이학석사 학위논문

Verification of the usefulness of anti-AQP5 autoantibodies as a biomarker for Sjögren's syndrome

항-아쿠아포린5 자가항체의 쇼그렌 증후군 바이오마커로서의 유용성 검증

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ABSTRACT

Verification of the usefulness of anti-AQP5 autoantibodies as a biomarker for Sjögren's syndrome

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Background

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease characterized by dryness of mouth. A

pathogenic feature of SS is characterized as polyclonal B cell hyperactivity evidenced by hypergammaglobulinemia and multiple autoantibodies, that include anti-Ro/SSA and anti La/SSB. Main components of diagnostic criteria consist of histological examinations of minor salivary glands for lymphocytic infiltration (focus score) and serology. However, presence or extensiveness of lymphocytic infiltration in salivary glands does not always indicate disease severity or degree of secretory dysfunction. SS is greatly under-recognized in clinical practice, mostly due to diverse symptomatic expressions making the initial diagnosis difficult. In fact other potential factors that contribute to SS dryness are currently under investigation. Antibodies to AQP5 have been proposed to contribute to secretory dysfunction in SS. Previous studies have identified the presence of autoantibodies to AQP5 in the sera from SS. Although the anti-AQP5 autoantibodies were also detected in control sera,

the levels of anti-AQP5 IgG were significantly higher in the SS samples (n=112) than in the healthy control samples (n=53) with sensitivity of 0.73 and a specificity of 0.68. The identification of disease associated biomarkers will therefore help to clarify the complex pathogenesis of SS.

Methods

A Case-controlled cross-sectional study was performed using sera from primary SS patients (n = 111 from SICCA cohort), non-autoimmune controls (NA, n = 43 from SICCA cohort) and autoimmune controls (n = 35 for RA patients and n = 35 for SLE patients). The presence of anti-AQP5 autoantibodies was screened using a cell-based indirect immunofluorecence (IIF) assay and ELISA assay.

Results

By IIF using patients serum alone, the screening of anti-AQP5

IgG in SS and NA groups resulted in sensitivity of 0.595 and specificity of 0.721. Pre-incubation of serum with epitope peptide E1 improved both sensitivity and specificity, resulting in 0.613 and 0.767, respectively.

To establish the best screening method for anti-AQP5 autoantibodies, ELISA assay technique was used. Using the assay, 224 sera were evaluated. As results, the levels of anti-AQP5 were significantly higher in the SS patients than other autoimmune disease patients with sensitivity of 0.856 and specificity of 0.707 detected by ELISA using peptide E1. Using peptide A, sensitivity and specificity were 0.892 and 0.637 respectively, excluding the case of RA and SLE. However the combination of three peptides did not improve the assay sensitivity nor specificity significantly.

The detection of anti-AQP5 was analyzed in comparison with clinical parameters used for SS diagnosis. ANA was significantly associated with anti-AQP5 frequency in SS group

(detected by ELISA). However there were no significant associations or only weak associations between anti-AQP5 autoantibodies and SS diagnosis factor.

Conclusion

Further studies are needed to conclude the comparison in performance of AQP5 marker to existing SS diagnose marker. Yet this study may be contributed to further development in improving diagnostic assays for diagnosis and treatment of SS.

Keywords : Sjogren's syndrome, Autoimmune disease, AQP5, Autoantibody, Biomarker

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CONTENTS

1. Introduction

Sjögren's syndrome (SS) is an autoimmune disease of unknown etiology. It is characterized by inflammation and dysfunction of salivary glands and lacrimal glands, causing the characteristic symptoms of xerostomia and xerophthalmia [1]. Immunological abnormalities such as antinuclear antibodies (ANAs), autoantibodies to SSA or SSB, and hypergammaglobulinemia are often detected in SS patients by laboratory tests. The 2002 modified European-American diagnostic criteria considered signs and symptoms of dryness as key elements of diagnosis and was most frequently used in clinical settings [2]. In 2016, criteria proposed by Sjögren's International Collaborative Clinical Alliance (SICCA) focused mainly on objective tests (signs) (Table 1) [3]. Main components of diagnostic criteria include both serology and histological examinations of labial salivary glands for focal

lymphocytic sialadenitis (FLS). However, the presence or extensiveness of lymphocytic infiltration in salivary glands does not always indicate the disease severity or degree of secretory dysfunction [4]. SS is considered as a heterogeneous autoimmune disease that posses both organ-specific and systemic features encompassing a wide spectrum of clinical/serological abnormalities and scattered complications [5, 6].

SS is known as one of the most common autoimmune disease in adults. However, SS is greatly under-recognized in clinical practice, mostly due to diverse symptomatic expressions, making the initial diagnosis difficult [7]. Therefore, other potential factors contributing to SS dryness are currently being studied. Up to date, autoantibodies to aquaporin (AQP) 5 were identified in the sera from SS. Although the anti-AQP5 autoantibodies were detected also in some control sera, the levels of anti-AQP5 IgG were

significantly higher in the SS samples than in the healthy control samples with sensitivity of 0.73 and a specificity of 0.68 [8]. Interestingly, antibodies to AQP5 have been proposed to contribute to secretory dysfunction in SS [9]. Therefore, the anti-AQP5 autoantibodies may reflect the disease activity of SS.

The aims of this study were to determine the usefulness of anti-AQP5 autoantibodies in non-Korean SS cohort and to optimize the screening method. In addition, the relationships of anti-AQP5 autoantibodies with other criteria used in the diagnosis of SS (SSA, SSB, ANA, RF, Focus score, Unstimulate Salivary flow rate, Ocular staining) were analyzed.

Table 1

2016 ACR/EULAR criteria for the diagnosis SS

Item	Score
Labial salivary gland biopsy showing focus score ≥ 1 focus/4 mm^2	3
Anti-SSA positive	3
Ocular staining score ≥ 5	1
Schirmer's test ≤ 5 mm in 5 mins	1
Unstimulated whole salivary flow ≤ 0.1 ml/min	1
positive : score ≥ 4	

2. Materials and Methods

2.1 Serum samples

This study was done in compliance with the Helsinki Declaration after approvals from the Institutional Review Board of Seoul National Hospital (IRB number : S-02017004). The study was also conformed to the STROBE guidelines. For this case-control study, sera from SS (n=111) patients and non-autoimmune control (NA) (n=43) subjects were obtained from Sjögren's International Collaborative Clinical Alliance (SICCA, USA), SLE (n=35) and RA (n=35) patients were obtained from the Department of Rheumatology Seoul St. Mary's Hospital. Demographic and clinical characteristic of subject group are summarized in Table 2. SICCA registry samples include Asian female with ages ranging from 22 to 79 years (mean age years 51.17 ± 13.9 years), while SLE and RA samples include Korean female. All SS samples fulfilled

the 2016 American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) criteria and were obtained at the time of diagnosis before starting treatment.

