Doctoral Thesis in Medicine

Characterization of salivary proteins identified as potential biomarkers for systemic lupus erythematosus through proteomic analysis

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-ABSTRACT-

Characterization of salivary proteins identified as potential biomarkers for systemic lupus erythematosus through proteomic analysis

Objectives: Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by pathogenic autoantibodies and uncontrolled inflammatory response. There are few reliable biomarkers available for diagnosis and monitoring the disease. We tried to find and characterize specific protein components in saliva of patients with SLE for their use as biomarkers in future.

Material and Methods: Salivary proteins were prepared from 11 samples from patients with SLE and healthy controls (HC), and were subjected to 2-dimensional gel electrophoresis (2-DE). The spots with greater than 2 fold change in intensity were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MS) analysis. The relative and absolute amounts of the several candidate proteins in saliva of patients with SLE and rheumatoid arthritis (RA) and HC were analyzed using western blotting and enzyme-linked immunosorbent assay.

Results: Proteomic analysis using 2-DE and MS identified 20 differentially expressed protein spots in the saliva of SLE patients compared in that of HC. Among them, proteins with more than two-fold differences in expression were found as immunoglobulin gamma-3 chain C (IGHG3), immunoglobulin alpha-1 chain C region, protein S100, lactotransferrin, leukemia-associated protein 7, and 8-oxoguanine DNA glycosylase. salivary IGHG3 levels were increased in SLE (3.0 ± 1.4 pg/mL) compared to those in RA (1.5 ± 0.7 pg/mL, $p < 0.001$) or HC (1.2 ± 0.5 pg/mL, $p < 0.001$), and salivary lactotransferrin levels were increased in SLE (5.0 ± 1.7 pg/mL) compared to those in RA (3.1 ± 1.6 pg/mL, $p < 0.001$) or HC (2.3 ± 1.7 pg/mL, $p < 0.001$). Salivary lactotransferrin levels were correlated with complement 3 (r = 0.27, $p = 0.01$) and complement 4 (r = 0.27, $p = 0.02$). The follow up study, the patient with increased salivary IGHG3 had significantly different in changes of hemoglobin (−0.85 ± 0.87 vs 0.42 ± 0.99 /μL, $p = 0.02$) and changes of complement 3 (−7.67 ± 14.15 vs 7.0 ± 9.34, $p = 0.02$) compared to those not. In addition, the patients with increased salivary lactotransferrin had significantly different in changes of ESR (−2.0 ± 4.6 vs 8.4 ± 9.56, $p = 0.02$) compared to those not, and salivary lactotransferrin levels negatively correlated with complement 3 levels (r = −0.5, $p= 0.02$).

Conclusion: Salivary IGHG3 and lactotransferrin levels were significantly increased in patients with SLE compared to those in patients with RA or HC, and could be used as potential biomarkers of SLE.

Key Indexing Terms: Systemic lupus erythematosus, saliva, immunoglobulin gamma-3 chain C, lactotransferrin

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by the presence of pathogenic autoantibodies and uncontrolled inflammatory response leading to diverse clinical manifestations (Lisnevskaia et al., 2014). The disease status of SLE including manifestations and disease activity is changing over time, however, very little information is avaliable about further progression of SLE. Anti-dsDNA antibody and complements are being used as diagnostic marker of SLE (Lloyd and Schur, 1981; Linnik et al., 2005; Pan et al., 2014). Anti-dsDNA antibodies induce apoptosis by engaging intracellular DNA, and cross-react with α-actin present in the glomeruli of SLE patients with renal disease (Isenberg et al., 2007). While anti-dsDNA antibody is found in 60-83% of SLE patients, studies investigating association of anti-dsDNA with disease flares within a few weeks or months, have reported contradictory results (Kavanaugh and Solomon, 2002). Complement deficiencies in classic pathway have been known to confer susceptibility to SLE, and the activation of complement system leads to the deficiency during SLE flares (Truedsson et al., 2007). However, complement level is not a reliable indicator of active SLE as their levels show considerable variation, do not reflect the consumption in tissue, nor do they indicate the presence of autoantibodies to complement proteins (Walport, 2002). Serologically active clinically quiescent (SACQ) SLE patients have a high titer of anti-dsDNA antibodies and low complement levels with no or low disease activity (Gladman et al., 2003; Steiman et al., 2015). Change in the expression levels of these

biomarkers are often non-specific and thus not reliable indicators of SLE progression. Until now, the studies looking for a biomarker for SLE have carried out, resulting in various candidate materials including cytokines, immune cells, autoantibodies, or genetic markers.

Proteomics is used to detect protein or peptide present in body fluids or tissues. Two-dimensional gel electrophoresis (2-DE) with mass spectroscopy (MS) is a reliable method for identification and characterization of proteins constituents in tissues and body fluids including blood, urine, or saliva, with some advantages of high sensitivity and specificity (Huang et al., 2009; Leitner and Lindner, 2009). Thus, 2-DE/MS has been exploited to discover novel biomarkers for diagnosis or disease monitoring in rheumatic diseases (Goeb et al., 2009; Kazemipour et al., 2015).

Saliva is a body fluid that can be obtained repeatedly in a non-invasive, risk-free manner (Kaufman and Lamster, 2002). A large part of proteins in saliva are derived from salivary glands, and some portions such as proteins are originated from blood. The similar compositions of saliva and blood make saliva samples suitable for detecting not only oral diseases but also systemic diseases (Lamy and Mau, 2012). Proteomic analysis using MS indicated that the presence of acid in saliva samples and gender differences affect the results of salivary proteome research (Xiao et al., 2017). Salivary biomarkers have proved promising in Sjogren's syndrome (SS), an autoimmune disorder involving salivary gland and sharing common characteristics with SLE (Tzioufas and Kapsogeorgou, 2015). Proteomic analysis demonstrated a difference in salivary composition between

primary and secondary SS (Baldini et al., 2011). When the levels of salivary α-enolase, β-2 microglobulin and immunoglobulin k light chain were compared between patients with SS, other autoimmune diseases with sicca symptoms (typical manifestations in SS such as dry mouth or dry eye) and healthy controls (HC). Hu S et al. identified salivary autoantibodies including anti-SSA and anti-SSB that could serve as biomarkers of SS (Hu et al., 2011).

