Autoantibody and Biopsy Grading Are Associated with Expression of ICAM-1, MMP-3, and TRAIL in Salivary Gland Mononuclear Cells of Chinese Patients with Sjögren's Syndrome

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ABSTRACT. *Objective.* Sjögren's syndrome (SS) is an inflammatory autoimmune disease. We investigated important factors associated with the expression of inflammation-related molecules in minor salivary gland (MSG) mononuclear cells in patients with SS.

Methods. Thirty-four patients with SS with a MSG biopsy grading of either grade III (10 patients) or grade IV (24 patients) were enrolled. The age, sex, autoantibodies, cell infiltration, and intercellular adhesion molecule-1 (ICAM-1), matrix metalloproteinase-3 (MMP-3), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), or CXCR3 expression were also analyzed.

Results. Ten of the 34 patients with SS were diagnosed with secondary SS; in these patients, the diagnosis of rheumatoid arthritis was confirmed in 8 and systemic lupus erythematosus in 2. TRAIL and ICAM-1 were overexpressed in patients with antinuclear antibodies (ANA) > 1:160, compared to those with titer < 1:160 (45.1 ± 4.4 vs 41.2 ± 3.9, p = 0.021, and 15.2 ± 5.7 vs 10.8 ± 3.3, p = 0.018, respectively). Higher erythrocyte sedimentation rate (ESR; $\geq$ 20) was associated with higher TRAIL expression and CD20 cell infiltration in contrast to lower ESR (< 20; p < 0.05). ICAM-1, TRAIL, and MMP-3 were expressed more predominantly in anti-SSA-positive than in anti-SSA-negative patients with SS. There was a significant difference in CD20 cell infiltration and MMP-3 expression between primary SS and secondary SS. Biopsy of a grade IV showed a significantly increased expression of TRAIL (44.9 ± 4.5 vs 40.8 ± 3.6, p = 0.013) and MMP-3 (62.7 ± 6.3 vs 54.4 ± 7.3, p = 0.003) in mononuclear cells as compared to those of grade III.

Conclusion. In our study, pathologic grading with a higher grade (grade IV) and the presence of SSA or a higher titer of ANA were significantly associated with the overexpression of TRAIL, MMP-3, or ICAM-1 in the salivary gland mononuclear cells in patients with SS. (J Rheumatol First Release April 1 2009; doi:10.3899/jrheum.080733)

Key Indexing Terms:
SJÖGREN'S SYNDROME   MINOR SALIVARY GLAND BIOPSY
ANTINUCLEAR ANTIBODY   TRAIL   MATRIX METALLOPROTEINASES

Sjögren's syndrome (SS) is a chronic autoimmune disease, characterized clinically by dry eye and dry mouth, serologically by the presence of autoantibodies, including anti-SSA or anti-SSB, and pathologically by focal lymphocyte infiltration[1,2].

The increased CD4 T lymphocyte infiltration in the minor salivary gland (MSG) biopsy tissue of patients with SS may contribute to the inflammation and apoptosis of epithelial cells through Fas-FasL and cell-to-cell interactions[3-6]. The lymphocyte aggregation or formation of a germinal center results from the abnormal B cell differentiation and proliferation[7]. In addition to the CD4 T cells or B

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Accepted for publication November 11, 2008.

cells, research has also demonstrated that dendritic cells and cytotoxic T lymphocytes (CD8 T cells and natural killer cells) participate in the epithelial cell destruction found in MSG of patients with SS[3]. The increased autoantibodies, including rheumatoid factor (RF), anti-SSA or anti-SSB, and the hypergammaglobulinemia in patients with SS are mainly produced *in situ* from the increased B and plasma cells in the salivary glands[7-9].

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), belonging to the TNF family, is a protein that can induce apoptotic cell death and develop T cell cyto-toxicity[10,11]. A previous study found that TRAIL receptor (TRAIL-R1) or TRAIL-R2 was overexpressed on the ductal epithelial cells in patients with severe SS[12]. This increased TRAIL in SS may be responsible for the destruction of epithelial cells.