2.2 Design of Peptides

Three peptides corresponding to extracellular epitopes of AQP5 were selected from the previous study [10]. For ELISA, the peptides were synthesized with biotin at the N-terminal (Table 3). All peptides were synthesized at Peptron (Daejeon, Korea).

2.3 Cell cultures

Madin-Darby canine kidney (MDCK) cell line was obtained from Korean cell line bank (KCLB, Seoul, Korea). Cells were maintained in DMEM medium with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The MDCK cells

were cultured at 37℃ in a water-saturated atmosphere of 95% air and 5% CO_2. MDCK cells expressing full length human AQP5 (MDCK-AQP5) were maintained in DMEM with 10% FBS and 1% penicillin and streptomycin in the presence of 2 ㎎/㎖ G418.

2.4 Indirect immunofluorescence assay (IIF)

A mixture of nontransfected MDCK and MDCK-AQP5 cells at 1:1 was plated onto collagen-coated coverslips of 12 mm diameter. After stimulation with 0.5 mM cAMP for 24 h, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and subjected to antigen retreival by incubation in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 105℃ for 20 min. After blocking with 5% bovine serum albumin (BSA) in PBS, the cells were incubated with mouse monoclonal antibodies raised against a peptide near the C-terminus of

human AQP5 (Santa cruz, Dallas, USA) along with human serum (1:100 dilution). For each serum sample, sera preincubated overnight with 10 ㎍/㎖ synthetic peptide A, C2 or E1 in RIA buffer (10 mM Tris-HCL, 350 mM NaCl, 1% BSA, 1% Triton X-100, 10% Horse serum, pH 7.6) were stained in parallel. Subsequently, the cells were stained with Alexa Fluor 488-conjugated Rat anti-mouse IgG (Jackson immunoresearch, West Grove, USA) and Alexa Fluor 555-conjugated goat anti-human IgG (Invitrogen, Waltham, USA). At least 3 areas of AQP5-expressing cells were randomly selected, based on the mouse anti-AQP5 antibody stain, and imaged sequentially for the staining with human IgG using a confocal microscope (Carl Zeiss, Oberkochen, Germany). After coding the images, the relative intensities of the anti-AQP5 human IgG signals were blindly determined by decreasing the brightness of the red signal until all the signals of the AQP5 stain disappeared.

2.5 Modified Enzyme-linked immunosorbent assay (ELISA)

The 96-well Maxisorp microtiter plates were precoated with 1 ㎍/㎖ avidin diluted in PBS at 4℃ for overnight . The wells were washed 3 times with PBS added 0.1% Tween 20 and blocked with Superblock blocking buffer (Thermo, Waltham, USA) at RT for 1 h. Subsequently, 100 ㎕ of 2 ㎍/㎖ biotinylated peptide antigen diluted in 1% BSA in PBS was added to each well and incubated 1 h at RT. Sera diluted in 1% BSA in PBS (1:50, 1:100) were then incubated in duplicates for 1 h, RT. After 3 times of wash, HRP-conjugated goat anti-human IgG (SouthernBiotech, Birmingham, USA) was added and incubated for 1 h. For quantification of bound antibodies, HRP activity was determined with 3,3'5,5'-tetramethylbenzidine dihydrocholoride (Merck, Kenilworth, USA). The absorbance was measured at 450 nm. A standard curve was prepared by making serial dilutions of the commercial IgG within a range of

concentrations near the expected concentrations of the samples (50 ng/㎖ to 0.78125 ng/㎖). The concentration of autoantibodies against peptides was calculated using the standard curve and the OD value obtained from the Antigen-peptide bound well subtracted with the OD value obtained from incubation with avidin alone. Non-coated peptide wells used to determine background levels and subtracted prior to data analysis.

2.6 Statistical analysis

With the levels of anti-AQP5 IgG detected in the SS and non-autoimmune control groups and SLE/RA, a receiver operating characteristic (ROC) analysis by nonparametric method and the Mann Whitney *U* test were performed. Because some groups did not pass the normality test, the difference was also analyzed by Mann Whitney *U* test. Association between SS criteria factors and the presence of

autoantibodies were examined using chi-squared test. Statistics were performed using SPSS (SPSS Inc., Chicago, USA) and Graphpad Prism (LaJolla, CA, USA).

Table 2

Demographic and clinical characteristics of non-autoimmune controls, SS, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) patients

	NA (n=43)	SS (n=111)	SLE (n=35)	RA (n=35)
Age (years)	53.09±14.9	50.42±13.6	30.49±9.4	57.03±12.4
anti - SSA+	0	102	20	0
anti - SSB+	15	69	6	0
RF+	0	75	0	27
ANA+	0	75	34	14
UWS flow rate≤0.1 mℓ/min	15	84	-	-
Focus score≥1	0	80	-	-
Max Ocular staining score≥5	0	105	-	-

- : Data is not available
NA : non-autoimmune controls
SSA : Sjögren's syndrome-related antigen A
SSB : Sjögren's syndrome-related antigen B
RF : rheumatoid factor
ANA : anti-nuclear antibody

Table 3

Sequences of Selected Peptides and Their positions within the AQP5 Protein.

Peptide Name	Position	Sequence	Type	Amino Acids
A	Extracellular loop A	WPSALPT	Linear	aa 35 to 41
C2	Extracellular loop C	NNNTTQG	Linear	aa 123 to 129
E1	Extracellular loop E	G**C**SMNPARSFG**C**	Cyclic	11 181 to 191
A-ⓑ	Extracellular loop A	biotin-WPSALPT-NH2	Linear	aa 35 to 41
C2-ⓑ	Extracellular loop C	biotin-NNNTTQG-NH2	Linear	aa 123 to 129
E1-ⓑ	Extracellular loop E	biotin-G**C**SMNPARSFG**C**	Cyclic	11 181 to 191

* A double bond was formed between the 2 cysteins in bold. Underlined cysteins were artificially added to form a double bond.