So far, very few studies have analyzed salivary samples from patients with SLE. We analyzed saliva samples by 2-DE with MS to find a differential composition of salivary peptides and concentrations of salivary proteins in SLE patients compared to that in HC or patients with rheumatoid arthritis (RA). The clinical relevance of the differentially expressed peptides and proteins were analyzed in patients with SLE.

II. MATERIALS AND METHODS

A. Study participants

This study was conducted in two steps. In the first step, 2-DE proteome analysis was performed to analyze differences in salivary protein composition between SLE patients and HC. In the next step, differential proteins identified by 2-DE were validated by western blotting and ELISA. Clinical characteristics of patients with SLE and HC in the first stage, and patients with SLE, RA and HC in the second stage, are shown in Table 1.

There were two participant groups in this study- the first step of 2-DE proteomic analysis was conducted with 11 samples from patients with SLE and 11 samples from HC. The second step was performed with a large sample of patients with SLE, RA, and HC, validating the concentration of proteins in the three groups. All enrolled patients with SLE, met the revised American College of Rheumatology classification criteria (Tan et al., 1982). Age and sex-matched patients with RA and HC who had no history of autoimmune or inflammatory disorders participated. Medical histories and clinical manifestations were collected by a chart review and blood test results including complete blood count, erythrocyte sedimentation rate (ESR), anti-nuclear antibody, complements 3 and 4, and anti-dsDNA antibody levels, were obtained.

In the second step, to validate the concentrations of candidate salivary proteins, 95 patients with SLE and 58 patients with RA were included, along with 64 HC (Data was not shown). Patients with RA were enrolled as a disease control group

to analyze if specific proteins were differentially expressed between SLE and RA, which are both chronic autoimmune diseases. In the second step which validated the proteins, the mean age of patients with SLE, RA, and HC were 39.7 ± 9.9, 41.0 ± 7.8, and 38.7 ± 7.4 years, respectively, and not different significantly. Among patients with SLE, 34 (35.8%) had positive levels of anti-dsDNA antibody, 27 (30.7%) had muco-cutaneous involvement, 33 (37.5%) had arthritis, 31 (35.2%) had nephritis, and mean systemic lupus erythematosus disease activity index (SLEDAI) was 4.2 ± 4.4. Among patients with RA, 45 (77.6%) had rheumatoid factors and their mean disease activity score 28 (DAS28) was 3.3 ± 1.15.

Table 1. Clinical characteristics of patients with systemic lupus erythematosus and rheumatoid arthritis, and healthy controls.

	SLE	RA	HC
Number	105	58	88
Age, years	39.3 ± 10.1	41.0 ± 7.8	37.5 ± 7.7
Sex (F/M)	97/8	51/7	82/6
Leukocyte, /μL	5,165.4 ± 2364.9		
Hemoglobin, /μL	12.2 ± 2.4		

Platelet, x 10^3/μL	221.8 ± 76.1
Lymphocyte, /μL	1482.9 ± 691.9
ESR, mm/h	16.4 ± 18.0
Complement 3, mg/dL	85.2 ± 26.9
Complement 4, mg/dL	18.6 ± 8.9
Anti-dsDNA Ab (+), n (%)	41 (39.0)
Rheumatoid factor (+), n (%)	45 (77.6)
Mucocutaneous involvement, n (%)	35 (33.3)
Arthritis, n (%)	37 (35.2)
Nephritis, n (%)	33 (31.4)
Serositis, n (%)	4 (3.8)
Hematologic involvement, n (%)	20 (19.0)
SLEDAI	4.3 ± 4.3

DAS-28	3.3 ± 1.15

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HC, healthy controls; ESR, erythrocyte sedimentation rate; dsDNA, double-strand deoxyribonucleic acid; Ab, antibody; SLEDAI, systemic lupus erythematosus disease activity index; DAS28, disease activity score including 28 joints. All values presented as number (%) or mean ± standard deviation.

B. Saliva sample collection

Saliva was collected from all participants between 9:00 and 11:00 am because salivary proteins are known to show diurnal variations. Subjects were not allowed to eat, drink, smoke, or perform oral hygiene procedures for at least 1 hour prior to the sample collection. Saliva was collected for 5 minutes after having the subjects rinse their mouth with water (Navazesh, 1993). No agents were used to stimulate saliva secretion. Subjects were asked to keep their mouths closed and expectorate saliva into a tube once per minute.

Each saliva sample was immediately treated with protease inhibitors to preserve the integrity of the protein constituents and centrifuged at 3,000 rpm for 15 minutes at 4°C. After removing the clear supernatant, the samples were aliquoted and stored at –20°C until further use. The samples from 11 patients with SLE or 11 HC were pooled equally, to remove intra-class variations that were detected between patients in 2-DE analyses. 1 ml of sample was concentrated 10 times by Amicon-3K, 14000 g, for 20 minutes at 4°C. Salivary samples were mixed with 500 μL of TCA/acetone (90%v/v)-dithiothreitol (DTT) mixture, and precipitated overnight at

-20°C. After centrifugation at 10,000 rpm, 10°C for 10 minutes, the supernatant was collected. Samples were pretreated with 250 μL of rehydration buffer and followed by centrifugation at 10,000 rpm for 10 minutes at 10°C to remove any insoluble material. Protein concentration of the samples was estimated by Bradford protein assay methods (Bio-Rad, Hercules, CA, USA).

C. Liquid chromatography tandem mass spectrometry (LC-MS)

1. Separation and in-gel protein digestion

2-DE proteomic analysis was performed on the saliva samples from 11 patients with SLE and 11 HC, which separated the sample into numerous spots with different concentrations. Twenty spots were selected and analyzed by liquid chromatography tandem-mass spectrometry (LC-MS) to analyze proteins with high specificity (Shushan, 2010). Gel pieces containing protein spots were destained, reduced, alkylated and digested with modified sequencing grade trypsin (Sigma, MO, USA), as previously described (Park et al., 2015). Peptide mixtures were lyophilized and stored at -80°C for further LC/MS analysis.