Matrix metalloproteinase-3 (MMP-3) is becoming an important disease activity marker in ankylosing spondylitis (AS)[13,14]. The tissue remodeling and/or destruction of the extracellular matrix mainly rely upon MMP and its inhibitor. Increased MMP-3 and MMP-9 expression in acini and duct cells, but not in lymphocytes in the MSG biopsy, has been identified in patients with SS[15]. The local excessive product from epithelial cells may be responsible for sialadenitis in patients with SS[15,16]. It has been reported that the increased surface expression of SSA or SSB antigen was TNF-α-mediated[7,17]. In our study, we focused on the MMP-3 expression in mononuclear cells, rather than acinar or ductal cells.

The recruitment of large mononuclear cell infiltrations in patients with SS may result from the increased chemokines, E-selectin, intercellular adhesion molecule-1 (ICAM-1), or vascular cell adhesion molecule-1 (VCAM-1), etc., from mononuclear cells or fibroblasts[18-21]. Both ICAM-1 and VCAM-1 have been shown to be important for lymphocyte migration from the blood vessels to the inflammatory tissues. Different classes of chemokines, such as CXCL13, CXCL21, and CXCL12, may attract different cell populations (B, T, dendritic cells, etc.)[9]. Recent studies have demonstrated that Th1 cells mainly express chemokine receptors CXCR3+ rather than CCR4–, and Th2 cells express CXCR3– rather than CCR4+[22,23]. In SS with interstitial pneumonia, CXCR3+ cells are predominantly expressed in lung tissue[24].

Primary SS is a common rheumatic disease in Chinese, and only one immunopathology study on the salivary gland has been reported[25]. Western reports in the past 20 years have shown that the immunopathologic studies were focused mainly on the epithelial cells or ductal cells, and less on the infiltrated mononuclear cells. In this study, factors including RF, anti-SSA or anti-SSB antibody, antinuclear antibodies (ANA), erythrocyte sedimentation rate (ESR), and biopsy grading were investigated to determine which may be associated with the expression of TRAIL, CXCR3, ICAM-1, or MMP-3 in the mononuclear cells of the MSG in Chinese patients with SS.

MATERIALS AND METHODS

Patients. During a 2-year period, salivary gland tissues were obtained from 34 patients with SS with a MSG biopsy grading of at least grade III, 32 women and 2 men. There were enough inflammatory cells to be studied if the biopsy grading was more than grade III, and this was the reason we enrolled patients with moderate or severe inflammation in the MSG. The diagnosis of SS was based on the American-European Consensus Group criteria[26]. All 34 patients had clinical evidence of dry eye and dry mouth for at least for 3 months. Besides Schirmer's test or sialoscintigraphy, our patients with SS had at least one of 2 positive: SSA antibody or biopsy (grade III or grade IV). Ages ranged from 22 to 81 years (mean 53). The study was approved by the institutional ethics committee of Taipei Veterans General Hospital. All patients were required to sign a consent form to participate in this research.

We separated the 34 patients into different groups, primary versus secondary SS, RF+ versus RF–, ESR $\geq$ 20 mm/h versus ESR < 20, ANA $\geq$ 1:160 versus ANA < 160, anti-SSA(+) versus anti-SSA(–), and grade 4 versus grade 3 sialadenitis.

MSG biopsy grading by H&E stain. Salivary gland specimens obtained from oral surgeries were fixed in 10% formalin and processed through paraffin embedding and sectioning[27,28]. After H&E staining, the amount of lymphocyte infiltration was graded as 0, 1, 2, 3, 4. We enrolled only those with grades 3 and 4. Grade 3 was confirmed by one focus (1/4 mm^2) of lymphocyte aggregation (> 50 lymphocytes/focus) and grade 4 was > 2 foci of lymphocyte aggregation.