3. Results

3.1 Higher levels of anti-AQP5 IgG were detected in the SS sera by IIF

The staining protocol was first optimized with-using the pooled sera. The 1:100 dilution was chosen to screen 43 NA and 111 SS samples for anti-AQP5 IgG (Fig 1). First, to observe the similarity from the previous data on non-Korean cohort, the experiment was conducted in the same method as previous studies. MDCK-AQP5 cells were double stained with mouse anti-AQP5 antibodies and sera. The anti-AQP5 IgG was detected in all NA and SS samples; however, a clear difference in titers was observed (p = 0.0074, Fig 2a). Using the cutoff value from ROC curve, 66 SS samples and 12 control samples were positive for anti-AQP5 IgG, resulting in a sensitivity 0.595 and a specificity 0.721 (Fig 2e, Table 4). In order to test if the use of epitope peptides can increase the

detection sensitivity and specificity, sera was preincubated with each epitope peptide and screened in parallel. The intensity of AQP5 staining significantly decreased upon peptide A, C2 and E1 in both SS and NA samples (Fig 2a). Although the intensity of staining in both samples was decreased, the intensity in SS remained higher compared to NA (Fig 2b, c, d).

These results indicate that the anti-AQP5 autoantibodies from the SS group recognized multiple extracellular epitopes more frequently than those from the NA groups. In addition, blocking effect of peptide was significantly effective in both SS and NA groups.

Analyzing ROC curve, both specificity and sensitivity were increased when blocking method with peptide E1 was used compared to when conventional IIF method was used (Fig 2f, Table 4). Among three peptides, E1 differentiated the most in between SS and NA samples with sensitivity 0.613 and

specificity 0.767 (Table 4).

As seen (Fig 2d), the most effective antibody reactivity was found in peptide E1 ($p < 0.0001$) when it was used to differentiate between SS groups and NA control groups. Thus, E1 was selected for further analysis. Due to shortage in the available amount of sera, SLE and RA samples were not subjected to IIF experiment.

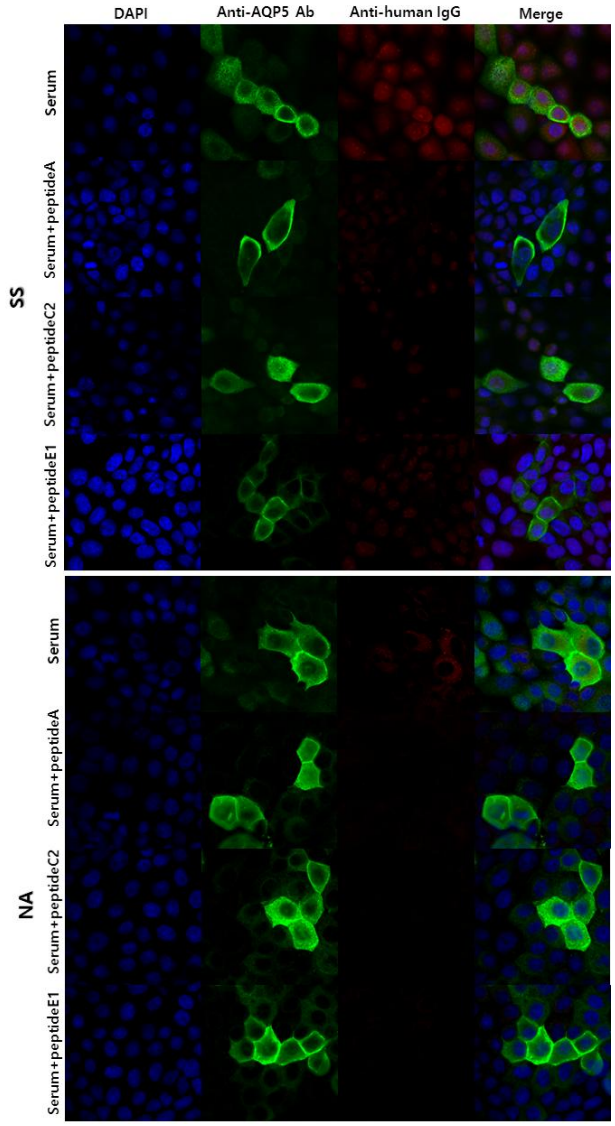
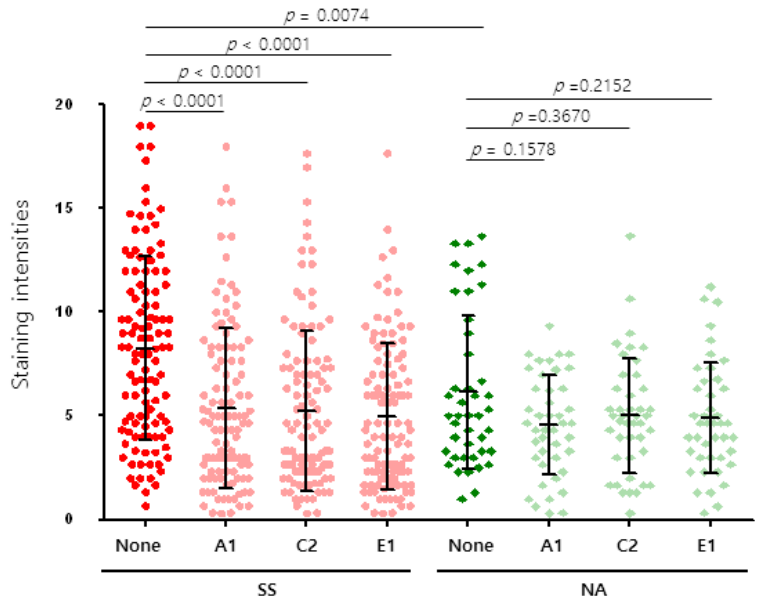


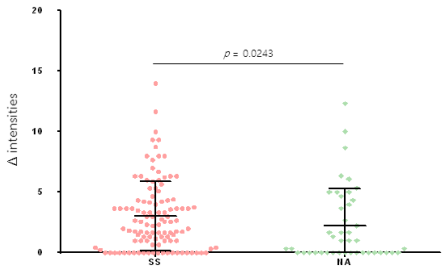
Figure 1

MDCK Cells over-expressing AQP5 were stained with anti-AQP5 antibodies and 1:100 dilution of either control and SS sera, followed by Alexa Fluor 488-conjugated Rat anti-mouse IgG(green) and Alexa Fluor 555-conjugated goat anti-human IgG (red).