2. Mass spectrometry

Each sample was resuspended in 0.1% trifluoroacetic acid (TFA) and injected in a Zorbox 300SB-C18 75 μm i.d × 15 cm column (Agilent, Santa Clara, CA, USA) via trap column. The peptides were separated in an acetonitrile gradient with at a flow rate of 200 nl/min with an UltiMate 3000 nano HPLC system (Dionex, Sunnyvale, CA, USA) and applied on-line to a LTQ (Thermo Fisher, Waltham,

MA, USA) ion-trap mass spectrometer. The mobile phase gradient was started with increase from 5 to 40% buffer within 110 minutes, thereafter increasing it to 80% buffer in 1 minute, followed by 80% buffer isocratic for 15 minutes. The main working liquid-junction ESI ion source parameters were as follows: ion spray voltage 1.6 kV, capillary voltage 24 V, and capillary temperature 200°C. Spectra were collected in full scan using dynamic exclusion criteria. LC-MS runs were analyzed using the software DeCyder MS (version 2.0; GE Healthcare, Uppsala, Sweden) (Khang et al., 2014). Peptide peaks were detected with an average peak width of 1 minute and matched with a mass accuracy of at least 0.6 Da and a maximum time window of 4 minutes. Next, the abundance of individual peptides in the respective gradient fraction was calculated by peak integration.

3. Protein identification using human protein database

Data were manually inspected and overlapping peaks were discarded. The threshold level for differentially expressed proteins was defined as at least 2-fold increase or decrease in spot intensity that was significant. MS spectra that correlated to peptide peaks were searched against the Uniprot Human database using the MASCOTTM v2.3 (Matrix Science, London, UK). For quantitative protein profiling, only proteins identified by multiple peptides with significant MASCOT score ($p < 0.05$) were considered.

D. Western blotting analysis

Immunoglobulin gamma-3 chain C region (IGHG3), immunoglobulin alpha-1 chain C region (IGHA1), protein S100-A8 (S100A8), lactotransferrin, leukemia-associated protein 7, and 8-oxoguanine DNA glycosylase (OGG1) were analyzed by western blot with rabbit anti-Human IGHG3 polyclonal (MBS248789, MyBiosource, San Diego, CA, USA), rabbit anti-Human IGHA1 polyclonal (MBS9206028, MyBiosource), and rabbit anti-Human, rat S100A8 polyclonal (MBS127619, MyBiosource), mouse anti-Human lactoferrin monoclonal (ab10110, Abcam, Cambridge, UK), rabbit anti-Human OGG1 polyclonal (NB100-106, Novusbio, Centennial, CO, USA). Each extract was loaded on 10% (for IGHG3) and 15% (for IGHA1, S100/A8) acrylamide gel, and transferred to PVDF membrane. The membranes were incubated with blocking antibody (goat anti-rabbit antibody, A120-101P, Bethyl Laboratories, Mongomery, TX, USA) diluted at 1:10,000 (IGHG3, S100/A8) and 1:5,000 (IGHA1). All the tests were carried out in triplicate, and each protein determined by optical density of specific immunoreactive bands.

E. Enzyme-linked immunosorbent assay

The levels of salivary IGHG3 and lactoferrin were measured in patients with SLE and RA, and HC by enzyme-linked immunosorbent assay (ELISA). Human IGHG3 ELISA kit (ab137981, Abcam) and human lactoferrin ELISA kit (ab108882, Abcam) were used to perform experiment in duplicates in accordance with the manufacturer's instructions.

F. Statistical analysis

Difference in expressions of salivary IGHG3, IGHA1, S100A8, lactoferrin, leukemia-associated protein 7, and OGG1 as determined by western blotting and concentrations of salivary IGHG3 and lactoferrin measured by ELISA, in patients with SLE and RA, and HC, were compared by a two-sample Wilcoxon rank-sum (Mann-Whitney) test. Correlations between levels of salivary IGHG3, lactoferrin and clinical features in patients with SLE were determined using the Spearman rank correlation technique. Area under the curve (AUC), sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR–) for SLE were calculated from the receiver operating characteristics (ROC) curve of salivary proteins. LR+ is defined as sensitivity/(1-specificity), and LR– is defined as (1-sensitivity)/ specificity. A p-values < 0.05 were considered statistically significant. All computations were performed by using the Statistical Package for the Social Sciences version 22.0 (IBS Corp, Armonk, NY, USA) and MedCalc version 14.10.2 (MedCalc software, Ostend, Belgium).

G. Ethical consideration

All subjects provided the informed consent, which has been regulated by the institutional review of board of our hospital (BMR-SMP-13-199).

III. RESULTS

A. Salivary protein identification

1. Composition of salivary peptides in patients with SLE

2-DE analysis identified differential expression pattern of salivary protein between patients with SLE and HC (Figure 1). Twenty separated spots showed different concentrations between the two gels. From LC-MS analysis and quantitative protein profiling, each protein name, fold change and ANOVA scale between two groups, and peptide sequences were determined (Table 2). Among 20 spots showing fold change values higher than 1.5, 4 spots were identified as alpha-amylases, and 2 spots were identified as protein S100, 8-oxoguanine DNA glycosylase (OGG1) and protein S100/A8. Other spots were identified as immunoglobulin gamma-3 chain C region (IGHG3), immunoglobulin alpha-1 chain C region (IGHA1), lactotransferrin, leukemia-associated protein 7, prolactin-inducible protein immunoglobulin gamma-1 chain C region, cystatin-D, and Zinc-alpha-2-glycoprotein.

