Predictive significance of CCL21 and CXCL13 levels in the minor salivary glands of patients with Sjögren's syndrome

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Abstract

Objective

To investigate whether CCL21 and CXCL13 expression levels in the minor salivary gland are associated with the laboratory and clinical manifestations of Sjögren's syndrome (SS).

Methods

Sociodemographic data on 106 SS patients were obtained and the glandular and extraglandular manifestations of the disease were documented. In addition, minor salivary gland biopsies were performed and the patients' laboratory findings were analysed. European League Against Rheumatism SS disease activity index (ESSDAI) values of SS disease activity at the time of biopsy and the SS disease damage index (SSDDI) values were also recorded. An immunohistochemical approach was used to semiquantitatively measure the CCL21 and CXCL13 expression in the minor salivary glands.

Results

The minor salivary glands of SS patients stained positively for CCL21 and CXCL13 in 46.2% (49/106) and 70.7% (75/106) of all cases, respectively. Higher-level expression of CCL21 and CXCL13 was associated with increases in ESR, IgG and rheumatoid factor levels, as well as anti-SS-A and -SS-B titers. A higher focus score and ESSDAI value at the time of biopsy were also associated with these chemokines. In patients with extraglandular manifestations of SS, the prevalence of lymphadenopathy increased with increasing CCL21 levels.

Conclusion

The expression levels of CCL21 and CXCL13 within the lymphocytic infiltrates of SS patients were associated with several laboratory features of the disease as well as lymphadenopathy and the extent of clinical disease activity. CCL21 and CXCL13 levels can therefore serve as useful markers to predict the disease activity and prognosis of patients with SS.

Key words

Sjögren's syndrome, chemokine, salivary gland, disease activity

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Introduction

Sjögren's syndrome (SS) is a chronic inflammatory disease characterised by the accumulation of lymphocytes in variant organs, resulting in sicca symptoms and extraglandular manifestations (1, 2). Ectopic lymphoid structures, found in a number of chronic inflammatory diseases including SS (3-5), consist predominantly of T and B cells and a network of high endothelial venules and follicular dendritic cells (FDC) (6, 7). The presence of ectopic lymphoid tissue is closely related to the expression of cytokines and chemokines (8).

Chemokines control inflammatory-cell migration and are divided into two functional groups (9). Inducible chemokines recruit neutrophils, monocytes, and immature or activated lymphocytes and are produced in peripheral inflamed tissue. Constitutive lymphoid-homing chemokines regulate leukocyte trafficking under physiologic conditions and are produced in primary and secondary lymphoid organs. CCL21 and CXCL13 are constitutive chemokines that play important roles in the formation of immune cell aggregates and in T- and B-cell clustering, which together results in an FDC network of so-called "lymphoid chemokines." CCL21 induces the homing of T cells (10), while CXCL13 determines the positioning of B cells in specific follicles and facilitates B-cell maturation (11). CCL21 and CXCL13 within lymphocytic infiltrates characteristic of the specific pathologic condition were shown to contribute to the formation and maintenance of ectopic lymphoid tissues (12, 13). However, whereas the roles of lymphoid chemokines in many chronic inflammatory diseases are well-known (6, 7), the relationship of similar networks to the clinical findings of SS is still unclear. In the current study, we investigated whether CCL21 and CXCL13 expression levels in the minor salivary glands of SS patients were associated with the laboratory and clinical manifestations of the disease.

Patients and methods

Patients and tissue samples

From January 2006 to April 2013, 106 patients who fulfilled the revised criteria proposed by the American-European Consensus group were evaluated (14). All patients also met the histopathologic criteria for a diagnosis of SS: ≥1 lymphocytic focus containing at least 50 mononuclear cells per 4 mm^2 (focus score) (15). This study was approved by the Institutional Review Board of Chonnam National University Hospital (CNUH-2015-049), Republic of Korea. Although informed consent was waived due to the retrospective design of the study, additional data collection carried out during the study preserved the patients' anonymity and confidentiality.

