immunoblotting and immunoprecipitation of (U) small nuclear ribonucleoproteins in the analysis of sera of patients with mixed connective tissue disease and systemic lupus erythematosus. A cross-sectional, longitudinal study. Arthritis Rheum 1986;29:986-96.
36. Snowden N, Hay E, Holt PJL, Berstein R. Clinical course of patients with anti-RNP antibodies. J Rheumatol 1993;20:1256-8.
37. de Rooji DJ, Habets WJ, van de Putte LB, et al. Use of recombinant RNP peptides 70K and A in an ELISA for measurement of antibodies in mixed connective tissue disease: a longitudinal follow up of 18 patients. Ann Rheum Dis 1990;49:391-5.