Immunohistochemical staining. Serial sections were stained with the following monoclonal antibodies: anti-CD3 (clone SP7; Lab Vision, UK), anti-CD4 (Clone 4B12; Lab Vision), anti-CD8 (Clone SP16; Lab Vision), anti-CD20 (polyclone; Lab Vision), anti-MMP-3 (clone 10D6; R&D Systems, USA), anti-ICAM-1 (Clone 23G12; Novocastra, UK), anti-TRAIL (Clone 75411; R&D Systems), and anti-CXCR3 (clone 4980; R&D Systems). Staining procedures were performed according to the described 3-step method[29]. Briefly, each 5 μm MSG tissue sample was incubated with primary antibody for 60 min. After washing with phosphate-buffered saline (PBS), the sections were incubated with a biotinylated anti-mouse secondary antibody for 15 min, using a streptavidin-labeled peroxidase complex kit (ABC; Signet). The color reaction was developed using diaminobenzidine (DAB) as a chromogen, and finally, the slides were stained with hematoxylin. Negative controls were performed using irrelevant isotype-matched monoclonal antibodies. The endogenous peroxidase was blocked with 1% hydrogen peroxide.

Microscopic analysis. Expression of ICAM-1, CXCR3, TRAIL, and MMP-3 in mononuclear cells of MSG biopsy tissues was analyzed by microscopic examination. At least 4 consecutive high-power fields were observed and counted. The percentage of positive cells relative to total cells was calculated for each sample, counted for at least 80, and up to 200 cells[30]. All profiles of the inflammation-related proteins (ICAM-1, CXCR3, TRAIL, and MMP-3) were compared between RF+ and RF–, ESR $\geq$ 20 and ESR < 20, ANA $\geq$ 1:160 and ANA < 160, anti-SSA(+) and anti-SSA(–), grade 4 sialadenitis and grade 3 sialadenitis, and primary and secondary SS.

Laboratory evaluation. ESR was measured by the Westergren method. Anti-SSA and SSB were measured using the immunodiffusion method. ANA was determined by immunofluorescence, and RF was measured by nephelometry.

Statistical analysis. Statistical analysis was carried out using SPSS version 13 software (SPSS, Chicago, IL, USA). The Mann-Whitney U-test was used, as a nonparametric method, to analyze group differences. To take into consideration the effect of multiple testing, the Sharpened Bonferroni method (step-down sequential) was used to

adjust for individual alpha level, while the overall level of significance was set at 0.05.

RESULTS

Clinical and laboratory analysis. Thirty-four patients with SS were included in our study. The mean age was 53.3 ± 14.7 years. The female:male ratio was 16:1. Of the 34 patients with SS, 22 had primary SS and 12 secondary SS. Of the 12 patients with secondary SS, rheumatoid arthritis (RA) was diagnosed in 8 and systemic lupus erythematosus (SLE) in 4. All patients, except for 6, presented a positive Schirmer's test (both OD and OS below 5 mm/5 min).

ESR ranged from 0 to 120 mm/h (mean 45.5 mm/h). The anti-SSA antibodies were positive in 50% and anti-SSB were positive in 33% of all patients with SS. Twenty-two patients (64.7%) had an ANA titer ≥ 1:160 and 12 patients (35.3%) had a titer < 1:160. RF was positive (≥ 40 U/ml) in 16 (47%) and negative (< 40 U/ml) in 18 patients (53%).

The light microscopic examinations of salivary gland biopsies are shown in Figure 1. In our study, we measured only the inflammation-related molecules in infiltrated inflammatory cells of salivary glands. Among the 34 patients, 10 were classified as grade III (Figure 1A) and 24 were grade IV (Figure 1B), which was confirmed by one pathologist and one research assistant.

Immunohistochemical finding. We stratified the 34 patients with SS into 2 groups based on the presence or absence of RF or SSA, primary or secondary SS, levels of ANA or ESR, and biopsy grading, to determine the difference in inflammation-related molecule expression in the inflammatory cell populations. The pathologic findings of the immunohistochemical staining are shown in Figure 2.