Clinical and laboratory manifestations

Baseline demographic, clinical, and laboratory data were collected at the time of minor salivary gland biopsy. An experienced rheumatologist assessed the clinical disease symptoms, including dry eye, dry mouth, enlargement of the parotid glands, and extraglandular manifestations. Ophthalmological examination was performed by one of the co-authors (KCY). Gastro-esophageal reflux was diagnosed by gastroscopy in symptomatic patients. Autoimmune thyroiditis was diagnosed by laboratory findings as hypothyroidism with increased autoantibody titers, including those of anti-thyroid peroxidase and anti-thyroglobulin antibodies. Interstitial lung disease and pulmonary fibrosis were assessed based on simple chest radiograph or high-resolution computed tomography. Renal involvement was defined as active urine sediment, proteinuria >500 mg/day, a glomerular filtration rate <60 mL/min, or renal tubular acidosis. Urinalysis was routinely performed in all patients. Radiography, including abdominal plain radiography, ultrasonography, and computed tomography, was performed both in symptomatic patients and routinely to evaluate nephrocalcinosis. Serositis was defined as pleural effusion, as seen on radiography, or pericardial effusion, detected during echocardiography. Carpal tunnel syndrome was defined as complaints of an abnormal sensation in the relevant anatomic area confirmed in a nerve conduction study. Lymphoma was diagnosed based on lymph node biopsy. The European League Against Rheu-

matism SS disease activity index (ESSDAI) (16) and the SS disease damage index (SSDDI) (17) were used to assess disease activity and the degree of tissue or organ damage, respectively.

Laboratory profiles, including white blood cell (WBC) count, lymphocytes, haemoglobin, platelets, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), immunoglobulin G (IgG), IgA, IgM, and complement (C3, C4, CH50) levels, were measured at the time of labial salivary gland biopsy. Autoantibodies against SS-A/Ro and SS-B/La were determined by ELISA; rheumatoid factor (RF) was assessed by nephelometry at the time of labial salivary gland biopsy.

Immunohistochemistry and measurements of chemokine expression

Formalin-fixed, paraffin-embedded, 3-μm-thick tissue sections placed on poly-L-lysine-coated slides were deparaffinised in xylene and rehydrated gradually in ethanol. Microwave antigen retrieval in citrate buffer was done prior to immunohistochemical staining; endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The primary antibodies used in the immunohistochemistry analysis were goat anti-human CCL21 polyclonal antibody (1:100; R&D Systems, Minneapolis, MN, USA, catalogue number AF366) and goat anti-human CXCL13 polyclonal antibody (1:100; R&D Systems, catalog number AF801). The secondary antibody was rabbit anti-gout biotin (Dako, Carpinteria, CA, USA). The primary antibodies were reacted at room temperature. A subsequent reaction was performed using a biotin-free system (Envision peroxidase detection system; Dako) to reveal possible false-positive staining due to endogenous biotin. After incubation of the sections in a chromogenic solution containing diaminobenzidine (Dako), they were counterstained with haematoxylin. Tissue sections sequential to those used to assess the foci were stained for CCL21 and CXCL13 to correlate expression with the number of lymphocytic infiltrates. Lymphocytic aggregates were considered positive for CCL21 or CXCL13 if more than a single cell stained positively. All sections were analysed independently by two observers blinded to the patients' data; discrepancies were resolved by consensus. A self-devised scoring system was used to semiquantitatively assess the level of CCL21 and CXCL13 expression, by calculating the percentage of positive cells within infiltrating lymphocytes. In this system, grade 0 was defined as no positive cells, and grades 1, 2, and 3 were defined as