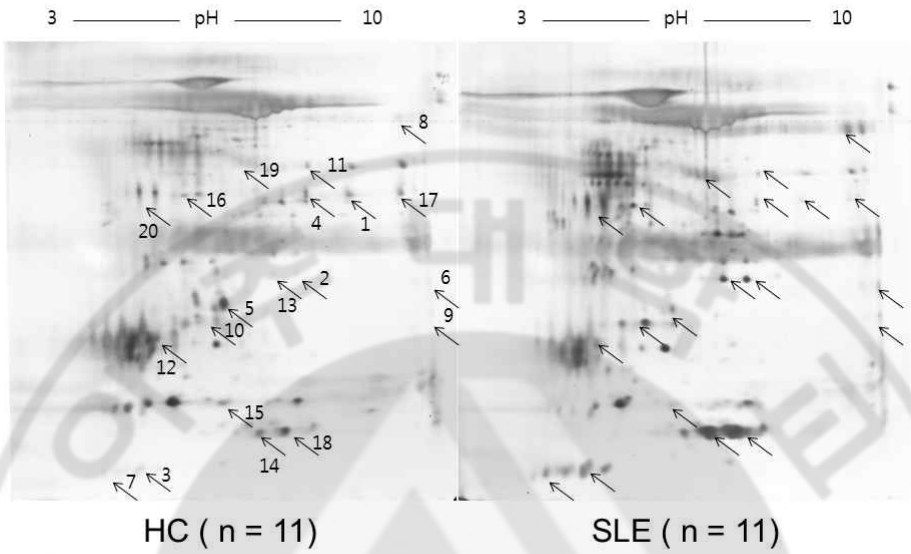


Figure 1. Representative 2D map of patients with systemic lupus erythematosus (SLE) (A) and healthy controls (HC) (B). A total of 150 μg of whole saliva proteins was separated by 2D using 18 cm pH 3 to 10 L strip (IPG) and 12.5% SDS-PAGE. Proteins were detected by silver staining. The map was analysed by Image master 2D platinum software. Spot numbers indicate all the proteins identified by MS and refer to the number reported in Tables 2.

Table 2. List of salivary peptides with different concentration on 2D electrophoresis analysis between patients with systemic lupus erythematosus and health controls

Spot	Increased protein name	Fold of variation SLE vs HC	Anova, *p*-value

2	Ig gamma-3 chain C region	4.101	0.000158
3	Protein S100	3.894	0.0045
6	8-oxoguanine DNA glycosylase	3.516	0.00637
7	Protein S100	2.947	2.48E-08
8	Lactotransferrin	2.827	4.06E-07
9	8-oxoguanine DNA glycosylase	2.794	0.00653
13	Ig gamma-1 chain C region	1.869	0.0323
14	Protein S100-A8	1.722	0.000208
16	Zinc-alpha-2-glycoprotein	1.629	0.00215
18	Protein S100-A8	1.512	0.0346
19	Carbonic anhydrase 6	1.425	0.0275
20	BPI fold-containing family A member 2	1.356	0.00212

Spot	**Decreased protein name**	**Fold of variation SLE vs HC**	**Anova, p-value**
1	Alpha-amylase 1	-4.228	0.000691
4	Alpha-amylase 1	-3.676	0.0372
5	Ig alpha-1 chain C region	-3.532	0.00511

10	Leukemia-associated protein 7	-2.176	0.0178
11	Alpha-amylase 1	-1.967	0.0402
12	Prolactin-inducible protein	-1.903	0.004
15	Cystatin-D	-1.638	0.00478
17	Alpha-amylase 1	-1.580	0.0319

SLE, systemic lupus erythematosus; HC, healthy controls; deoxyribonucleic acid

2. Differential expressions of salivary proteins in patients with SLE and RA, and HC

Western blot analysis was carried out with 14 samples of patients with SLE, 12 samples of patients with RA, and 8 samples of HC that were age matched (Figure 2). Of the patients with SLE, disease duration was 6.6 ± 5.7 years, SLEDAI was 8.5 ± 4.6, C3 levels was 59.3 ± 25.8 mg/dL C4 levels was 9.4 ± 4.5 mg/dL, and ESR was 31.4 ± 24.5 mm/h. Among the patients with SLE, 10 patients possessed anti-dsDNA antibody, 5 patients had muco-cutaneous symptom, 5 patients had arthritis, 6 patients had active lupus nephritis, and 5 patients had hematologic involvement. Of the 8 patients with RA, mean disease duration was 5.8 ± 7.6 years, and mean DAS28 was 2.49 ± 1.23.

Salivary IGHG3 and lactotransferrin were highly expressed in patients with SLE compared to patients with RA or HC ($p < 0.01$), as determined by western blot analysis. Salivary IGHA1 was not expressed different in patients with SLE compared to those with RA ($p = 0.09$) or HC ($p = 0.09$). Expressions of salivary

S100A8 and leukemia-associated protein 7 were not different in patients with SLE compared in those with RA (p = 0.2 and p = 0.62) and in HC (p = 0.39 and p = 0.48). Expression of salivary OGG1 was increased in patients with RA compared with that in patients with SLE (p = 0.02) or HC (p = 0.02).

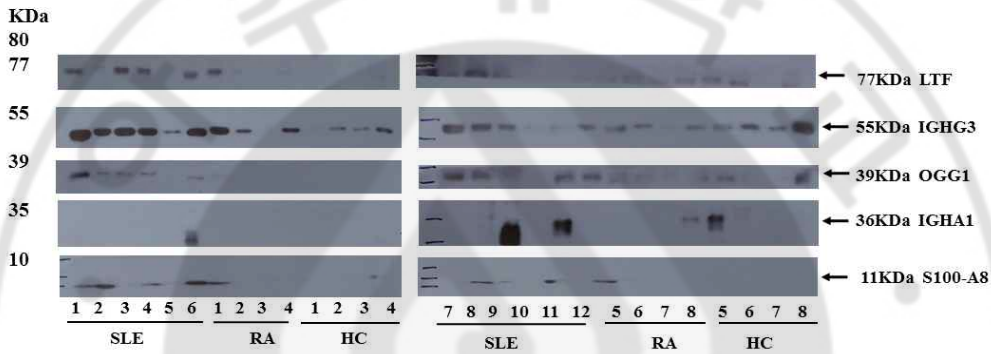


Figure 2. Expressions of salivary proteins between patients with systemic lupus erythematosus and rheumatoid arthritis, and health controls. LTF, lactotransferrin; IGHG3, immunoglobulin gamma-3 chain C region; OGG1, 8-oxoguanine DNA glycosylase; IGHA1, immunoglobulin alpha-1 chain C region

B. Validation of salivary proteins

1. Increased levels of salivary IGHG3 and lactotransferrin in patients with SLE

IGHG3 and lactotransferrin in saliva samples were measured using ELISA test (Figure 3). The levels of salivary IGHG3 were elevated (3.0 ± 1.4 pg/mL) in patients with SLE compared to patients with RA (1.5 ± 0.7 pg/mL, p < 0.001) and HC (1.2 ± 0.5 pg/mL, < 0.001). The levels of salivary lactotransferrin were

elevated (5.0 ± 1.7 pg/mL) in patients with SLE compared to those in patients with RA (3.1 ± 1.6 pg/mL, $p < 0.001$) and HC (2.3 ± 1.7 pg/mL, $p < 0.001$).