Biopsy grading. MMP-3 and TRAIL were overexpressed in grade IV, compared to grade III, of all 34 patients with SS (62.7 ± 6.3 vs 54.4 ± 7.3, p = 0.003; and 44.9 ± 4.5 vs 40.8 ± 3.6, p = 0.013, respectively). When we divided our patients into primary and secondary SS, patients with pri-
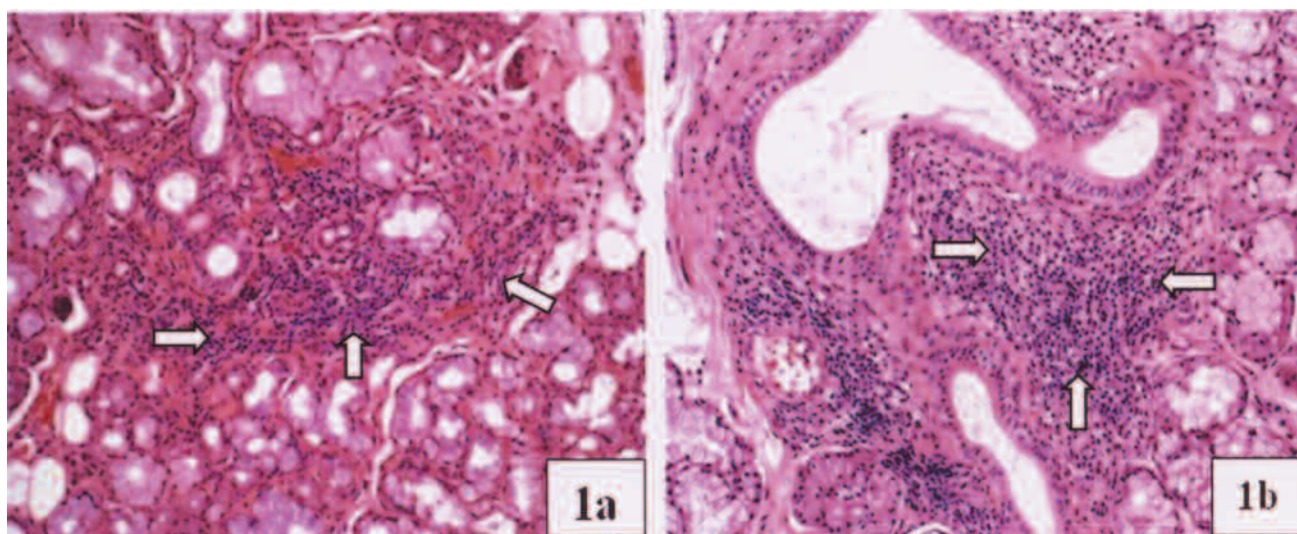

Figure 1. Lymphocyte infiltration (arrows) in minor salivary gland (H&E stain). Panel a: grade 3 sialadenitis; panel b: grade 4 sialadenitis (200× magnification).
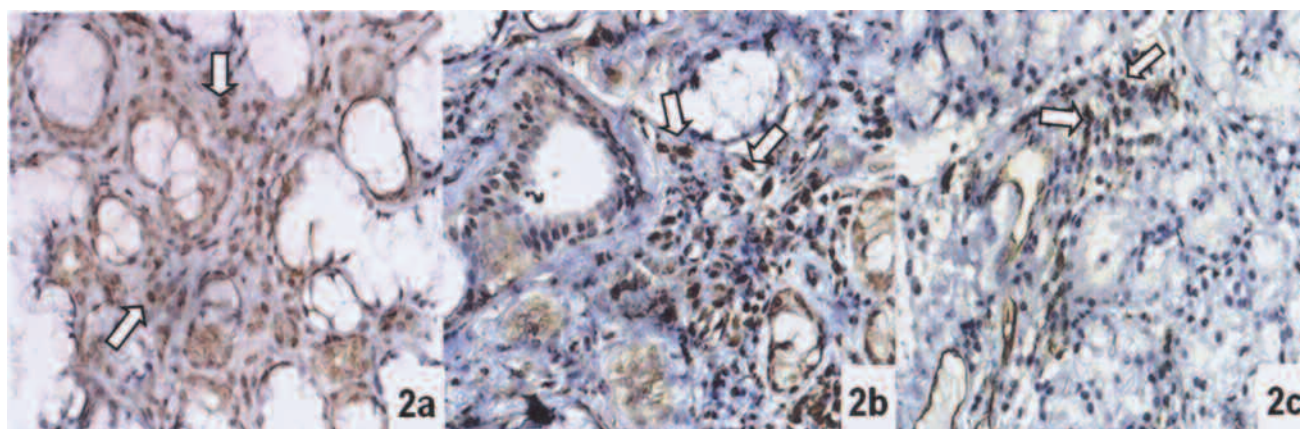