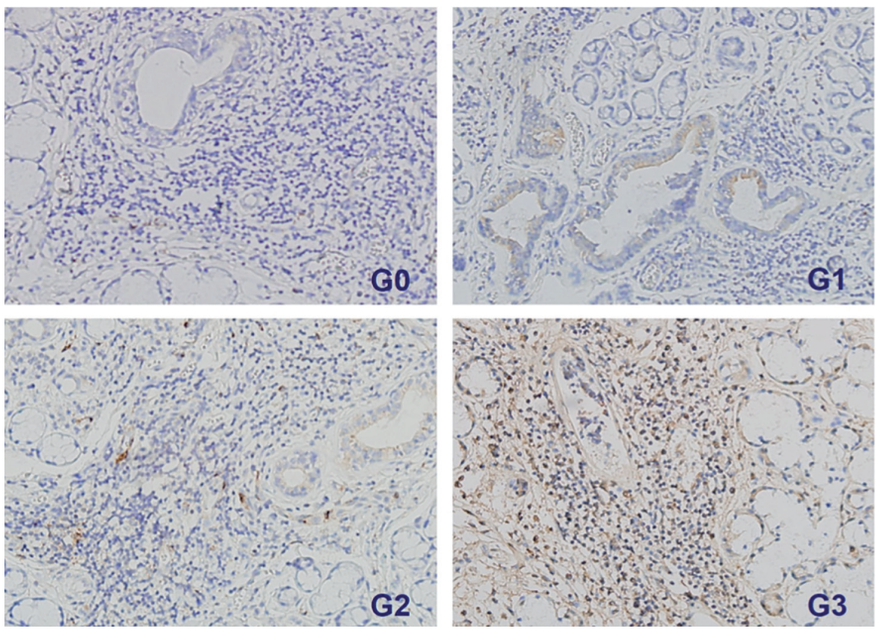

Fig. 1. Grades defined by the CCL21 expression level. Tissue sections (3 μm thick) from labial salivary gland biopsies in patients with Sjögren's syndrome (SS) were stained immunohistochemically for CCL21 (brown). CCL21 expression levels were defined semiquantitatively as follows: no positive cells, grade 0; 1–33% positivity, grade 1; 34–66% positivity, grade 2; and > 67% positivity, grade 3. Original magnification ×200.

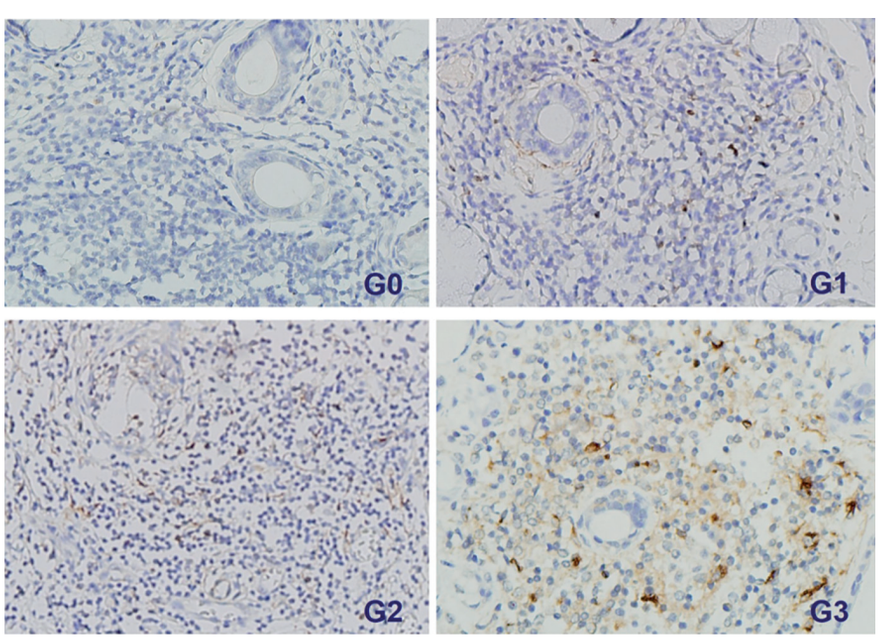

Fig. 2. Grades defined by the CXCL13 expression level. Tissue sections (3 μm thick) from labial salivary gland biopsies of patients with SS were stained immunohistochemically for CXCL13 (brown). CXCL13 expression levels were defined semiquantitatively as follows: no positive cells, grade 0; 1–33% positivity, grade 1; 34–66% positivity, grade 2; and > 67% positivity, grade 3. Original magnification ×200.

Table I. Baseline demographic characteristics and clinical manifestations at the time of minor salivary gland biopsy of 106 patients with Sjögren's syndrome.

Age (years)	45.4 ± 11.3	WBC (/mm^3)	5,389 ± 1,999
Sicca symptom onset age (years)	42.8 ± 10.9	Lymphocyte (/mm^3)	1,736 ± 654.1
Females (%)	103/106 (97.2)	Hgb (g/dL)	12.2 ± 1.5
ESSDAI	5.65 ± 3.69	Platelet (×10^3/mm^3)	231.6 ± 67.9
SSDDI	0.94 ± 0.92	ESR (mm/h)	41.6 ± 28.1
		CRP (mg/dL)	0.39 ± 0.67
Glandular manifestation		IgG (mg/dL)	2,025 ± 648.5
Ocular symptom (%)	102/106 (96.2)	C3 (mg/dL)	99.1 ± 24.3
Oral symptom (%)	101/106 (95.3)	C4 (mg/dL)	21.6 ± 10.3
Schirmer's test positive rates (%)	79/93 (84.9)	CH50 (U/mL)	50.6 ± 15.9
BUT positive rate (%)	95/96 (99.0)	Anti-SS-A/Ro (%)	92/106 (86.8)
Salivary scan positive rates (%)	94/100 (94.0)	Anti-SS-B/La (%)	38/106 (35.8)
Enlargement of parotid glands (%)	10/106 (9.4)	RF (%)	47/101 (46.5)

Unless otherwise specified, values are given as means ± SD.
ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index; SSDDI: Sjögren's syndrome disease damage index; BUT: Tear film break-up time; WBC: white blood cell; Hgb: haemoglobin; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; Ig: immunoglobulin; RF: rheumatoid factor.