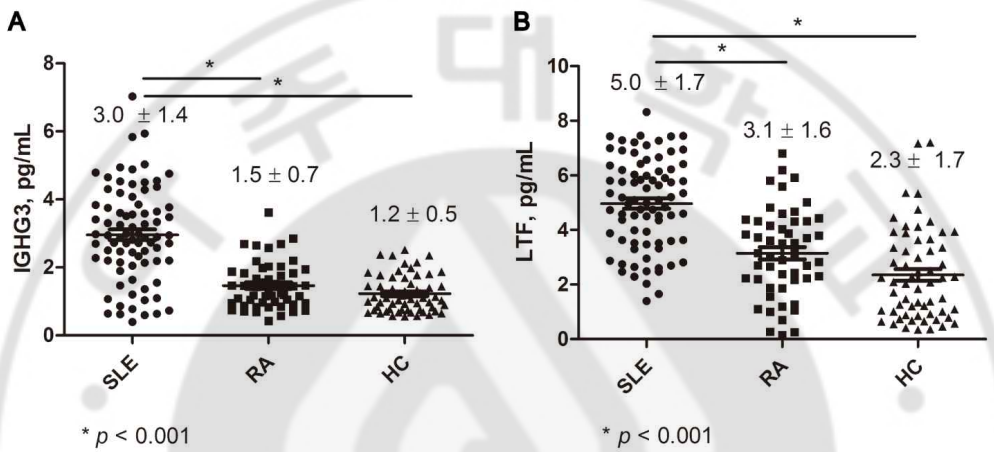


Figure 3. The concentrations of salivary immunoglobulin gamma-3 chain C region (IGHG3) and lactotransferrin (LTF) in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), and health controls (HC). Data are expressed as the mean ± SD. A Mann Whitney test was used to perform the statistical analysis.

In the ROC analysis of salivary IGHG3 and LTF, the AUC were 0.835 (95% CI 0.776-0.883, $p < 0.001$), 0.838 (95% CI 0.778-0.887, $p < 0.001$), respectively (Fig. 4). With a cut-off value of 2.69 pg/mL, the sensitivity and specificity of salivary IGHG3 were 73.3% and 90.4% for diagnosis of SLE, respectively. Also, the sensitivity and specificity of salivary LTF were 69.5% and 85.1% with a cut-off value of 5.59 pg/mL.

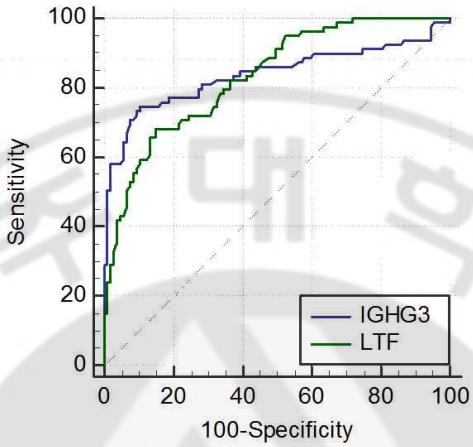


Figure 4. Receiver operating characteristics (ROC) curves for salivary immunoglobulin gamma-3 chain C region (IGHG3) and lactotransferrin (LTF) in patients with systemic lupus erythematosus and healthy controls . In the ROC curve, the area under the curve (AUC) was 0.835 for salivary IGHG3 (95% CI 0.776-0.883; $p < 0.001$) and 0.838 for salivary LTF (95% CI 0.778-0.887, $p < 0.001$).

2) Salivary lactotransferrin correlated with complement levels

Correlation analysis was performed between clinical characteristics and expression of IGHG3 and lactotransferrin (Table 3). Although C3 levels were correlated minimally with salivary IGHG3 (r = 0.22, $p = 0.05$), most serum markers and clinical manifestations did not. Complement levels correlated with salivary lactotransferrin (C3; r = 0.27, $p = 0.01$, C4; r = 0.27, $p = 0.02$).

Table 3. Correlation of salivary biomarkers and clinical features in patients with systemic lupus erythematosus

	IGHG3		Lactotransferrin	
	r	*p*-value	r	*p*-value
Age	-0.04	0.56	0.17	0.02
ESR	0.01	0.97	-0.19	0.1
Complement 3	0.22	0.05	0.27	0.01
Complement 4	0.19	0.1	0.27	0.02
Anti-dsDNA Ab (+)	0.1	0.39	-0.14	0.21
Mucocutaneous involvement	-0.52	0.65	-0.11	0.34
Arthritis	0.07	0.56	-0.05	0.66
Nephritis	-0.5	0.66	0.05	0.67
Serositis	0.07	0.55	0.2	0.08
Hematologic disease	-0.01	0.93	0.06	0.6
SLEDAI	-0.11	0.36	-0.08	0.46

IGHG3, Immunoglobulin gamma-3 chain C region; ESR, erythrocyte sedimentation rate; dsDNA, double-strand deoxyribonucleic acid; Ab, antibody; SLEDAI, systemic lupus erythematosus disease activity index.