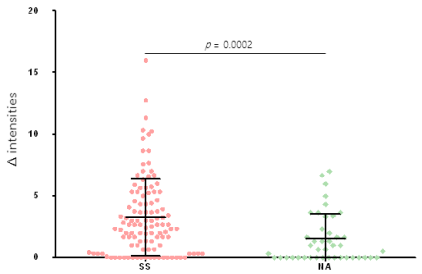
(a)


p = 0.0074
p < 0.0001
p < 0.0001
p < 0.0001
p =0.2152
p =0.3670
p = 0.1578
Staining intensities
20
15
10
5
0
None
A1
C2
E1
None
A1
C2
E1
SS
NA

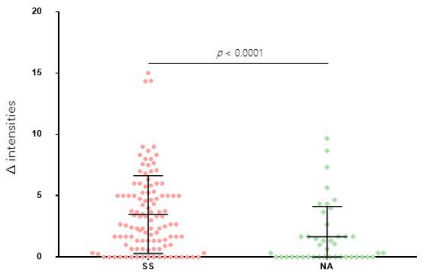
(b)


p = 0.0243
Δ intensities
20
15
10
5
0
SS
NA

(c)


p = 0.0002
Δ intensities
20
15
10
5
0
SS
NA

(d)


p < 0.0001
Δ intensities
20
15
10
5
0
SS
NA

(e) (f)

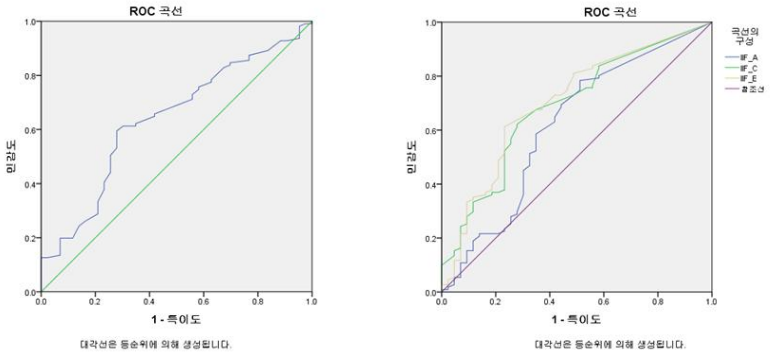


Figure 2

Higher levels of anti-AQP5 IgG were detected in the SS and NA sera by IIF.

(a) The intensities of the red signals for anti-AQP5 IgG were expressed by the magnitude of brightness that was reduced until the staining of AQP5 disappeared.

(b) Subtracted intensity of sera preincubated with peptide A

(c) Subtracted intensity of sera preincubated with peptide C2

(d) Subtracted intensity of sera preincubated with peptide E1

(e) A ROC curve for the levels of anti-AQP5 IgG is shown.

(f) A ROC curve for the levels of anti-AQP5 IgG preincubated with peptides is shown.

Table 4

Sensitivity and Specificity of IIF methods

	AUC (confidence interval)	*p*	Sensitivity	Specificity	Accuracy
Serum	0.638 (0.542-0.735)	0.008	0.595	0.721	0.658
Serum + peptide A	0.613 (0.508-0.718)	0.030	0.775	0.488	0.632
Serum + peptide C2	0.686 (0.594-0.779)	0.000	0.622	0.721	0.671
Serum + peptide E1	0.703 (0.609-0.796)	0.000	0.613	0.767	0.690

3.2 Higher levels of anti-AQP5 IgG were detected in the SS sera by ELISA

To establish the best screening method for anti-AQP5 autoantibodies, ELISA assay was developed, which systematically screened sera for the presence of IgG, specifically to epitope peptides (Fig 3). To maintain the structure of peptides in solution, peptides were bound to avidin-coated wells through biotin conjugated to the N-terminus of peptides (Fig 3). Using these assays, 224 sera were screened for IgG antibodies that bind to each epitope peptide A, C2, E1 and mixture of them. The levels of IgG to each antigen were significantly higher in SS group than in control groups using both 1:50 and 1:100 dilution of sera (Fig 4a, b). To compare the accuracy between ELISA and IIF, ROC analysis was performed only for the SICCA registry samples. As a result, the highest accuracy was obtained from peptide E1 when it was used at serum diluted 1:50 on the basis of

AUC (Table 5). Using the cutoff value from the ROC curve, 76 SS samples (68.5%) and 16 NA samples (37.2%) were positive for Peptide E1 at serum diluted 1:50, resulting in a sensitivity 0.856 and a specificity 0.558 (Fig 4c, Table 5).

Both SLE and RA samples were resulted in the highest accuracy in peptide A at serum diluted 1:100 based on AUC. Using the cutoff value from ROC curve, 99 SS samples (89.2%), 28 NA samples (65.1%), 9 SLE samples (25.7%), 4 RA samples (11.4%) were positive for peptide A at serum diluted 1:100, resulting in a sensitivity 0.892 and a specificity 0.637 (Fig 4d, Table 6, 7). However combining the three peptides did not improve the assay sensitivity nor specificity significantly (Table 5, 6).

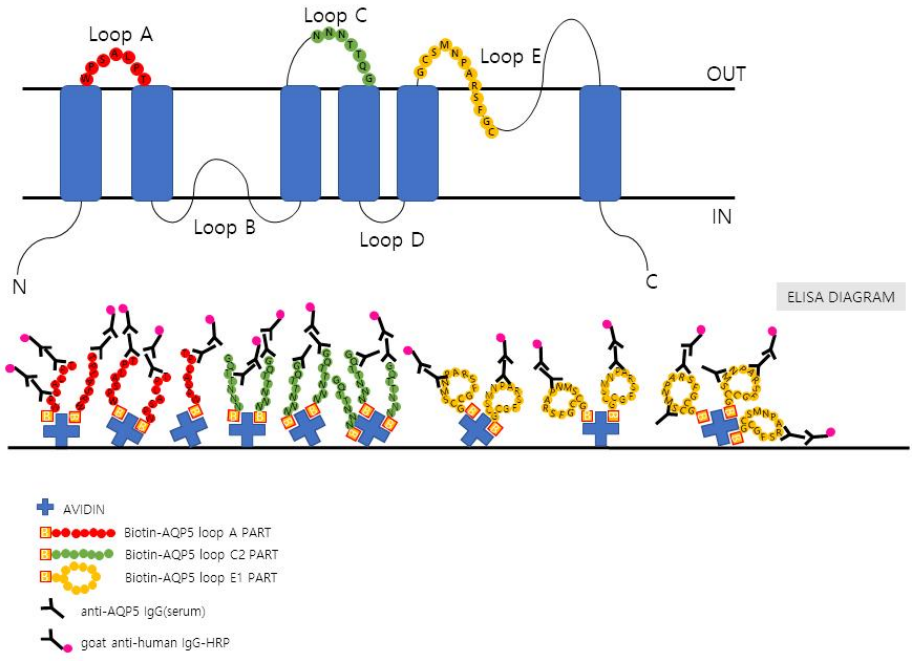
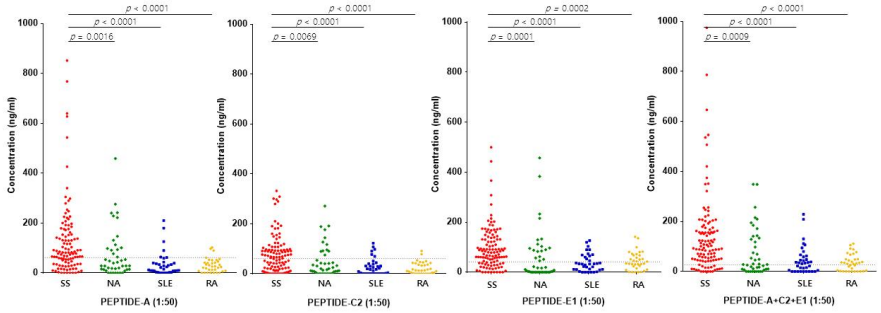


