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Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects

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Background and Objective: Salivary proteomics technology can be used to evaluate the disease progession of periodontitis and the systemic screening of proteomes of saliva from subjects with aggressive periodontitis has not been available. The objective of this preliminary study was to compare the proteomic profile of whole unstimulated saliva of subjects with generalized aggressive periodontitis (GAgP) with that of healthy volunteers to identify proteins, the levels of which were significantly altered between the two groups.

Material and Methods: Whole unstimulated saliva was obtained from five subjects with GAgP and five healthy subjects, and proteins were separated using two-dimensional gel electrophoresis. Proteins, the levels of which were significantly different between the two groups, were identified by computer image analyses and subsequent electrospray ionization tandem mass spectrometry.

Results: Eleven proteins that exhibited a different level in the GAgP group vs. the control group were identified. Compared with whole saliva of healthy control subjects, the levels of serum albumin, immunoglobulin (Ig) γ2 chain C region, Ig α2 chain C region, vitamin D-binding protein, salivary α-amylase and zinc-α2 glycoprotein were increased in whole unstimulated saliva of GAgP subjects, while those of lactotransferrin, elongation factor 2, 14-3-3 sigma, short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor and carbonic anhydrase 6 were decreased.

Conclusion: Comparison of the proteomic profile of whole unstimulated saliva of GAgP subjects with that of healthy control subjects revealed at least 11 differential proteins. The approach applied herein might be helpful to aid understanding of the etiology of GAgP.

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Based on the classification used by the American Academy of Periodontology, three types of periodontitis are currently recognized, namely aggressive periodontitis (AgP), chronic periodontitis and periodontitis associated with systemic disease (1). Aggressive periodontitis, including generalized AgP (GAgP) and localized AgP, is a multifactorial disease, which is comprised of a heterogeneous group of infectious diseases characterized by a complex host–microbial interaction in the periodontium (1). The aggressive nature of AgP and its early onset depend on the bacterial etiology, host susceptibility, hereditary and environmental factors, and often also behavioral factors (1). Failure to treat AgP appropriately can result in the loss of attachment or destruction of the periodontal ligament and loss of adjacent supporting bone (1). Therefore, besides the identification of AgP biomarkers, it is crucial to investigate and understand the complex etiology of the disease.

Saliva is an important component of the host oral immune defense system and may be an effective source for AgP biomarkers because it can reflect changes in oral and systemic health. Since the 1990s, saliva has been used in disease diagnosis in internal medicine (2). Subsequently, saliva has been used in the diagnosis of periodontal disease (3–5), and changes in the saliva protein composition upon the development of periodontitis have been documented (6). This led to the recommended use of periodontal disease-related markers in the diagnosis of periodontal disease, such as host proteins (enzymes and immunoglobulins), phenotypic markers (epithelial keratin), host cells, hormones and bacteria (3–5). Protein and bacterial markers found in saliva can be identified using Western blot analyses, enzyme-linked immunosorbent assays (ELISAs) and polymerase chain reaction (PCR) methods, but these methods are only suitable for small samples and are expensive, time consuming and laborious. However, besides plaque, blood and serum, saliva can also exhibit molecular changes upon the development of periodontitis that can contribute to a better understanding of the molecular etiology of the disease (7,8).