3. Changes in salivary IGHG3 and lactotransferrin expression during follow-up

Among the patients with SLE, 21 patients were enrolled for the follow-up study, from whom saliva samples were obtained for the second time 1 to 2 years after first saliva samples were collected. The concentration of salivary IGHG3 increased significantly (1.74 ± 0.77 vs 3.21 ± 1.6 pg/mL, p = 0.004), and the concentration of salivary lactotransferrin decreased marginally (5.15 ± 1.73 vs 4.1 ± 1.56 pg/mL, p = 0.08) (Figure 5). Clinical features were compared between two categories of patients- those showing and those not showing changes in salivary IGHG3 levels. Hemoglobin and complement 3 levels were found to show changes in the patients with increased IGHG3 (p = 0.02 for both) (Table 4). The change in hemoglobin levels in the patients with increased IGHG3 was -0.85 ± 0.87/μL and that of patients with decreased IGHG3 was 0.42 ± 0.99/μL (p = 0.02). The change in complement 3 levels in the patients with increased IGHG3 was -7.67 ± 14.15 mg/dL and that of patients with decreased IGHG3 levels was 7.0 ± 9.34 mg/dL. The change of ESR in the patients with increased lactotransferrin level was -2.0 ± 4.6 mm/h and that of the patients with decreased lactotransferrin level was 8.4 ± 9.56 mm/h (p = 0.02). In correlation between the change of salivary proteins and clinical features, the change of complement 3 levels was correlated with the change of salivary lactotransferrin level (Figure 6).

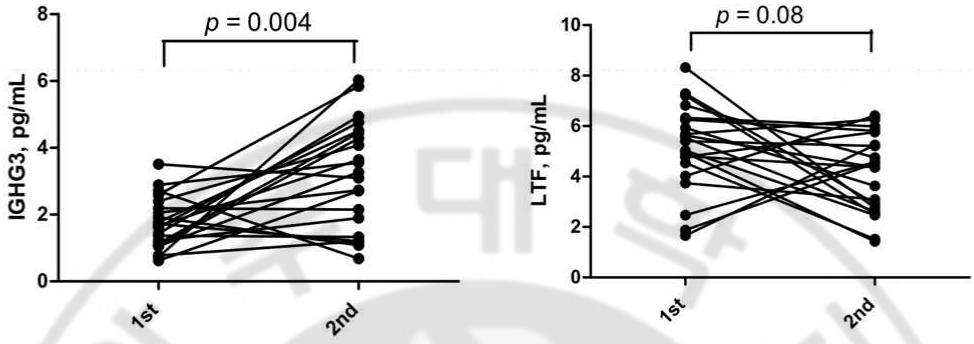


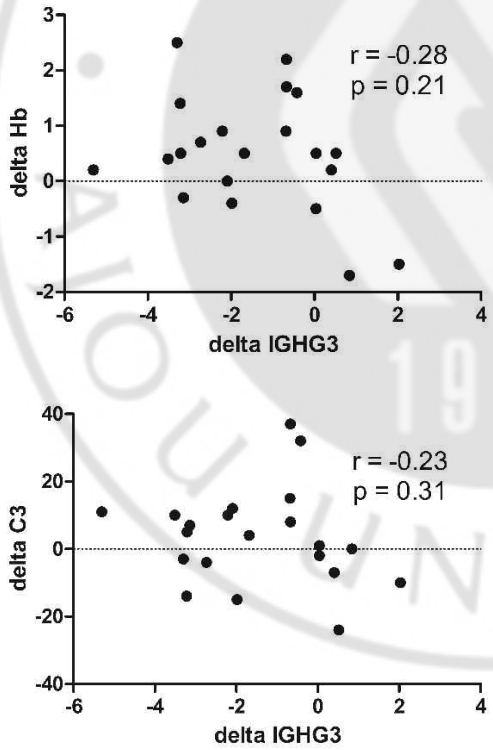
Figure 5. Changes of salivary immunoglobulin gamma-3 chain C region (IGHG3) and lactotransferrin (LTF) in patients with systemic lupus erythematosus (SLE) after 1-2 years. Wilcoxon signed ranks test were calculated.

Table 4. Comparison of clinical features according to the changes of salivary proteins in follow-up study

	Salivary IGHG3			Salivary lactotransferrin		
	▲ (n=15)	▼ (n=6)	*p*	▲ (n=6)	▼ (n=15)	*p*
Δ WBC, /μL	-245.3 ± 1,891.9	-1,966.7 ± 2,163.9	0.13	-1,000.0 ± 2,746.6	-632.0 ± 1,850.1	0.91
Δ Hb, /μL	-0.85 ± 0.87	0.42 ± 0.99	0.02	-0.73 ± 0.87	-0.39 ± 1.14	0.46
	6.07 ± 10.94	3.83 ± 5.53	0.79	-2.0 ± 4.6	8.4 ± 9.56	0.02

Δ ESR, mm/hr						
Δ C3, mg/dL	-7.67 ± 14.15	7.0 ± 9.34	0.02	-14.67 ± 16.51	1.0 ± 11.16	0.06
Δ C4, mg/dL	1.07 ± 11.13	-3.67 ± 9.5	0.67	4.83 ± 15.89	-2.33 ± 7.56	0.91

IGHG3, Immunoglobulin gamma-3 chain C region; WBC, white blood cells; Hb, hemoglobin; ESR, erythrocyte sedimentation rate; C, complement



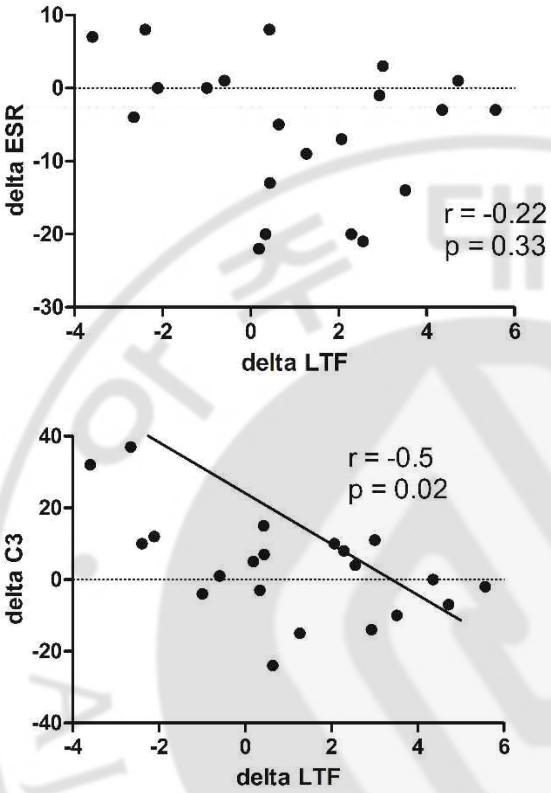


Figure 6. Correlation between the changes of salivary immunoglobulin gamma-3 chain C region (IGHG3) and lactotransferrin (LTF) with the changes of serologic marker for disease activity in patients with systemic lupus erythematosus (SLE). Hb, hemoglobin; C, complement; ESR, erythrocyte sedimentation rate. Spearman's correlations were calculated.