Figure 2. Expression of TRAIL (a), MMP-3 (b), and ICAM-1 (c) (arrows) in minor salivary gland mononuclear cells of patients with Sjögren's syndrome (400× magnification).

mary SS who had grade 4 sialadenitis revealed a significantly higher expression of MMP-3 than those had grade 3 sialadenitis (p = 0.016). Meanwhile, MMP-3 expression was also higher in grade 4 than in grade 3 for patients with secondary SS, although the result did not reach statistical significance (p = 0.182; Table 1).

ANA titer. The relationship between the ANA titer (≥ 1:160 and < 160) and the immunopathologic findings of the salivary gland tissues was investigated. TRAIL and ICAM-1 showed a significant difference between high ANA titer and low ANA titer (45.1 ± 4.4 vs 42.1 ± 3.9, p = 0.021; and 15.2 ± 5.7 vs 10.8 ± 3.3, p = 0.018, respectively; Table 2).

Presence of RF and anti-SSA. There was no significant difference in the CD20 cell population between positive RF and negative RF only. In contrast to RF, patients with positive anti-SSA revealed a significantly higher expression of ICAM-1, TRAIL, and MMP-3 on infiltrating inflammatory cells, when compared to patients with negative anti-SSA (p value for MMP-3 < 0.005, for ICAM-1, TRAIL, p < 0.05; Table 3).

Primary versus secondary SS. There was a significant difference in CD20 cell infiltration (21.6 ± 7.8 in primary SS vs 26.9 ± 7.9 in secondary SS, p = 0.028) and MMP-3 expression (58.2 ± 6.5 in primary SS vs 63.8 ± 8.1 in secondary SS, p = 0.017) between the 2 groups (Figure 3).

ESR level. We separated ESR into 2 groups: a high ESR group (ESR ≥ 20 mm/h) and a low ESR group (ESR < 20). CD20 (26.9 ± 8.2 vs 19.6 ± 8.2, p = 0.006) and TRAIL expression (45.4 ± 4.5 vs 41.9 ± 4.0, p = 0.036) were significantly more increased in the high ESR group than in the low ESR group, respectively (Figure 4).

DISCUSSION

SS is a common autoimmune disease in Caucasians[31-33], and is characterized by the presence of serum autoantibodies (RF, ANA, anti-SSA, and anti-SSB), hyperimmunoglobulinemia, and mononuclear cell infiltration in the salivary glands.

The MSG biopsy in our 70 cases disclosed grade 0 in 14

Table 1. Expression of MMP-3 and TRAIL on salivary gland mononuclear cells in 34 patients with Sjögren's syndrome (SS) with different grades (grade 3 vs grade 4 sialadenitis).

	Grade 3		Grade 4		p*	S[†]/NS[†]
	Mean, %	SD	Mean, %	SD		
TRAIL	40.8	3.6	44.9	4.5	0.013	S[†]
MMP-3	54.4	7.3	62.7	6.3	0.003	S[†]
Primary SS (n = 22)	53.8	6.9	60.8	4.9	0.016	S[†]
Secondary SS (n = 12)	56.8	11.1	65.3	7.4	0.182	NS[†]

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; MMP: matrix metalloprotease. * Mann-Whitney U-test. [†]S/NS: statistically significant (nonsignificant) after Sharpened Bonferroni adjustment for individual type I error probability.

Table 2. The association of ANA titer (ANA < 160, ANA ≥ 160) with expression of ICAM-1, and TRAIL in salivary gland mononuclear cells of patients with Sjögren's syndrome.