Table II. Comparison of clinical manifestations and laboratory and histological findings by CCL21 level.

	CCL21 level				*p*-value
	G0 (n=57)	G1 (n=31)	G2 (n=11)	G3 (n=7)	
Extraglandular manifestation					
Arthralgia/arthritis (%)	38 (66.7)	19 (61.3)	6 (54.5)	6 (85.7)	0.852
Raynaud's phenomenon (%)	12 (21.1)	3 (9.7)	2 (18.2)	4 (57.1)	0.244
Lymphadenopathy (%)	4 (7.0)	5 (16.1)	3 (27.3)	3 (42.9)	0.003
GER (%)	6 (10.5)	4 (12.9)	3 (27.3)	0 (0)	0.763
Autoimmune thyroiditis (%)	6 (10.5)	2 (6.5)	1 (9.1)	1 (14.3)	0.994
Photosensitivity (%)	5 (8.8)	2 (6.5)	1 (9.1)	0 (0)	0.520
ILD/pulmonary fibrosis (%)	5 (8.8)	0 (0)	0 (0)	1 (14.3)	0.582
Renal involvement (%)	3 (5.3)	1 (3.2)	0 (0)	1 (14.3)	0.797
Serositis (%)	2 (3.5)	0 (0)	0 (0)	1 (14.3)	0.559
Psychosis (%)	2 (3.5)	1 (3.2)	0 (0)	0 (0)	0.480
Carpal tunnel syndrome (%)	1 (1.8)	1 (3.2)	0 (0)	0 (0)	0.755
Lymphoma (%)	0 (0)	1 (3.2)	0 (0)	0 (0)	0.738
Sclerodactyly (%)	1 (1.8)	0 (0)	0 (0)	0 (0)	0.439
Laboratory manifestation					
WBC (/mm^3)	5,573 ± 2,366	4,900 ± 1,031	5,590 ± 1,811	5,728 ± 2,264	0.935
Lymphocyte (/mm^3)	1,795 ± 738.3	1,622 ± 504.3	1,900 ± 629.3	1,500 ± 503.3	0.621
Haemoglobin (g/dL)	12.4 ± 1.5	12.0 ± 1.5	12.1 ± 1.8	12.2 ± 1.3	0.466
Platelet (×10^3/mm^3)	228.8 ± 62.4	224.0 ± 60.4	273.4 ± 103.6	222.7 ± 67.4	0.928
ESR (mm/h)	34.1 ± 27.7	46.9 ± 23.0	55.3 ± 29.5	57.3 ± 34.9	<0.001
CRP (mg/dL)	0.29 ± 0.27	0.38 ± 0.76	1.02 ± 1.44	0.33 ± 0.29	0.641
IgG (mg/dL)	1,870 ± 612.5	2,042 ± 491.9	2,261 ± 668.6	2,674 ± 949.4	0.002
C3 (mg/dL)	95.3 ± 24.3	100.9 ± 23.4	116.6 ± 23.7	95.6 ± 21.2	0.099
C4 (mg/dL)	21.6 ± 10.7	21.0 ± 8.51	26.1 ± 14.5	18.9 ± 6.17	0.981
CH50 (U/mL)	49.1 ± 17.3	52.2 ± 14.9	52.6 ± 14.4	53.9 ± 8.64	0.433
RF (IU/mL)	32.1 ± 69.1	33.2 ± 80.6	30.8 ± 39.1	49.8 ± 43.1	0.015
Anti-SS-A/Ro (U/mL)	136.4 ± 92.2	184.7 ± 47.5	137.0 ± 79.8	200.0 ± 0.00	0.027
Anti-SS-B/La (U/mL)	23.6 ± 48.4	60.2 ± 78.0	67.5 ± 85.1	81.4 ± 85.3	0.002
Focus score	1.67 ± 1.02	2.23 ± 0.88	2.05 ± 0.72	2.14 ± 0.90	0.011
ESSDAI	4.86 ± 3.10	5.61 ± 3.58	7.18 ± 4.42	10.1 ± 4.14	0.005
SSDDI	0.98 ± 0.86	0.97 ± 1.20	0.82 ± 0.41	0.71 ± 0.76	0.342