Proteomic research and technology has greatly advanced studies in molecular medicine. The emergence of proteomics technology and theory are facilitating the development of disease diagnosis using saliva biomarkers (9). Ryu *et al*. (10) performed an extensive proteomic study and compared the proteomes of saliva samples obtained from subjects with Sjögren's syndrome, an autoimmune disease characterized by lymphoplasmocytic infiltration of the salivary and lacrimal glands, with those of saliva samples from healthy control subjects and identified several putative biomarkers. One of the main strengths of proteomics is the qualitative and quantitative study of the differences between proteomes in different conditions, including different genotypes, diseases and drug influences (11–13). Application of proteomics technology to saliva samples led to the identification of biomarkers that can be used in the diagnosis of systemic diseases. Previous research on periodontitis showed that the IgA content in saliva of subjects with periodontitis was higher than that in saliva of healthy control subjects (7). Sandholm *et al*. (14) also demonstrated that the concentration of IgG in saliva is more important than the concentration of IgA because the specific antibody against periodontitis causative bacteria is the determinative factor to limit and control the infection. In that study, an increased IgG content in whole saliva was also seen in 34% of the subjects with moderate periodontitis and in 57% of the subjects with severe periodontitis (14). The concentration of IgG antibody associated with infection by *Actinobacilus actinomycetemcomitas*, a pathogenic bacterium specifically correlated with AgP (15,16), increased in 55% of untreated young subjects with periodontitis (14). This percentage, however, decreased to 28% after treatments, such as subgingival scaling and root planing (14). It has also been reported that the concentration of *Porphyromonas gingivalis* IgG antibody increased in subjects with rapidly progressing periodontitis (17). These results showed that the examination of immune proteins in saliva using proteomics technology can be used to evaluate the disease progress of periodontitis, which also indicates the possibility of using the proteomics technology to examine saliva to diagnose AgP and to increase understanding of the etiology of the disease. To our knowledge, no reports describing the systemic screening of proteomes of saliva from subjects with AgP are yet available. In the present study, we compared the proteomes of whole unstimulated saliva from five subjects with GAgP with that of healthy subjects and identified 11 proteins, the levels of which were different among the two groups. This preliminary study provides a basis for applying proteome technology to analyze saliva samples, which may contribute to the identification of novel biomarkers for GAgP and to our understanding of the etiology of the disease.

Material and methods

Subjects

Subjects with GAgP are generally characterized as follows: (1) age < 30 years; (2) more than three teeth affected in addition to the involvement of the first molar and incisor teeth; and (3) generalized interproximal attachment loss on affected teeth. Subjects with GAgP ($n = 5$) were randomly selected from the outpatients at the Department of Periodontology at No. 9 People's Hospital, Shanghai Jiaotong University Medical College. Three of the subjects who participated in this study were male and two were female, and their average age was 24.8 ± 3.83 years. All patients met the revised criteria for diagnosis by the American Academy of Periodontology (1). Healthy subjects ($n = 5$) were recruited from the students of the School of Stomatology, Shanghai Jiaotong University. There were three male subjects and two female subjects, with an average age of 24 ± 0.71 years. The common inclusion criteria for subjects with GAgP and healthy subjects were no systemic systemic diseases, females were not pregnant or nursing, no initial therapy

was done within 6 months before the study was undertaken, no antibiotics were taken during the past 3 months, no decayed teeth, non-smoking and no alcohol usage. The five healthy subjects had < 10% gingival bleeding sites, the probing depth was < 3 mm, and the sites with a > 2 mm attachment loss did not exceed 1%. X-ray scans did not reveal any bone absorption.

Saliva sample preparation

Whole unstimulated saliva samples were collected following the Rhodus improved method (18). A volume of 1.2 mL saliva was collected from each of the fasting subjects between 09.00 and 11.00 h, and the samples were immediately stored on ice to prevent protein degradation. Protease inhibitor cocktail (100 μL/1 mL saliva; Sigma-Aldrich, St Louis, MO, USA) was added to the samples immediately after collection. Samples were centrifuged at 4°C at 1300*g* for 5 min. Then, the supernatant was collected and centrifuged again at 20,000*g* for 30 min. Finally, 500 μL supernatant was collected and stored at −80°C. An equal amount of individual samples in each group (5 GAgP patients or 5 control subjects, respectively, for the comparative analysis) were pooled and placed into a 3 kDa ultrafiltration tube for the centrifugation. The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA) using bovine γ-globulin as an internal control (Sigma-Aldrich).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed three times, independently, for each subject of the GAgP group and the healthy control group. The 2-DE protocol was adopted from Ryu *et al*. (10) with minor modifications. For isoelectrofocusing, protein samples (100 μg each) obtained from the GAgP and healthy control groups were dissolved in 250 μL rehydration buffer (8 M urea, 2% Chaps, 18 mM dithiothreitol and 0.5% carrier ampholyte). The samples were electrophoresed on a SE-600 2-DE system (Amersham, UK) at 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 6 h and 500 V for 4 h. After focusing, strips of the two samples were equilibrated for 15 min in a solution containing 6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% sodium dodecylsulphate (SDS), 30% (w/v) glycerol, bromophenol blue and dithiothrietol. Strips were then applied onto an upright 12.5% SDS gel and electrophoresed at 15 mA for approximately 30 min. After electrophoresis, the gels were moved for silver nitrate staining and Coomassie Brilliant Blue (R-350) staining according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Dye images were collected using a GS-710 Typhoon scanner (Bio-Rad), and Imagemaster (GE Healthcare) was used for the quantitative spot analysis of the 2-D gel images.