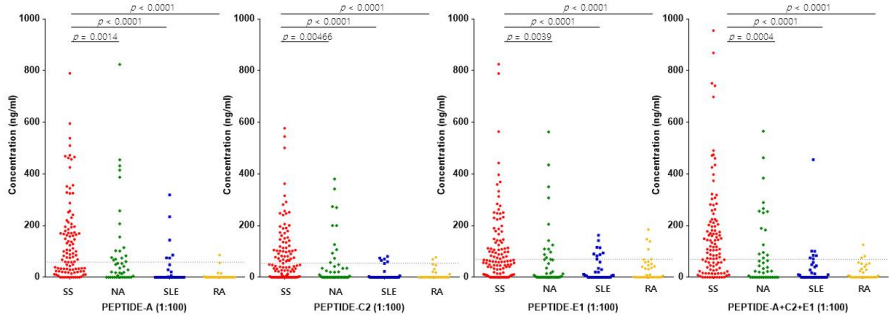
Figure 3

General scheme of ELISA assay and structure AQP5.

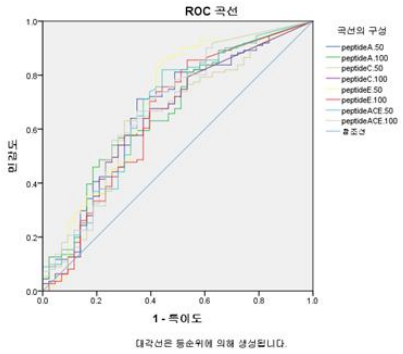
(a)


p < 0.0001
p < 0.0001
p = 0.0016
PEPTIDE-A (1:50)
p < 0.0001
p < 0.0001
p = 0.0069
PEPTIDE-C2 (1:50)
p = 0.0002
p < 0.0001
p = 0.0001
PEPTIDE-E1 (1:50)
p < 0.0001
p < 0.0001
p = 0.0009
PEPTIDE-A+C2+E1 (1:50)
Concentration (ng/ml)
SS
NA
SLE
RA

(b)


p < 0.0001
p < 0.0001
p = 0.0014
PEPTIDE-A (1:100)
p < 0.0001
p < 0.0001
p = 0.00466
PEPTIDE-C2 (1:100)
p < 0.0001
p < 0.0001
p = 0.0039
PEPTIDE-E1 (1:100)
p < 0.0001
p < 0.0001
p = 0.0004
PEPTIDE-A+C2+E1 (1:100)
Concentration (ng/ml)
SS
NA
SLE
RA

(c)


ROC 곡선
곡선의 구성
peptideA.50
peptideA.100
peptideC.50
peptideC.100
peptideE.50
peptideE.100
peptideACE.50
peptideACE.100
참조선
민감도
1 - 특이도
대각선은 동순위에 의해 생성됩니다.

(d)

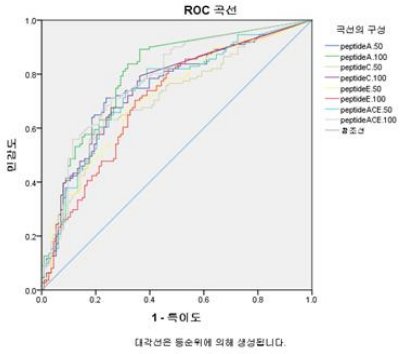

ROC 곡선
곡선의 구성
peptideA.50
peptideA.100
peptideC.50
peptideC.100
peptideE.50
peptideE.100
peptideACE.50
peptideACE.100
참조선
민감도
1 - 특이도
대각선은 동순위에 의해 생성됩니다.

Figure 4

IgG reactive with AQP5 epitope peptides determined by ELISA. Concentrations IgG reactive with A, C2, E1 or a mixture of A, C2 and E1 epitope peptides were measured by ELISA using sera diluted 1:50 (a) or 1:100 (b)

(c) ROC curves for the levels of anti-AQP5 IgG binding to different epitope peptides are shown.

(d) ROC curves for the levels of anti-AQP5 IgG binding to different epitope peptides are shown (SLE and RA patients included).

Table 5

Sensitivity and Specificity of ELISA in SICCA Cohort

Peptide	Sera dilution	AUC	Sensitivity	Specificity	Accuracy	*p*	confidence interval
A	1:50	0.665	0.712	0.651	0.6814	0.002	0.565-0.764
C2	1:50	0.640	0.631	0.698	0.66415	0.007	0.540-0.740
E1	**1:50**	**0.695**	**0.856**	**0.558**	**0.707**	**0.000**	**0.593-0.798**
ACE	1:50	0.671	0.820	0.558	0.689	0.001	0.570-0.772
A	1:100	0.664	0.811	0.465	0.638	0.002	0.567-0.762
C2	1:100	0.645	0.676	0.605	0.640	0.005	0.544-0.746
E1	1:100	0.649	0.739	0.581	0.660	0.004	0.544-0.753
ACE	1:100	0.682	0.667	0.651	0.659	0.000	0.583-0.781