Unless otherwise specified, values are given as means ± SD.
GER: gastro-esophageal reflux; ILD: interstitial lung disease; WBC: white blood cell; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; Ig, immunoglobulin; RF, rheumatoid factor; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index; SSDDI: Sjögren's syndrome disease damage index.

1–33%, 34–66%, and >67% positivity, respectively (Fig. 1-2). The percentage of chemokine expression was assessed twice by a trained observer blinded to the patients' information. A weighted Cohen's κ was calculated to assess the intraobserver agreement; the κ indices for CCL21 and CXCL13 were 0.96 and 0.95, respectively. In the case of disagreement, the biopsy tissues were reviewed by another independent observer and the grade was defined by reaching a consensus between the two observers.

Statistical analysis

Demographic, laboratory, and clinical manifestations were summarised using the mean and standard deviation for continuous variables and numbers and percentages for categorical variables. Associations between extraglandular manifestations and CCL21 and CXCL13 expression levels were assessed using a χ^2 test for trend. The Jonckheere-Terpstra test was used to evaluate whether the laboratory values and indexes for the clinical features differed significantly with increasing CCL21 and CXCL13 expression. Significant parameters were analysed using Spearman's tests for correlation. All statistical analyses were performed using SPSS for Windows software (v. 18.0; SPSS Inc., Chicago, IL, USA). A *p*-value <0.05 was considered to indicate statistical significance.

Results

The minor salivary glands of SS patients in this study stained positively for CCL21 (46.2%; 49/106) and CXCL13 (70.7%; 75/106). The baseline demographic, glandular, and laboratory data of the 106 SS patients at the time of minor salivary gland biopsy are summarised in Table I. The mean age of the 103 (97.2%) female and 3 (2.8%) male patients was 45.4±11.3 years and the mean age at the onset of sicca symptoms was 42.8±10.9 years. Most of the patients had glandular subjective symptoms and positivity on objective tests. Parotid gland enlargement was noted in 10 patients (9.4%). The mean IgG level (2,025±648.5mg/dL) was higher than the normal value. Anti-SS-A/Ro

Table III. Comparison of clinical manifestations and laboratory and histological findings by CXCL13 level.