Protein identification by electrospray ionization tandem mass spectrometry

The separation and identification of the digested proteins was conducted using a Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled with a Surveyor HPLC system (ThermoScientific, San Jose, CA, USA). First, a Microcore RP column (C18; 15 cm × 150 μm; ThermoHypersil, San Jose, CA, USA) was used to separate the protein digests. Solvent A was 0.1% (v/v) formic acid and solvent B was 0.1% (v/v) formic acid in 100% (v/v) acetonitrile. The gradient was held at 2% solvent B for 15 min, and increased linearly to 98% solvent B over 90 min. The peptides were eluted from the C18 microcapillary column at a flow rate of 150 μL/min before being directly electrosprayed into an LCQ-Deca mass spectrometer (ThermoFinnigan, San Jose, CA, USA) with the application of a spray voltage of 3.2 kV and with a capillary temperature of 200°C. The full scan ranges from 400 to 2000 m/z. Protein identification using tandem mass spectrometry raw data was performed using SEQUEST software (University of Washington, licensed to Thermo Finnigan) based on the database of the International Protein Index (IPI; human, V3.15.1). A relative molecular mass of 57 (57 Da) was added to the average molecular mass of cysteines in tandem mass spectrometry data searching. Both *b* ions and *y* ions were were also included in the database search. Protein identification results were filtered using cross-correlation score (Xcorr) (Charge + 1, Xcorr ≥ 1.9; Charge + 2, Xcorr ≥ 2.2; Charge + 3, Xcorr ≥ 3.75) and the delta correlation value (≥0.1).

Enzyme-linked immunosorbent assays

Lactoferrin, IgA2 and albumin concentrations were determined in aliquots of individual subject samples by ELISA using the manufacturer's recommended protocols (Dakopatts, Glostrup, Denmark). Twenty new subjects (GAgP, $n = 10$; healthy control, $n = 10$) were recruited in ELISA used for validation of the candidate proteins. There were five male (age 24.8 ± 3.00 years) and five female subjects (age 23.4 ± 3.34 years) in each group.

Statistical analyses

The amounts of protein were expressed as means ± SD. The difference in amount of protein between the control group and the GAgP group was tested by Student's paired *t*-test for three representative proteins, i.e. lactoferrin, IgA2 and albumin. All statistics were two-tailed and performed using SAS software (version 9.1.3; SAS Inc., Cary, NC, USA). The significance level was defined as $p < 0.05$.

Results

Two-dimensional gel electrophoresis of whole unstimulated saliva from GAgP subjects and healthy control subjects

The 2-DE gels run on protein samples obtained from GAgP subjects were compared with those from healthy control subjects and analyzed using the Imagemaster software. Twenty-one different protein spots were observed

between the GAgP group and the healthy control group. Eleven differential proteins were identified by comparison of the electrospray ionization tandem mass spectrometry peptide data of these different spots with those in the human protein database (item position index (IPI) V3.15.1), which includes some unnamed and hypothetical proteins. The remaining proteins failed to match any protein in the database, suggesting that they may represent presently unknown and/or uncharacterized proteins. Figure 1 shows a representative 2-DE gel of a protein profile derived from whole unstimulated saliva from a GAgP subject. Identified differential proteins are listed in Table 1.