IV. DISCUSSION

Salivary 2-DE proteomic analysis showed that the densities of twenty spots were significantly different between patients with SLE and HC. These spots were revealed as IGHG3, S100A8, IGHA1, lactotransferrin, 8-oxoguanine DNA glycosylase, and leukemia-associated protein 7. In immunoblotting experiments, the expressions of salivary IGHG3 and lactotransferrin were significantly higher in patients with SLE compared to that in patients with RA and HC.

Saliva studies including proteomics have been conducted to discover disease biomarkers not only in oral disorders, but also in systemic diseases (Abdul Rehman et al., 2017; Kaur et al., 2018). Several cytokines in the saliva are upregulated in oral cancer, and cortisol levels in saliva are used to quantify the degree of psychological stress, or to assess cortisol excess or deficiency (Blair et al., 2017). Although further studies are being performed, salivary cytokines from B cells are expected to be a useful biomarker in Sjogren's syndrome (Navarro-Mendoza et al., 2018). However, salivary studies have not been extensively performed in SLE.

The role of immunoglobulin G (IgG) in pathogenesis of SLE is known. Interaction of anti-dsDNA-containing IgG and pleural mesothelial cells induced the synthesis of proinflammatory cytokines, suggesting the pathogenic mechanism of serositis in SLE (Guo et al., 2004). High level of serum IgG was a poor prognostic factor in autoimmune hepatitis combined with SLE, and the deposition of IgG has been shown to cause tissue damage (Lim et al., 2016; Fang et al., 2018). Serum IgG was correlated with serologic activity and predict flares in lupus nephritis, and IgG

induced the expression of calcium/calmodulin-dependent protein kinase IV, which is highly expressed on podocytes in lupus nephritis and leads to renal damage (Ichinose et al., 2016; Yap et al., 2016). IGHG3, also known as IgG3, is a constant region of immunoglobulin heavy chains. IGHG3 can combine IgG Fcγreceptor (FcγR) of neutrophilic granulocyte and macrophages. FcγRI on the surface of dendritic cells promotes the antigen presentation of dendritic cells to T cells (Nimmerjahn and Ravetch, 2008). Aberrant expression of FcγR for IgG has been observed in patients with arthritis and SLE, and FcγR is involved in antigen presentation and immune-complex-mediated maturation of dendritic cells, regulation of B-cell activation and plasma cell survival in SLE (Bave et al., 2003). The role of FcγR and the effect of its gene polymorphisms on the susceptibility or manifestations of SLE has been examined (Dong et al., 2014; Vigato-Ferreira et al., 2014; Jeon et al., 2015). A study of patients with autoimmune hemolytic anemia showed that the levels of IGHG3 on red blood cells (RBC) were associated with frequency of RBC transfusion after diagnosis (Lai et al., 2014). As an immunoprotein, increased IGHG3 expression in RBC might lead to more severe hemolysis. Although it is necessary to investigate the mechanism of salivary IGHG3 level elevation in patients with SLE, our results suggest that salivary IGHG3 may be helpful as a differential diagnostic biomarker for SLE. Moreover, it showed the reliability of the sensitivity and specificity of salivary IGHG3 for diagnosis of SLE. IGHA1, also known as IgA1, is abundant in sero-mucous secretion such as saliva. IGHA1 was enriched in the cellular fractions obtained from plasma cells of patients with autoimmune diseases including SLE (Streicher et al., 2014). Anti-dsDNA

antibody IgA level was found to be increased in patients with lupus nephritis, and was higher in active disease (Villalta et al., 2013). IgA to Epstain-Barr virus was found in SLE, and IgA against Epstain-Barr virus capsid antigen was associated with disease flare (Chen et al., 2005; Draborg et al., 2012). The levels of salivary IgA1 were not different in patients with SLE or RA, and HC.

Lactotransferrin, also known as lactoferrin, is a multifunctional glycoprotein of the transferrin family which is found in mucosal secretions and secondary granules of polymorphonuclear leukocytes (Gonzalez-Chavez et al., 2009). It not only plays a role in protection against microorganisms, but also in immunomodulation, inflammation, and anticancer activity through its interactions with the host immune system (Legrand et al., 2005, 2006). Lactoferrin-specific IgG autoantibodies were found in serum of patients with RA and SLE (Caccavo et al., 2005). Release of surface-expressed lactoferrin from polymorphonuclear neutrophils modulates T helper cell type 1(Th1)/Th2 cytokine production, and low levels of lactoferrin in patients with SLE leads to abnormal Th1/Th2 production (Li et al., 2006).

Lactoferrin-containing immune complexes induced production of pro-inflammatory cytokines from monocytes and monocytes-derived macrophages, and had the ability to induce human macrophages into pro-inflammatory, M1–like phenotype (Hu et al., 2017; Gao et al., 2018). Inflammatory M1 macrophages are known to mediate severe and non-resolving inflammation in SLE (Iwata et al., 2012). Salivary lactoferrin has a role in antimicrobial activity against bacteria, viruses and fungi, and its levels are elevated in periodontal disease (Fabian et al., 2012; Glimvall et al., 2012; Rocha Dde et al., 2012). In this study, the levels of salivary lactoferrin

were elevated in only patients with SLE, and not in patients with RA and HC. Salivary lactotransferrin had a reliable level of sensitivity and specificity for diagnosis of SLE.