	CXCL13 level				*p*-value
	G0 (n=31)	G1 (n=28)	G2 (n=28)	G3 (n=19)	
Extraglandular manifestation					
Arthralgia/arthritis (%)	24 (77.4)	16 (57.1)	19 (67.9)	10 (52.6)	0.144
Raynaud's phenomenon (%)	4 (12.9)	7 (25.0)	7 (25.0)	3 (15.8)	0.642
Lymphadenopathy (%)	2 (6.5)	3 (10.7)	6 (21.4)	4 (21.1)	0.070
Autoimmune thyroiditis (%)	2 (6.5)	5 (17.9)	2 (7.1)	1 (5.3)	0.690
GER (%)	5 (16.1)	3 (10.7)	4 (14.3)	1 (5.3)	0.369
Photosensitivity (%)	2 (6.5)	3 (10.7)	2 (7.1)	1 (5.3)	0.828
ILD/pulmonary fibrosis (%)	2 (6.5)	2 (7.1)	1 (3.6)	1 (5.3)	0.704
Renal involvement (%)	1 (3.2)	2 (7.1)	1 (3.6)	1 (5.3)	0.883
Serositis (%)	0 (0)	2 (7.1)	1 (3.6)	0 (0)	0.996
Psychosis (%)	1 (3.2)	1 (3.6)	0 (0)	1 (5.3)	0.996
Carpal tunnel syndrome (%)	0 (0)	1 (3.6)	0 (0)	1 (5.3)	0.378
Lymphoma (%)	0 (0)	0 (0)	1 (3.6)	0 (0)	0.535
Sclerodactyly (%)	0 (0)	1 (3.6)	0 (0)	0 (0)	0.760
Laboratory manifestation					
WBC (/mm^3)	6,229 ± 2,692	5,293 ± 1,491	4,950 ± 1,809	4,805 ± 1,049	0.014
Lymphocyte (/mm^3)	2,003 ± 849.1	1,732 ± 540.2	1,514 ± 548.0	1,632 ± 441.0	0.041
Haemoglobin (g/dL)	12.4 ± 1.7	12.5 ± 1.5	12.0 ± 1.4	11.9 ± 1.5	0.158
Platelet (×10^3/mm^3)	260.1 ± 78.0	217.6 ± 67.1	230.6 ± 59.0	207.4 ± 44.0	0.022
ESR (mm/h)	29.2 ± 25.0	39.5 ± 22.5	50.0 ± 29.2	52.2 ± 31.9	<0.001
CRP (mg/dL)	0.27 ± 0.29	0.33 ± 0.34	0.53 ± 1.00	0.47 ± 0.86	0.968
IgG (mg/dL)	1,719 ± 439.6	2,106 ± 828.7	2,168 ± 506.6	2,155 ± 713.7	0.004
C3 (mg/dL)	102.2 ± 27.6	97.3 ± 26.7	94.3 ± 20.8	103.8 ± 19.6	0.560
C4 (mg/dL)	22.7 ± 10.3	22.2 ± 12.3	20.7 ± 11.3	20.4 ± 4.7	0.565
CH50 (U/mL)	48.8 ± 18.7	46.9 ± 18.4	51.3 ± 13.0	58.3 ± 6.29	0.177
RF (IU/mL)	36.7 ± 89.9	30.5 ± 38.5	42.5 ± 77.1	50.5 ± 54.1	0.004
Anti-SS-A/Ro (U/mL)	115.7 ± 90.0	156.6 ± 75.2	172.9 ± 76.8	189.1 ± 45.8	0.001
Anti-SS-B/La (U/mL)	15.8 ± 38.6	31.4 ± 52.9	58.6 ± 76.8	79.6 ± 87.7	0.001
Focus score	1.32 ± 0.83	2.00 ± 1.09	2.30 ± 0.83	2.11 ± 0.81	<0.001
ESSDAI	4.45 ± 3.17	5.11 ± 3.12	6.68 ± 4.17	7.00 ± 3.90	0.006
SSDDI	0.90 ± 0.98	0.96 ± 0.69	1.04 ± 1.17	0.84 ± 0.77	0.911

Unless otherwise specified, values are given as means ± SD.
GER: gastro-esophageal reflux; ILD, interstitial lung disease; WBC, white blood cell; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; Ig: immunoglobulin; RF: rheumatoid factor; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index; SSDDI: Sjögren's syndrome disease damage index.

Table IV. Correlation between chemokine levels and laboratory findings, focus score, and clinical index.

	CCL21 level	CXCL13 level
WBC	-0.009	-0.245*
Lymphocyte	-0.047	-0.198*
Platelet	0.007	-0.219*
ESR	0.327**	0.308**
IgG	0.360**	0.348**
RF	0.217*	0.322**
Anti-SS-A/Ro	0.302**	0.327**
Anti-SS-B/La	0.246*	0.286**
Focus score	0.255**	0.367**
ESSDAI	0.268**	0.269**

*p<0.05, **p<0.01.
WBC: white blood cell; ESR: erythrocyte sedimentation rate; Ig: immunoglobulin; RF: rheumatoid factor; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index.

Ab and anti-SS-B/La Ab were positive in 86.8% (92/106) and 35.8% (38/106) of the patients, respectively.

Data on the laboratory, histological, and clinical manifestations of the patients at the time of biopsy according to CCL21 level are shown in Table II. Among the extraglandular manifestations, the prevalence of lymphadenopathy increased with increasing CCL21 levels in four patients (7.0%) with grade 0, five patients (16.1%) with grade 1, three patients (27.3%) with grade 2, and three patients (42.9%) with grade 3 positivity (p=0.003). Among the laboratory manifestations, higher-level CCL21 expression was associated with increases in the ESR (p<0.001,) IgG (p=0.002) and RF (p=0.015) levels, and anti-SS-A/Ro (p=0.027) and -SS-B/La (p=0.002) titers at the time of biopsy. Pathologically, the focus score increased across all four grades: 1.67±1.02 in grade 0, 2.23±0.88 in grade 1, 2.05±0.72 in grade 2, and 2.14±0.90 in grade 3 (p=0.011). The ESSDAI increased with increasing CCL21 levels as follows: 4.86±3.10 in grade 0, 5.61±3.58 in grade 1, 7.18±4.42 in grade 2, and 10.1±4.14 in grade 3 (p=0.005), whereas the SSDDI did not differ according to the expression level. An analysis of the ESSDAI domains associated with CCL21 showed that higher CCL21 expression was associated with the lymphadenopathy (p=0.005), glandular (p=0.016), and biological domains (p<0.01) (data not shown).