Identification of differential proteins

Compared with whole saliva of healthy control subjects, the levels of serum albumin, Ig γ2 chain C region, Ig α2 chain C region, vitamin D-binding protein, salivary α-amylase and zinc-α2 glycoprotein were increased in whole unstimulated saliva of GAgP subjects, and the levels of lactotransferrin, elongation factor 2, 14-3-3 sigma, short palate, lung and nasal epithelium carcinoma-associated protein 2 (PLUNC2) precursor and carbonic anhydrase 6 were decreased (Table 1).

Confirmation of selected representative differential proteins by ELISA

The purpose of the ELISA experiment was to confirm the reliability of the proteomic study. Three representative proteins (i.e. lactoferrin, IgA2 and albumin) that were shown to be associated with AgP (7,8) were selected and used in the ELISAs. The levels of these proteins were evaluated in saliva samples of control and GAgP subjects (Table 2). Overall, the amount of protein for the GAgP group was greater than that for the control group, except for lactoferrin ($p < 0.001$). The outcome of the ELISAs not only demonstrates the reliability of the proteomic findings, but also provides additional evidence regarding the evolvement of those proteins in AgP pathogenesis.

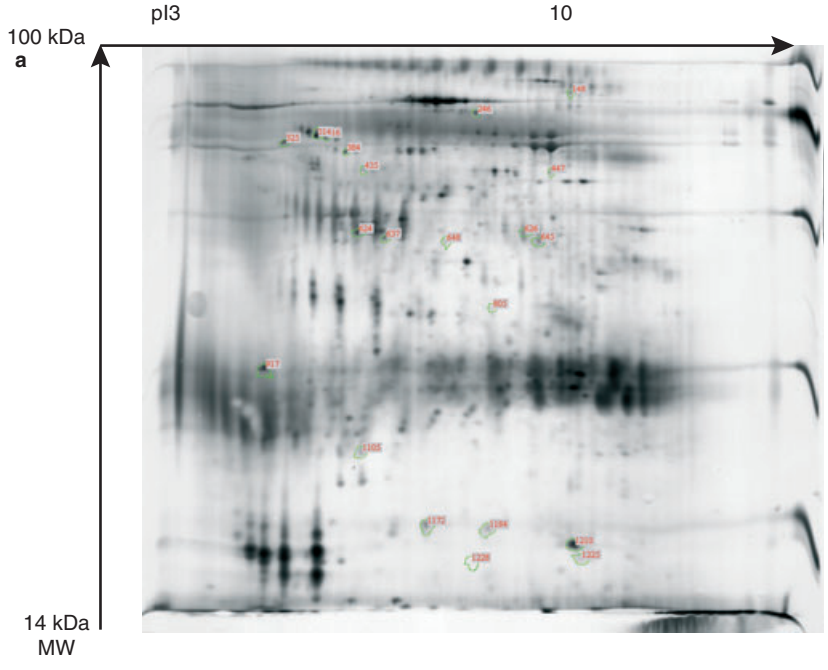

Fig. 1. Two-dimensional gel electrophoresis of unstimulated saliva samples obtained from a GAgP subjects. Protein spots, the levels of which were different in the GAgP group compared with saliva samples of healthy control subjects, are indicated. The apparent molecular weight and isoelectric point are indicated on the vertical and horizontal axis, respectively. The red numbers represent random ID by a appropriate software. pI, isoelectric point isoelectric focusing with pH range 3–10 in the horizontal dimension.

Discussion

In this study, we compared the proteomic profiles of whole unstimulated saliva from subjects with GAgP with those from healthy subjects and identified 11 proteins from the subjects with GAgP, the level of which was altered compared with that in the healthy subjects.