	ANA < 160 (n = 12)		ANA ≥ 160 (n = 22)		p*	S[†]/NS[†]
	Mean, %	SD	Mean	SD		
ICAM-1	10.8	3.3	15.2	5.7	0.018	S[†]
TRAIL	41.2	3.9	45.1	4.4	0.021	S[†]

ANA: antinuclear antibodies; ICAM: intercellular adhesion molecule; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; * Mann-Whitney U-test. [†]S/NS: statistically significant (nonsignificant) after Sharpened Bonferroni adjustment for individual type I error probability.

Table 3. The relationship between serum SSA antibodies and expression of MMP3, ICAM-1, or TRAIL in salivary gland mononuclear cells of patients with Sjögren's syndrome.

	SSA– (n = 15)		SSA+ (n = 15)		p*	S[†]/NS[†]
	Mean, %	SD	Mean	SD		
MMP-3	55.9	6.4	64.1	6.8	0.002	S[†]
ICAM-1	12.1	3.9	16.7	5.6	0.013	S[†]
TRAIL	41.8	4.3	45.4	4.6	0.026	S[†]

For abbreviations, see Table 2. * Mann-Whitney U-test. [†]S/NS: statistically significant (nonsignificant) after Sharpened Bonferroni adjustment for individual type I error probability.
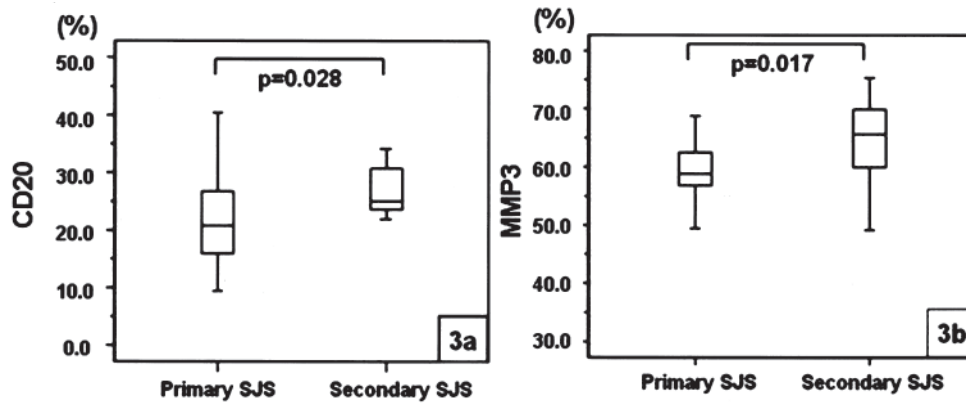
Figure 3. Percentage of CD20-positive cells (a) and MMP-3 expression (b) in salivary gland mononuclear cells was significantly higher in patients with secondary Sjögren's syndrome than with primary SS. p = 0.028, p = 0.017, respectively.
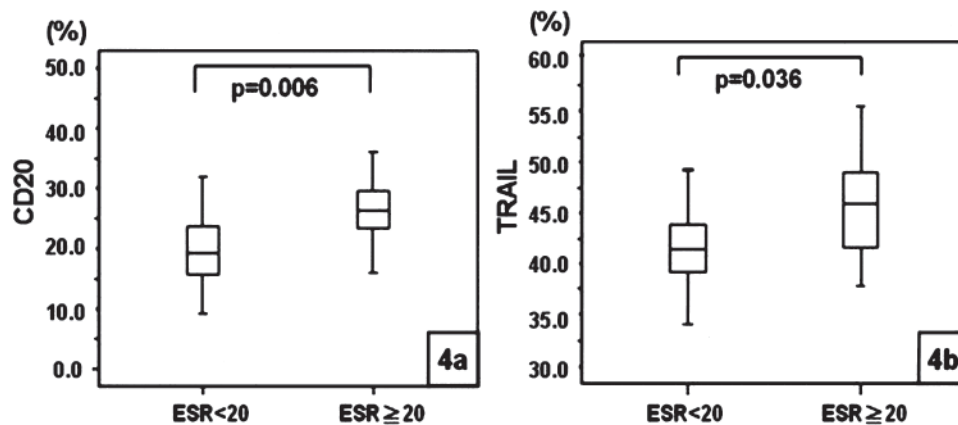