The patients' laboratory, histological, and clinical data with respect to CXCL13 level at the time of biopsy are provided in Table III. The extraglandular manifestations did not differ significantly according to the expression levels of this cytokine. An analysis of the laboratory findings showed an association between the higher-level expression of CXCL13 and decreases in WBC (p=0.014), lymphocyte (p=0.041), and platelet (p=0.022) numbers and increases in ESR (p<0.001), IgG (p=0.004), RF (p=0.004), and anti-SS-A/Ro (p=0.001) and -SS-B/La (p=0.001) titers at the time of biopsy. The focus score increased with an increase in the level of CXCL13 as follows: 1.32±0.83 in grade 0, 2.00±1.09

in grade 1, 2.30±0.83 in grade 2, and 2.11±0.81 in grade 3 (p<0.001). The ESSDAI increased to 4.45±3.17 in grade 0, 5.11±3.12 in grade 1, 6.68±4.17 in grade 2, and 7.00±3.90 in grade 3 (p=0.006) patients, whereas expression-level-related changes in the SSDDI were not observed. An analysis of the domains of ESSDAI associated with CXCL13 level showed that higher-level CXCL13 expression was associated with the haematological (p=0.039) and biological domains (p<0.001) (data not shown).

Correlations between CCL21 and CXCL13 expression levels and clinical findings are shown in Table IV. WBC (r = -0.245, p<0.05), lymphocyte (r= -0.198, p<0.05) and platelet (r= -0.219, p<0.05) counts correlated negatively with the CXCL13 but not the CCL21 level. However, the latter correlated positively with the ESR (r=0.327, p<0.01), IgG (r=0.360, p<0.01) and RF (r=0.217, p<0.05) levels, anti-SS-A/Ro (r=0.302, p<0.01) and anti-SS-B/La (r=0.246, p<0.05) titers, the focus score (r=0.255, p<0.01), and the ESSDAI value (r =0.268, p<0.01) at the time of biopsy. Correlations between CXCL13 and the ESR (r=0.308, p<0.01), IgG (r=0.348, p<0.01) and RF (r=0.322, p<0.01) levels, anti-SS-A/Ro (r=0.327, p<0.01) and anti-SS-B/La (r=0.286, p<0.01) titers, the focus score (r=0.367, p<0.01), and the ESSDAI value (r=0.269, p<0.01) at the time of biopsy were also positive.

In our study, 10 patients (9.4%) had secondary SS, including 2 patients with rheumatoid arthritis and 8 with systemic lupus erythematosus. There were no statistically significant differences in the demographic, pathological, laboratory, or clinical data of patients with primary *versus* secondary SS (data not shown).

Discussion

In this study, the minor salivary glands of 46.2% and 70.7% of the SS patients stained positively for CCL21 and CXCL13. Higher-level CCL21 expression was associated with increases in the ESR, IgG and RF levels, anti-SS-A/Ro and -SS-B/La titers, the focus score, and the ESSDAI value at the time of biopsy. Higher-level CXCL13 expression was associated with decreases in WBC, lymphocyte, and platelet counts and increases in the ESR, IgG and RF levels, anti-SS-A/Ro and -SS-B/La titers, the focus score, and the ESSDAI value at the time of biopsy. In patients with extraglandular manifestations of SS, the prevalence of lymphadenopathy tended to increase with increasing CCL21 levels.