Table 6

Sensitivity and Specificity of ELISA in SICCA, SLE and RA

Peptide	Sera dilution	AUC	Sensitivity	Specificity	Accuracy	*p*	confidence interval
A	1:50	0.748	0.712	0.761	0.736	0.000	0.683-0.813
C2	1:50	0.706	0.631	0.770	0.700	0.000	0.637-0.774
E1	1:50	0.714	0.685	0.673	0.679	0.000	0.646-0.781
ACE	1:50	0.741	0.820	0.611	0.715	0.000	0.675-0.806
A	**1:100**	**0.797**	**0.892**	**0.637**	**0.765**	**0.000**	**0.738-0.857**
C2	1:100	0.747	0.793	0.637	0.715	0.000	0.682-0.812
E1	1:100	0.705	0.820	0.531	0.675	0.000	0.637-0.773
ACE	1:100	0.783	0.712	0.743	0.728	0.000	0.722-0.843

3.3 Result of anti-AQP5 antibody assays in SICCA cohorts.

To compare the difference in screening methods, peptides of A, C2 and E1 were identified as potential substrates for anti-AQP5 autoantibodies detection. Among these several peptides resulted in significant antibody reactivity, peptide A and E1 were selected for further analysis. E1 resulted in sensitivity and specificity of 0.613 and 0.767 respectively in IIF assay while resulted in sensitivity and specificity of 0.856 and 0.558 respectively in ELISA assay with 1:50 diluted sera (Table 4, 5). Although these assays had similar effects to antibody detection, ELISA assay was confirmed to be more adequate method in calculating sensitivity and accuracy.

Next, the correlation between each method was analyzed. As seen, approximately 60% of the SS sera was reacted by IIF and modified IIF. Approximately 89% and 69% SS sera were reacted by ELISA using peptide A and E1 respectively (Table 7). In SS samples, the concordance rates between IIF and

ELISA remained approximately 50% throughout their comparisons: comparing with IIF to ELISA_A and ELISA_E and the other comparing with IIF_E to ELISA_A and ELISA_E. Although when comparing between ELISA and IIF resulted in a similar concordance rate, comparing between ELISA_A to ELISA_E and IIF to IIF_E resulted in a different concordance rate. In fact, ELISA showed a higher concordance rate than IIF. These findings confirm that the peptide E1 is the most adequate substrate for detecting AQP5 autoantibodies.

Table 7

Results of AQP5 antibody assays

	NA	SS	SLE	RA
IIF, N of positive (%)	12 (27.9%)	66 (59.5%)	-	-
IIF_E, N of positive (%)	10 (23.3%)	68 (61.3%)	-	-
ELISA_A, N of positive (%)	28 (65.1%)	99 (89.2%)	9 (25.7%)	4 (11.4%)
ELISA_E, N of positive (%)	16 (37.2%)	76 (68.5%)	10 (28.6%)	11 (31.4%)
Accordance between IIF vs. IIF_E	8/14 (57.1%)	51/83 (61.4%)	-	-
Accordance between IIF vs. ELISA_A	8/32 (25%)	56/109 (51.4%)	-	-
Accordance between IIF vs. ELISA_E	4/24 (16.7%)	46/93 (49.5%)	-	-
Accordance between IIF_E vs. ELISA_A	7/31 (22.6%)	59/108 (54.6%)	-	-
Accordance between IIF_E vs. ELISA_E	2/24 (8.3%)	48/96 (50%)	-	-
Accordance between ELISA_E vs. ELISA_A	12/32 (37.5%)	72/104 (69.2%)	5/13 (38.5%)	1/14 (7.1%)

* N of positive means higher than cut-off value

3.4 Association between anti-AQP5 autoantobodies and other SS diagnosis factor in SICCA cohort.

The presence of autoantibodies has always been considered as one of the criteria for the diagnosis of SS. According to previous studies, the presence of four autoantibodies (SSA, SSB, RF, ANA) indicated distinct clinical features of SS [10 , 11] . To further analyze the SS diagnosis, several different methods in detecting anti-AQP5 were compared with former clinical parameters. The clinical parameters included detecting only for SSA during serological tests. Thus, anti-AQP5 was compared specifically to SSA autoantibodies in this case.

In SICCA cohort that includes SS patients and NA subjects, anti-SSA autoantibodies were highly associated with given SS diagnostic factors (Table 8). Among the diverse methods detecting anti-AQP5 autoantibodies, the result by IIF using serum alone had association only with SSA and Dry eye. The

result by ELISA using peptide E1 had additional associations with SSA, ANA, FLS and Dry eye. When analyzing the comparison between SS and NA samples, NA samples did not reveal any correlation with SS diagnosis parameters (Data not shown). Contrastingly to NA patients, SS patients showed a significant association between, the autoantibodies detceted by ELISA using peptide E1 and ANA (Table 9).

Furthermore, the method of detecting anti-AQP5 autoantibodies was able to differentiate between SS and NA samples from SICCA Cohort. Yet, the relationship between anti AQP5 autoantibodies to SS diagnosis parameters was not significant enough to draw its conclusion.

Table 8

Chi-square between the seven disease associated diagnosis markers and clinical parameters in the SICCA cohort