Figure 4. Percentage of CD20-positive cells (a) and TRAIL expression (b) in salivary gland mononuclear cells of patients with Sjögren's syndrome were significantly increased in the high ESR group (ESR ≥ 20 mm/h) compared with the low ESR group (ESR < 20 mm/h). p = 0.006, p = 0.036, respectively.

patients, grade I in 10 patients, grade II in 10 patients, grade III in 10 patients, and grade IV in 24 patients. Since we have attempted to observe the expression of cytokines or chemokines in the mononuclear cells in MSG, we selected only cases that had either grade III or grade IV with sufficient mononuclear cells to investigate.

First, we wanted to know the association of RF and ANA with salivary gland inflammation. RF has been detected frequently in patients with primary SS, with a prevalence ranging from 38% to 61%[34,35]. Garcia-Carrasco, *et al* demonstrated the high prevalence of ANA (74%) in their 400 cases with primary SS[34]. In addition, primary SS patients with positive ANA and positive anti-ENA presented more severe glandular or extraglandular involvement, which included interstitial lung disease, vasculitis, Raynaud's phenomenon, neuropathy, etc.[34,35]. In our study, positive RF did not show a significant difference when compared to negative RF. In contrast, TRAIL and ICAM-1 were significantly increased

in expression in the high-titer ANA group (≥ 1:160) rather than in the low-titer ANA group (< 1:160).

ANA and RF were both included in the 6 criteria in the 1993 European criteria for SS. The prevalence of primary SS differed, ranging from 0.6% to 4.8%, depending on whether the investigators used the 1993 criteria, including ANA and RF, or the 2002 American-European Consensus Group (US-EU) criteria, which did not include ANA and RF, to survey primary SS[26,32,36-40]. In spite of the many negative results for RF or ANA observed in the MSG in this study, we still recommend keeping ANA and RF in the US-EU criteria in order to recognize primary SS earlier, as Kabasakal, *et al* concluded[31].

In primary SS, the positive rate is 40% for anti-SSA antibody and 26% for anti-SSB antibody, as reported by previous investigators[34]. However, the presence of SSA antibody was significantly associated with positive salivary gland findings characteristic for SS, and reduced salivary flow,

when compared to the negative SSA/SSB antibodies[41-43]. The presence of ENA (SSA/SSB) antibody carried more extraglandular manifestations and a higher risk of developing lymphoproliferative disorders[34]. In Taiwan, the serum anti-SSA antibody was detected in 74% and anti-SSB antibody in 65% of patients with primary SS[44]. Our study confirmed that SS with positive SSA/SSB presented an overexpression of MMP-3, ICAM-1, and TRAIL in mononuclear cells when compared to patients with negative SSA.

TRAIL can induce apoptosis of tumor cells, but not of normal cells. Failure to induce apoptosis of infiltrated mononuclear cells through the Fas-FasL or TRAIL-TRAIL-R system is important to the development of autoimmune disease[33,45,46]. The pathologic findings confirming the apoptosis-related mechanism in SS were evidenced by the overexpression of Fas antigen on ductal epithelial cells and FasL on mononuclear cells, caspase in lymphocytes, P53 antigen and its transcription target P21 in ductal cells, and the increased percentage of TUNEL apoptotic cells in the ducts and acina of the MSG in SS[3,6,11,45-50]. In SS, the pathways have been implicated by interactions between epithelial cells expressing the Fas molecules and surrounding T cells expressing FasL[6,33,45,46]. In this study, we did not measure the Fas and FasL or other apoptosis-related molecules (Bcl2, BAX, caspase, etc.). Matsumura, et al studied TRAIL in patients with SS and demonstrated that TRAIL was present in the infiltrating inflammatory cells, and both TRAIL-R1 and TRAIL-R2 were strongly expressed on the ductal epithelial cells[12]. Therefore, both TRAIL and TRAIL-R may be involved in the pathogenesis of lymphocytic sialadenitis through the TRAIL-induced apoptosis of epithelial cells. Our study measured TRAIL on infiltrating mononuclear cells only, and anti-SSA(+) was associated with the overexpression of TRAIL when compared to anti-SSA(–). Whether the presence of SSA(+) may cause more severe sialadenitis by mediating the TRAIL/TRAIL-R system or other apoptosis-related mechanism needs to be studied.