The results of this preliminary study showed an increased level of serum albumin in saliva of patients with GAgP, which is consistent with previous findings that the albumin content is significantly increased in whole saliva of subjects with GAgP (6,19). In addition, we observed increased levels of IgG2 and IgA2 in saliva of subjects with GAgP. It is commonly accepted that inflammation and damage of the periodontal tissue are accompanied by changes in the quality, quantity and specificity of certain antibodies. Secreted IgA2 (sIgA2) in saliva plays an important role in the resistance against pathogenic bacteria and viruses and is one of the major defense factors in saliva. In addition, IgG2 produced via T-helper 2 cell induction is a specific serum antibody against periodontal pathogens and is produced primarily in gingival tissues (14,20). The IgG level in the gingival crevicular fluid is similar to that in serum (14,20). These results indicate that the acquired immune response is enhanced in subjects with GAgP and that the local oral immune response is closely integrated with the systemic immune response.

Compared with the healthy control group, the level of some of the differential proteins was increased or decreased in whole saliva of subjects with GAgP. Proteins, the level of which was decreased, included elongation factor 2, carbonic anhydrase 6, PLUNC2 precursor, 14-3-3 sigma and lactotransferrin. Elongation factor 2 promotes the transfer of newly formed GTP-dependent protein from the A site to the P site of the ribosome

Table 1. Differentially expressed proteins in whole unstimulated saliva between GAgP and healthy control groups

Spot no.	Number of peptide fragments	Detected tryptic peptides	Xcorr	ΔCn	Protein	Molecular weight (actual/ theoretical)	Isoelectric points (actual/ theoretical)	Average ratio changes[a]
1210	1	R.KRGHVFEESQVAG TPMFVVK.A	2.217	0.195	Elongation factor 2	43/95	5/6.4	−6.5
645	3	R.TTLTGLDVQDM*LPR.N	3.643	0.528	Carbonic anhydrase 6 precursor	30/35	5.5/6.7	−3.6
		R.TTLTGLDVQDM*LPR.N	3.354	0.444				
		K.LENSLLDHR.N	2.443	0.329				
626	1	K.LENSLLDHR.N	2.519	0.450		48/35	7/6.7	−1.7
805	2	K.LKVDLGVLQK.S	2.337	0.271	PLUNC2 precursor	26/27	5.5/5.4	+3.3
		K.LKVDLGVLQK.S	2.741	0.327				
624	14	K.AEFAEVSK.L	2.247	0.221	Serum albumin	48/72	5.5/6.4	+1.5
		K.AEFAEVSK.L	2.720	0.238				
		K.DDNPNLPR.L	2.287	0.219				
		K.LVNEVTEFAK.T	3.780	0.475				
		K.LVNEVTEFAK.T	2.997	0.424				
		K.TCVADESAENCDK.S	4.428	0.521				
		K.TCVADESAENCDK.S	3.440	0.489				
		K.YICENQDSISSK.L	3.060	0.487				
		K.YICENQDSISSK.L	3.230	0.470				
		K.YICENQDSISSK.L	3.585	0.359				
		K.YICENQDSISSK.L	4.096	0.400				
		R.FKDLGEENFK.A	2.612	0.198				
		R.LSQRFPK.A	2.261	0.161				
		R.LSQRFPK.A	2.308	0.121				
1105	7	K.LVNEVTEFAK.T	2.543	0.426		30/72	5/6.4	+3.2
		K.LVNEVTEFAK.T	2.603	0.442				
		K.TCVADESAENCDK.S	3.130	0.516				
		K.TCVADESAENCDK.S	3.550	0.463				
		K.YLYEIAR.R	1.912	0.227				
		R.FKDLGEENFK.A	2.423	0.193				
		R.FKDLGEENFK.A	2.702	0.334				
1172	14	K.AAFTECCQAADK.A	2.302	0.120		23/72	6/6.4	+1.9
		K.CCTESLVNR.R	2.573	0.421				
		K.FQNALLVR.Y	2.630	0.223				
		K.FQNALLVR.Y	2.666	0.248				
		K.KVPQVSTPTLVEVSR.N	3.782	0.451				
		K.KVPQVSTPTLVEVSR.N	2.653	0.532				
		K.LVNEVTEFAK.T	2.571	0.432				
		K.LVNEVTEFAK.T	2.793	0.431				
		K.SLHTLFGDK.L	2.317	0.402				
		K.TCVADESAENCDK.S	3.190	0.509				
		K.VPQVSTPTLVEVSR.N	3.325	0.535				
		K.VPQVSTPTLVEVSR.N	3.670	0.500				
		K.YICENQDSISSK.L	3.760	0.416				
		K.YICENQDSISSK.L	2.245	0.413				