		SSA			IIF			IIF_E			ELISA_A			ELISA_E		
		n (%)		*p*	n (%)		*p*	n (%)		*p*	n (%)		*p*	n (%)		*p*
		-	+		-	+		-	+		-	+		-	+	
SSA	-				34 (44.7)	18 (23.1)	**0.006**	35 (46.1)	17 (21.8)	0.002	16 (59.3)	36 (28.3)	**0.003**	30 (48.4)	22 (23.9)	**0.003**
	+				42 (55.3)	60 (76.9)		41 (53.9)	61 (78.2)		11 (40.7)	91 (71.7)		32 (51.6)	70 (76.1)	
SSB	-	52 (100)	41 (40.2)	**<0.0005**	46 (60.5)	47 (60.3)	1	49 (64.5)	44 (56.4)	0.327	19 (70.4)	74 (58.3)	0.284	43 (69.4)	50 (54.3)	0.067
	+	0 (0)	61 (59.8)		30 (39.5)	31 (39.7)		27 (35.5)	34 (43.6)		8 (29.6)	53 (41.7)		19 (30.6)	42 (45.7)	
RF	-	49 (94.2)	30 (29.4)	**<0.0005**	43 (56.6)	36 (46.2)	0.202	45 (59.2)	34 (43.6)	0.056	20 (74.1)	59 (46.5)	0.011	39 (62.9)	40 (43.5)	0.022
	+	3 (5.8)	72 (70.6)		33 (43.4)	42 (53.8)		31 (40.8)	44 (56.4)		7 (25.9)	68 (53.5)		23 (37.1)	52 (56.5)	
ANA	-	47 (90.4)	32 (31.4)	**<0.0005**	44 (57.9)	35 (44.9)	0.111	46 (60.5)	33 (42.3)	0.025	19 (70.4)	60 (47.2)	0.035	46 (74.2)	33 (35.9)	**<0.0005**
	+	5 (9.6)	70 (68.6)		32 (42.1)	43 (55.1)		30 (39.5)	45 (57.7)		8 (29.6)	67 (52.8)		16 (25.8)	59 (64.1)	
Dry mouth	-	30 (57.7)	25 (24.5)	**<0.0005**	27 (35.5)	28 (35.9)	1	32 (42.1)	23 (29.5)	0.130	9 (33.3)	46 (36.2)	0.829	25 (40.3)	30 (32.6)	0.392
	+	22 (42.3)	77 (75.5)		49 (64.5)	50 (64.1)		44 (57.9)	55 (70.5)		18 (66.7)	81 (63.8)		37 (59.7)	62 (67.4)	
FLS	-	43 (82.7)	31 (30.4)	**<0.0005**	43 (56.6)	31 (39.7)	0.053	45 (59.2)	29 (37.2)	0.010	19 (70.4)	55 (43.3)	0.012	41 (66.1)	33 (35.9)	**<0.0005**
	+	9 (17.3)	71 (69.6)		33 (43.4)	47 (60.3)		31 (40.8)	49 (62.8)		8 (29.6)	72 (56.7)		21 (33.9)	59 (64.1)	
Dry eye	-	43 (82.7)	6 (5.9)	**<0.0005**	35 (46.1)	14 (17.9)	**<0.0005**	36 (47.4)	13 (16.7)		15 (55.6)	34 (26.8)	**0.006**	28 (45.2)	21 (22.8)	**0.005**
	+	9 (17.3)	96 (94.1)		41 (53.9)	64 (82.1)		40 (52.6)	65 (83.3)		12 (44.4)	93 (73.2)		34 (54.8)	71 (77.2)	

Table 9

Chi-square between the seven disease associated diagnosis markers and clinical parameters in the SS

		SSA			IIF			IIF_E			ELISA_A			ELISA_E		
		n (%)		*p*	n (%)		*p*	n (%)		*p*	n (%)		*p*	n (%)		*p*
		-	+		-	+		-	+		-	+		-	+	
SSA	-				3 (6.7)	6 (10)	0.736	2 (4.7)	7 (10.3)	0.478	1 (8.3)	8 (8.1)	1	3 (8.6)	6 (7.9)	1
	+				42 (93.3)	60 (90)		41 (95.3)	61 (89.7)		11 (91.7)	91 (91.9)		32 (91.4)	70 (92.1)	
SSB	-	9 (100)	41 (40.2)	<0.0005	15 (33.3)	35 (53)	0.052	16 (37.2)	34 (50)	0.241	4 (33.3)	46 (46.5)	0.542	16 (45.7)	34 (44.7)	1
	+	0 (0)	61 (59.8)		30 (66.7)	31 (47)		27 (62.8)	34 (50)		8 (66.7)	53 (53.5)		19 (54.3)	42 (55.3)	
RF	-	6 (66.7)	30 (29.4)	0.056	12 (26.7)	24 (36.4)	0.309	12 (27.9)	24 (35.3)	0.533	5 (41.7)	31 (31.3)	0.521	12 (34.3)	24 (31.6)	0.829
	+	3 (33.3)	72 (70.6)		33 (73.3)	42 (63.6)		31 (72.1)	44 (64.7)		7 (58.3)	68 (68.7)		23 (65.7)	52 (68.4)	
ANA	-	4 (44.4)	32 (31.4)	0.468	13 (28.9)	23 (34.8)	0.542	13 (30.2)	23 (33.8)	0.835	4 (33.3)	32 (32.3)	1	19 (54.3)	17 (22.4)	**0.002**
	+	5 (55.6)	70 (68.6)		32 (71.1)	43 (65.2)		30 (69.8)	45 (66.2)		8 (66.7)	67 (67.6)		16 (45.7)	59 (77.6)	
Dry mouth	-	2 (22.2)	25 (24.5)	1	8 (17.8)	19 (28.8)	0.260	10 (30.3)	17 (25)	1	2 (16.7)	25 (25.3)	0.727	6 (17.1)	21 (27.6)	0.341
	+	7 (77.8)	77 (75.5)		37 (82.2)	47 (71.2)		33 (76.7)	51 (75)		10 (83.3)	74 (74.7)		29 (82.9)	55 (72.4)	
FLS	-	0 (0)	31 (30.4)	0.60	12 (26.7)	19 (28.8)	0.833	12 (27.9)	19 (27.9)	1	4 (33.3)	27 (27.3)	0.736	14 (40)	17 (22.4)	0.069
	+	9 (100)	71 (69.6)		33 (73.3)	47 (71.2)		31 (72.1)	49 (72.1)		8 (66.7)	72 (72.7)		21 (60)	59 (77.6)	
Dry eye	-	0 (0)	6 (5.9)	1	4 (8.9)	2 (3)	0.220	3 (7)	3 (4.4)	0.675	0 (0)	6 (6)	1	1 (2.9)	5 (6.6)	0.663
	+	9 (100)	96 (94.1)		41 (91.1)	64 (97)		40 (93)	65 (95.6)		12 (100)	93 (94)		34 (97.1)	71 (93.4)	

4. Discussion

Previous study discovered the presence of anti-AQP5 autoantibodies in the sera of both healthy control subjects and SS patients with sensitivity of 0.73 and a specificity of 0.68. Since the control group represented the healthy population in previous studies, there was no need to measure the saliva flow rate, thus resulting in a higher sensitivity level. On the other hand, in this experiment, the control group represented the non-autoimmune disease patients in SICCA cohort, whose saliva flow rates had decreased, hence resulting in a lowered sensitivity level. In this study, anti-AQP5 autoantibodies from SS group recognized multiple extracellular epitopes more frequently than anti-AQP5 from control group. In order to generate appropriate screening methods, 154 sera by IIF assay and 224 sera by modified ELISA assay were screened. As a result, the finding

suggested that the serums from 111 SS patients from SICCA cohort contained IgG that bind to extracellular epitopes of AQP5.