Recent studies have shown that MMP-3 can be regarded as a disease activity marker for AS[13,14]. The serum MMP-3 level correlated not only with ESR and C-reactive protein, but also with the Bath AS Disease Activity Index (BAS-DAI). Our investigation has demonstrated that a higher biopsy grading (grade IV) is significantly associated with the overexpression of MMP-3 in infiltrated mononuclear cells of the MSG, particularly in patients with primary SS. Higher MMP-3 expression was also noted in grade 4 sialadenitis in secondary SS, but the result did not reach statistical significance. It may be due to the limited number of cases. In general, lip biopsy is less often performed in patients with secondary SS in clinical practice. As far as we know, only one study showed that the expression of MMP-3 and MMP-9 was increased in acinar and ductal cells, but was not detected in infiltrating mononuclear cells in MSG;

the expression of MMP-3 and MMP-9 also did not correlate with mononuclear cell infiltration[15]. A high MMP-9 expression in the salivary glandular cells was demonstrated by Wu, et al[16], but they did not measure it in mononuclear cells of the MSG. In our study, we demonstrated that MMP-3 expression was significantly higher in secondary SS than in primary SS. It remains unknown whether the overexpression of MMP-3 in the mononuclear cells indicates a higher disease activity in patients with SS, and why MMP-3 was overexpressed in secondary SS compared to primary SS; these questions deserve further study.

The recruitment of mononuclear cell infiltration in the MSG of patients with SS depends upon the distribution and function of adhesion molecules. Turkcapar, et al found that in salivary glands, ICAM-1 was expressed on vascular endothelial cells and mononuclear inflammatory cells[18]. In our study, the presence of anti-SSA antibody was significantly associated with the overexpression of ICAM-1 on mononuclear cells. The abundant mononuclear cell infiltration in the salivary glands indicated that ICAM-1 may play an important role. Our study suggests that the appearance of the anti-SSA antibody may associate with the expression of ICAM-1, which later can increase the movement of mononuclear cells from the intravascular to the extravascular salivary gland tissue.

During inflammation, chemokine (CXCR3) receptors are overexpressed on Th1 cells and the CXCR3 ligands (IP-10) on different cells[51,52]. In the SS salivary glands, many CXCR3-positive lymphocytes were observed around the duct[53]. It is suggested that CXCR3 can recruit activated T cells to sites on the MSG or other organs. Shimizu, et al studied the distribution of CXCR3 in interstitial pneumonia and found that CXCR3-positive cells infiltrated significantly in the fibrotic areas and lymphoid clusters of patients with SS[24]. However, in our study, CXCR3 on inflammatory cells was not significantly increased in SS patients with higher titer ANA or SSA. We may examine other chemokines or their receptors to determine the role of chemokines in the sialadenitis of SS.

In summary, a significant association was demonstrated between the presence of autoantibodies or a high class of sialadenitis and the overexpression of TRAIL, MMP-3, or ICAM-1 in infiltrating inflammatory cells in SS. Further study is required to determine how the autoantibody and biopsy grading influence the salivary gland inflammation.

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Correction

Chen W-S, Lin K-C, Chen C-H, et al. Autoantibody and biopsy grading are associated with expression of ICAM-1, MMP-3, and TRAIL in salivary gland mononuclear cells of Chinese patients with Sjögren's syndrome. J Rheumatol 2009;36:989-96. The name of a second corresponding author should have been given, as follows: Prof. C-Y. Tsai; E-mail: cytsai@vghtpe.gov.tw. We regret the error.

doi:10.3899/jrheum.080733C1