Table 1. Continued

Spot no.	Number of peptide fragments	Detected tryptic peptides	Xcorr	ΔCn	Protein	Molecular weight (actual/ theoretical)	Isoelectric points (actual/ theoretical)	Average ratio changes[a]
917	16	K.GAVEKGEELSCEER.N	3.261	0.371	14-3-3 protein sigma	24/28	4.5/4.7	−3.2
		K.GAVEKGEELSCEER.N	4.026	0.234				
		K.GAVEKGEELSCEER.N	3.305	0.415				
		K.SNEEGSEEKGPEVR.E	3.226	0.466				
		K.SNEEGSEEKGPEVR.E	3.329	0.484				
		K.SNEEGSEEKGPEVR.E	4.120	0.455				
		K.SNEEGSEEKGPEVR.E	3.330	0.437				
		R.SAYQEAMDISKK.E	2.379	0.235				
		R.VISSIEQK.T ! R.VLSSIEQK.S	2.383	0.143				
		R.VISSIEQK.T ! R.VLSSIEQK.S	2.561	0.266				
		R.VISSIEQK.T ! R.VLSSIEQK.S	2.214	0.306				
		R.VLSSIEQKSNEEGSEEKGPEVR.E	4.770	0.507				
		R.YLAEVATGDDK.K	3.683	0.402				
		R.YLAEVATGDDK.K	2.483	0.242				
		R.YLAEVATGDDKKR.I	4.300	0.531				
		R.YLAEVATGDDKKR.I	3.357	0.337				
435	11	K.YLTWASR.Q	2.577	0.381	Ig α2 chain C region	20/52	5/5.1	+3.1
		R.DASGATFTWTPSSGK.S	3.695	0.477				
		R.DASGATFTWTPSSGK.S	3.550	0.524				
		R.GFSPKDVLVR.W	2.322	0.361				
		R.GFSPKDVLVR.W	2.956	0.401				
		R.QEPSQGTTTFAVTSILR.V	3.990	0.588				
		R.QEPSQGTTTFAVTSILR.V	3.923	0.605				
		R.QEPSQGTTTFAVTSILR.V	4.740	0.450				
		R.VAAEDWK.K	2.358	0.27				
		R.WLQGSQELPREK.Y	2.735	0.331				
		R.WLQGSQELPREK.Y	2.653	0.353				
637	8	K.AYLEEECPATLR.K	3.441	0.419	Zinc-α2-glycoprotein	46/34	6/5.6	+2.2
		K.AYLEEECPATLR.K	3.352	0.477				
		K.AYLEEECPATLRK.Y	3.807	0.461				
		K.AYLEEECPATLRK.Y	3.478	0.399				
		K.NILDRQDPPSVVVTSHQAPGEK.K	3.928	0.489				
		K.NILDRQDPPSVVVTSHQAPGEK.K	4.792	0.484				
		R.FGCEIENNR.S	2.215	0.259				
		R.YSLTYIYTGLSK.H	4.482	0.443				
447	2	K.GPSVFPLAPCSR.S	2.645	0.254	Ig γ2 chain C region	58/45	7/7.5	+1.8
		R.STSESTAALGCLVK.D	2.643	0.282				