From previous studies, three different peptides were already chosen for screening assays: peptide A, C2, E1 (Table 1). These assays do not contain real autoantigens, but rather measure the reactivity of SS specific antibody to mimotopes of AQP5 origin. Previous results concluded that the presence of anti-AQP5 autoantibodies was correlated with unstimulated salivary flow rate. However in this study the relationship between anti-AQP5 and salivary flow rate was not recognized. There was no significant association between anti-AQP5 autoantibodies and SS diagnosis factor. Only a weak association was discovered, specifically between anti-AQP5 to ANA.

IIF is one of the most commonly used techniques for antibody detection. However, due to its long duration time in

IIF's screening method, modified ELISA can become more effective than IIF. The serologic tests used to diagnose Sjogren's syndrome are not specific to Sjogren's syndrome and are frequently detected in other autoimmune diseases. Thus, to diagnose SS accurately, the more SS-specific serological markers were required. Using the modified ELISA assay, there were more anti-AQP5 autoantibodies discovered in SS patients' sera compared to in other autoimmune diseases. Nevertheless, the presence of AQP5 autoantibodies did not have a significant association with given SS diagnosis factors. In fact, this study concludes that anti-AQP5 autoantibodies are not as efficient as SSA, the current diagnostic marker. Thus, further studies are needed to conclude the comparison in performance of AQP5 maker to existing SS diagnose marker. Meanwhile, this study may be contributed to further development in improving diagnostic assays for treatment of SS. These findings suggest the need

to further investigate on presence of antibodies to AQP5 in larger cohorts of patients with different autoimmune diseases. It is also necessary to observe whether NA patients in SICCA cohort can develop into SS later in their stages.

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항-아쿠아포린5 자가항체의 쇼그렌 증후군 바이오마커로서의 유용성 검증

1. 목 적

쇼그렌 증후군은 구강건조증과 안구건조증을 주 증상으로 하는 질병으로 자가면역반응에 의해 침샘과 눈물샘의 기능저하가 발생한다. 현재 쇼그렌 증후군의 진단 기준에는 질병의 원인을 설명하고 질병의 활성을 반영하는 진단마커가 없다. 아쿠아포린5는 침샘과 눈물샘의 선세포(acinar cell) 및 도관세포(duct cell)에 발현하는 물통로단백질로 물분자가 지질성분인 세포막을 통과하는 통로를 제공한다. 아쿠아포린5가 결핍된 생쥐는 타액분비가 크게 감소하는 것이 보고된 바가 있다. 이전 연구에서 쇼그렌 증후군 환자 혈청에서 항-아쿠아포린5 자가항체를 발견하였고 이는 낮은 비자극 타액분비율과 연관이 있었다. 항-아쿠아포린5 자가항체의 바이오마커로서의 유용성을 검증하기 위해서는 독립적인 쇼그렌 증후군 환자 코호트에서 관찰이 이루어져

야 하고 선행연구에는 포함되지 않은 질환 대조군으로 다른 자가면역 질환 환자에서 항-아쿠아포린5 자가항체의 존재 여부를 확인해야 한다. 본 연구의 목적은 질환 대조군을 포함한 단면연구에서 항-아쿠아포린5 자가항체의 특이성과 민감도를 산출하고 항-아쿠아포린5 자가항체와 쇼그렌 증후군 임상지표 간의 연관성을 조사하는 것이다. 또한, 항-아쿠아포린5 자가항체 검출에 있어서 간접면역형광법과 새로이 개발된 효소면역분석법의 효율을 비교하고자 한다.

2. 방 법

쇼그렌증후군 환자(n=111)의 혈청과 질환 대조군으로 전신성 루프스 환자(n=35)와 류마티스 관절염 환자(n=35)의 혈청, 비 자가면역질환 환자의 혈청(n=43)을 이용하여 실험을 진행하였다. 아쿠아포린5의 세포 외 부분인 Loop A, Loop C, Loop E 서열로 비오틴을 붙인 펩타이드를 제작하여 이에 결합하는 환자 혈청의 IgG의 양을 효소면역분석법를 통해 측정하였다. 또한 세포기반 간접면역형광법을 사용하여 자가항체를 검색하였다. 아쿠아포린5를 과발현하는 MDCK세포와 발현하지 않는 세포를 섞어 커버슬립에 배양 후 배양된 세포를 고정하

고 항원의 구조를 회복 시키는 과정을 거쳤다. 항-아쿠아포린5 항체와 실험군 또는 대조군 혈청으로 염색 후 각각의 일차항체를 다른 색의 형광이 부착된 이차항체로 염색하여 공초점주사현미경으로 관찰하였다. 항-아쿠아포린5 항체로 염색된 세포를 무작위로 선택해 사진을 찍은 후 사람 IgG에 의한 염색 양상을 사진 찍어 염색된 형광강도를 측정하였다.

실험군과 대조군에서 얻어진 항 아쿠아포린-5 자가항체 값에 대해 receiver operating characterisitic (ROC) 분석을 통해 쇼그렌 증후군에 대한 민감도와 특이도를 산출하고 쇼그렌 증후군 환자군 및 건조증 대조군에서 항-아쿠아포린5 자가 항체의 존재와 타액분비율 간의 연관성을 분석하고 항-아쿠아포린5 자가항체 존재와 기타 임상지표나 검사결과와의 연관성을 분석하였다.

3. 결 과

그동안 쇼그렌 증후군의 진단에 사용되는 혈청학적 검사 (항-SSA, 항-SSB, 항핵 자가항체)는 쇼그렌 증후군 특이적이지 않고 다른

자가면역질환에서도 빈번하게 검출되기 때문에 추가 검사를 필요로 한다. 본 연구에서는 새로이 개발된 효소면역분석법을 통해서 자가항체를 측정하였고, 간접면역형광법으로 자가항체를 측정하였다.

3. 결 론

아쿠아포린5 자가항체를 통해서 구강 건조증 환자를 포함하는 비 자가면역 대조군과 쇼그렌 증후군 환자를 구분하기에는 부족함을 느끼지만 다른 자가면역 질환 환자와는 특이성을 보였다.

아쿠아포린5 자가항체가 기존의 쇼그렌 증후군 진단 마커를 대체 할 수는 없지만, 다른 자가면역질환과 구분할 수 있는 바이오마커로 활용할 수 있을 것이다.

주요어 : 쇼그렌 증후군, 아쿠아포린5, 자가항체, 바이오마커

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