To the best of our knowledge, this is the first study of SS patients to compare their clinical features with the expression of chemokines in their minor salivary glands. Both CCL21 and CXCL13 play pivotal roles in inducing ectopic lymphoid neogenesis and in chronic inflammatory processes (12, 13, 18), by activating T-cell subsets and causing B cells to undergo maturation, clonal selection, and proliferation. Therefore, the increased expression of CCL21 and CXCL13 is closely associated with the formation of germinal centres, which may result in hypergammaglobulinaemia and the production of autoantibodies, through B-cell activation (19). Considering that the presence of germinal centres in salivary glands of SS patients is associated with the distinct features of the extraglandular manifestations of the disease, including autoantibodies (20-22), it could be assumed that the increased expression of CCL21 and CXCL13 in the minor salivary glands of SS patients would be correlated with several laboratory findings, including autoantibody levels. On the other hand, according to our results, increased CXCL13 expression was associated with reductions in WBC, lymphocyte, and platelet counts. The ability of CXCL13 to promote B-cell activity would suggest that autoantibodies against blood components lead to cytopenias in these patients. Taken together, these observations suggest a hypothesis in which the chronic inflammatory condition and the immune reactions stimulated by lymphoid chemokines induce increases in inflammatory markers, such as ESR, causing B cells to produce high levels of IgG and autoantibodies, which in turn leads to cytopenia in SS patients. Our study showed an association between the expression levels of lymphoid chemokines and the presence of lymphadenopathy but not the occurrence of lymphoma. In the salivary glands of SS patients with MALT lymphoma, CXCL13 and CCL21 are selectively associated with areas of reactive lymphoid proliferation, suggesting that these chemokines are involved in the regulation of malignant B-cell survival (23). In the ACCESS cohort, although chemokines in the salivary glands were not measured, serum levels of CXCL13 and CCL11 in primary SS patients were shown to be associated with the occurrence of lymphoma (24). In our study, only one patient was diagnosed with lymphoma, which prevented confirmation of this association. A longer follow-up of our study patients would allow an assessment of lymphoma development.

Interestingly, our results demonstrated an increase in the ESSDAI score at the time of biopsy, together with elevated expression of both CCL21 and CXCL13. It has been suggested that the higher the level of lymphoid chemokine expression in lymphocyte infiltrates, the greater the enhancement of inflammatory mechanisms, which would result in a higher ESSDAI score by affecting several glandular and extraglandular manifestations. A detailed analysis of the ESSDAI domain associated with CCL21 and CXCL13 level showed an association between higher-level CCL21 expression and lymphadenopathy as well as glandular and biological domains, and between higher-level CXCL13 expression and haematological and biological domains. These results suggest that CCL21 and CXCL13 modulate the various clinical manifestations of SS through their specific pathogenic functions and that the levels of these chemokines can serve as biomarkers for disease activity in SS patients. In the current study, the expression of lymphocytic chemokines was significantly associated with disease activity, but not with disease damage. It has been suggested that CCL21 and CXCL13 may affect the early stage of inflammation which can influence on disease activity, but not on disease damage which results from delayed and cumulative effects of these chemokines. Another possibility is that although less

likely to develop, SSDDI score of the patients included in this study was too low to show the association between the expression of these chemokines and SSDDI.

In our patients, a higher focus score in the minor salivary glands was associated with higher CCL21 and CXCL13 expression levels. Kramer *et al*. showed that in the salivary tissue of SS mouse models, CXCL13 increases with disease progression and that CXCL13 neutralisation reduces glandular inflammation in a non-obese diabetic (NOD) model (25). Barone *et al*. reported an association between CXCL13 and CCL21 in the salivary glands of SS patients and increased size of periductal inflammatory aggregates (13). Similarly, the expression of both lymphoid chemokines in lymphocyte infiltrates, even in the absence of germinal-centre-like aggregation, in the salivary glands of SS patients was also reported (13, 26). Overall, these observations suggest that CCL21 and CXCL13 promote lymphocyte recruitment to diseased salivary glands and thereby increase the degree of lymphocytic infiltration, as described by the focus score.

Our study had several limitations. First, it used a retrospective design and the possibility of recall-bias cannot be excluded, because the study was mainly based on a medical chart review. Second, we were unable to measure serum chemokine levels at the time of minor salivary gland biopsy. Simultaneous determination of serum chemokine levels may have enabled an assessment of the systemic effect of chemokine expression in the salivary glands. Interestingly, in a study in which saliva and serum CXCL13 levels were measured simultaneously, the levels reported in saliva did not correlate with those in serum (25). Further investigations are necessary to determine whether chemokine levels in serum reflect those in the salivary glands. Third, the limitations inherent to single-centre studies prevent general recommendations based on the results.

Nonetheless, taken together, our results are consistent with an association between the expression levels of CCL21 and CXCL13 within the lymphocytic infiltrates of SS patients and several laboratory features of the disease, as well as lymphadenopathy and the extent of clinical disease activity. Therefore, CCL21 and CXCL13 levels can serve as a useful biomarker for predicting disease activity and prognosis in patients with SS.

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