Table 1. Continued

Spot no.	Number of peptide fragments	Detected tryptic peptides	Xcorr	ΔCn	Protein	Molecular weight (actual/ theoretical)	Isoelectric points (actual/ theoretical)	Average ratio changes[a]
148	9	K.CGLVPVLAENYK.S	2.523	0.234	Lactotransferrin precursor	80/78	7/8.5	−1.7
		K.NLLFNDNTECLAR.L	2.829	0.493				
		R.DGAGDVAFIR.E	3.398	0.370				
		R.DGAGDVAFIR.E	3.681	0.293				
		R.KSEEEVAAR.R	3.050	0.358				
		R.SNLCALCIGDEQGENK.C	4.790	0.464				
		R.VPSHAVVAR.S	2.424	0.551				
		R.VPSHAVVAR.S	2.210	0.506				
		R.VVWCAVGEQELR.K	3.737	0.242				
384	4	K.SLGECCDVEDSTTCFNAK.G	3.941	0.535	Vitamin D-binding protein precursor	66/53	5.5/5.4	+1.7
		K.SLGECCDVEDSTTCFNAK.G	4.149	0.577				
		R.VCSQYAAYGEK.K	3.860	0.219				
		R.VCSQYAAYGEK.K	3.326	0.164				
1184	3	K.TGSGDIENYNDATQV.D	3.334	0.434	Salivary α-amylase precursor	26/58	6.5/6.5	+1.6
		R.WVDIALECER.Y	3.410	0.473				
		R.WVDIALECER.Y	3.368	0.418				

[a]Plus and minus indicate that the level of a particular protein spot was higher or lower in saliva protein samples from the aggressive periodontitis group compared with that from the control group, respectively.
XCorr, cross-correlation score; ΔCn, delta correlation value.

(21), but its role in saliva remains unknown. In fact, elongation factor 2 is a ubiquitous protein with a highly conserved function across organisms, including bacteria, fungi and humans, and the amino acid sequence of elongation factor 2 is highly conserved (more than 85%) among organisms (22). Therefore, it cannot be excluded, based on the current data, that elongation factor 2 identified in saliva was due to the oral presence of microorganisms. Considering that elongation factor 2 is the most abundant protein in the bacterial cell, it is not surprising that this protein is present in the human protein database. We showed that the level of elongation factor 2 in saliva of AgP patients was 6.5-fold lower than that in saliva of healthy subjects. Whether this indicates a weaker defense response in AgP patients compared with healthy control subjects remains to be determined. Carbonic anhydrase 6 is a protein secreted in saliva. Its decreased level in subjects with Sjögren's syndrome was found to be the result of salivary gland damage (10,23), but it remains unclear how this could be related to a decreased level in saliva of GAgP subjects. The parotid gland sectretes PLUNC2, which is a protein belonging to the PLUNC family (24). PLUNC can attach lipopolysaccharides and thereby affect and inhibit bacterial growth (25,26). Previous studies demonstrated that the expression of PLUNC in epithelial tissue was regulated by inflammatory or other stimulus (27). The mechanism underlying the decreased level of PLUNC2 in saliva of subjects with GAgP remains unknown, but might be to the result of an inhibitory immune response induced by the bacteria that cause GAgP. The level of 14-3-3 sigma was also decreased in saliva of GAgP subjects. This protein is known as the epithelial cell marker protein 1 and is primarily expressed in the keratinized epidermis. Evaluation of the mechanism of the decrease in its level in saliva of GAgP subjects needs further investigation. Lactotransferrin is an iron-binding protein and its antibacterial effect is achieved by competing for iron with bacteria, thereby inhibiting bacterial growth

Table 2. Comparison of the level of lactoferrin, IgA2 and albumin between the control and GAgP group

	Control group (n = 10)	GAgP group (n = 10)	p-value[a]
Lactoferrin (μg/mL)	8.9 ± 0.5	6.8 ± 0.8	< 0.001
IgA2 (μg/mL)	52.5 ± 5.1	118.4 ± 5.6	< 0.001
Albumin (μg/mL)	58.4 ± 4.7	89.3 ± 6.1	< 0.001

Data are presented as means ± SD.
[a]The statistical analysis was performed using Student's paired t-test.