Ca-binding S100 proteins are released from neutrophils and monocytes, and interact with pattern recognition receptors including toll-like receptor 4 (TLR4) and receptor for advanced glycation endproduct (RAGE), thereby enhancing immune cell activation (Kim et al., 2012; Narumi et al., 2015). Level of cell surface S100A8/A9 was increased in serum of patients with SLE and plasmacytoid dendritic cells in patient with SLE synthesize S100A8/A9, leading to amplification and persistence of inflammation (Lood et al., 2011). Serum S100A8/A9 levels were increased in inactive patients with SLE and those with cardiovascular disease (Tyden et al., 2013). Salivary S100A8 levels was higher in patients with SLE, and it was similar in patients with RA, suggesting that salivary S100A8 represents systemic inflammatory status as in serum.

The levels of salivary IGHG3 did not correlated with any clinical feature of SLE, while salivary lactotransferrin positively correlated with age and complement levels. Complement deficiency is indicative of active SLE, and salivary lactotransferrin showed a positive correlation with complement 3. This is contrary to the results of salivary lactotransferrin that is highly detected as a biomarker in patients with SLE, and further investigations are needed.

In the follow-up measurement, the concentrations of salivary IGHG3 were significantly increased in 21 patients with SLE after 1-2 years. Their mean level was lower than the mean value of total of 95 patients with SLE in the first

measurement, while they were enrolled randomly. Although salivary IGHG3 levels were increased during follow-up, their levels were not associated with disease activity markers. These results suggested that the number of patients in follow-up study was small, and inter-assay precision was considered a problem.

The limitations of the follow up study were that most of the patients were being administered standard management for SLE at the time of sample collection, so the difference in their disease activity was not large, and the administration of drugs may have affected protein components in the samples. The number of samples was not sufficient to analyze the difference in protein components according to clinical features. Furthermore, the number of follow-up patients was small.

This work revealed the differences in salivary protein composition in patients with SLE compared to that in HC. Salivary IGHG3 and lactotransferrin levels were significantly increased in patients with SLE compared to those in patients with RA or HC, and their concentrations were found to be useful indicators for disease discrimination. Moreover, the changes in the salivary lactotransferrin levels were correlated with the change in the blood complement 3 levels.

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-국문요약-

단백분석을 통해 전신홍반루푸스의 잠재적 생물표지자로서 확인된 타액 내 단백의 특성화

서론: 전신홍반루푸스는 자가항체의 생성과 전신에 발생하는 염증반응이 특징적인 자가면역질환으로 다양한 증상 및 질병활성도를 가진다. 전신홍반루푸스의 진단에서 항핵항체의 유무가 중요하지만 질환 특이적인 지표가 없으며 치료반응을 평가하는데 활용할 수 있는 생물표지자가 부족하다. 타액은 비침습적이고 반복적으로 수집할 수 있는 장점이 있으며 혈액 내 단백이 포함되어 있어 질병의 특징을 연구하는데 유용한 체액이다. 최근 많이 사용되는 단백분석기술을 통해 전신 질환에서 변화된 타액 내 단백 조성의 차이를 정밀하게 분석할 수 있다.

방법: 11명의 전신홍반루푸스와 건강대조군에게서 수집된 타액으로 2차원 전기영동을 시행하였고, 분리된 점들 중 차이를 보이는 것들을 질량분석기를 통해 단백성분을 밝혔다. 웨스턴블랏과 효소결합면역흡착측정법을 통해 전신홍반루푸스 환자와 류마티스관절염 환자, 건강대조군에게서 제공받은 타액 내 후보 단백성분들의 농도를 측정하였다. 전신홍반루푸스 환자들에게서 1-2년 뒤 다시 타액을 수집하여 타액 내 단백성분의 농도를 측정하여 농도의 차이와 임상양상의 차이간의 상관성을 분석하였다.

결과: 2차원 전기영동을 시행한 결과에서 전신홍반루푸스와 건강대조군의 타액 내 단백성분 20개의 점들 중 2배 이상 차이를 보이는 점들은 immunoglobulin gamma-3 chain C (IGHG3), Ig alpha-1 chain C region, protein S100, 락토트랜스페린, 8-oxoguanine DNA glycosylase, 그리고 leukemia-associated protein 7 이였다. 타액 내 IGHG3의 농도는 전신홍반루푸스 환자군 (3.0 ± 1.4 pg/mL)에서 류마티스관절염 환자 (1.5 ± 0.7 pg/mL, $p < 0.001$) 나 건강대조군(1.2 ± 0.5 pg/mL, $p < 0.001$)에 비해서 유의하게 높았고, 타액 내 락토트랜스페린의 농도도 전신홍반루푸스 환자군 (5.0 ± 1.7 pg/mL)에서 류마티스관절염 환자군(3.1 ± 1.6 pg/mL, $p < 0.001$)이나 건강대조군(2.3 ± 1.7 pg/mL, $p < 0.001$)에 비해서 유의하게 높았다. 타액 내 락토트랜스페린은 전신홍반루푸스 환자들의 보체 3 ($r = 0.27$, $p = 0.01$)과 보체 4 ($r = 0.27$, $p = 0.02$)와 양의 상관관계가 있었다. 1-2년 후 시행된 추적 연구에서는 타액 내 IGHG3의 농도가 증가된 군은 감소된 군에 비하여 헤모글로빈의 변화량 (−0.85 ± 0.87 vs 0.42 ± 0.99, $p = 0.02$)과 보체 3 의 변화량 (−7.67 ± 14.15 vs +7.0 ± 9.34, $p = 0.02$)에서 유의하게 차이를 보였다. 타액 내 락토트랜스페린의 농도가 증가된 군은 감소된 군에 비해 ESR의 변화량 (−2.0 ± 4.6 vs 8.4 ± 9.56, $p = 0.02$)에서 유의하게 차이를 가졌고, 타액 내 락토트랜스페린의 변화량과 보체 3의 변화량이 음의 상관관계를 보였다 ($r = -0.5$, $p = 0.02$).

결론: 타액 내 IGHG3와 락토트랜스페린은 전신홍반루푸스에서 상승되어 있으며, 이는 잠재적인 생화학적 지표로 가능성을 시사한다.