(28). The decreased level of lactotransferrin may indicate a large consumption of lactotransferrin as a defense against bacteria. Alternatively, the decrease of the level of lactotransferrin may be induced by the bacteria that cause GAgP. Interestingly, a negative correlation between *Actinobacillus actinomycetemcomitans* and lactoferrin in saliva of subjects with *A. actinomycetemcomitans*-associated periodontal disease suggested that low concentrations of lactoferrin favor bacterial growth (7).

The increased level of α-amylase in saliva of GAgP subjects is consistent with observations published in other reports (29,30). Taken together, the changes in the level of carbonic anhydrase 6, PLUNC2 and salivary α-amylase observed in saliva of GAgP subjects strongly suggests that the salivary gland is involved in the development process of the inflammation. Interestingly, we demonstrate for the first time a possible link between vitamin D-binding protein and GAgP. Vitamin D-binding protein promotes blood vessel and smooth muscle contraction, selectively promotes the chemotactic activity of neutrophil and mononuclear cells on complement 5a and complement 5a with the carboxy-terminal arginine removed, and increases their dissolving ability and effusion (31).

In this preliminary study, we identified 11 differential proteins. It is, however, important to remark that this number may not represent the complete protein population, the level of which is altered in saliva from GAgP subjects compared with that of healthy subjects, owing to limitations in the use of the technique. For example, we did not detect changes in the level of defensins or matrix metalloproteases. When multiple 2-DE gels are run using the same sample, it is technically difficult to obtain two identical gel patterns. Therefore, only those spots exhibiting a good repeatability were used for further identification, so it is likely that some important proteins were omitted. In addition, in traditional 2-DE, the pH of the immobilized pH gradient strips ranges between 3 and 10; hence, it is likely that proteins that appeared in the low or high pH range were not separated and detected. Defensins, for example, are acidic proteins with a molecular weight of only 3 kDa and will not be detected using traditional 2-DE. Furthermore, it cannot be excluded that some proteins may have been degraded during sample preparation. It is thus important to conduct additional studies using an expanded subject population combined with alternative approaches to characterize the complete collection of proteins, the levels of which are altered in saliva of GAgP subjects compared with the saliva of healthy control subjects.

Generalized AgP is a disease caused by multiple factors and its etiology is complex (32). The susceptibility of the host is the result of mutual interactions among bacteria, the immune system and environment, in which the host factor plays an important role (32). The proteomic profile of whole unstimulated saliva of subjects with GAgP contains proteins, the levels of which differ compared with the levels in saliva of healthy control subjects. While future studies involving an increased number of GAgP and healthy control subjects are required, as well as further characterization of the differential proteins identified herein, this preliminary study demonstrates that the use of proteome analysis may contribute to our understanding of the etiology of AgP, which is not yet well understood.

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References

1. Armitage GC, Mariotti A, Holmstrup P *et al*. 1999 International Workshop for a Classification of Periodontal Diseases and Conditions. Papers. Oak Brook, Illinois, October 30–November 2, 1999. *Ann Periodontol* 1999;**4:**1–112.
2. Quissell DO. Steroid hormone analysis in human saliva. *Ann N Y Acad Sci* 1993; **694:**143–145.
3. Kornman KS, Crane A, Wang HY *et al*. The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 1997;**24:**72–77.
4. Socransky SS, Haffajee AD, Smith C *et al*. Microbiological parameters associated with IL-1 gene polymorphisms in periodontitis patients. *J Clin Periodontol* 2000;**27:**810–818.
5. Aurer A, Jorgić-Srdjak K, Plancak D *et al*. Proinflammatory factors in saliva as possible markers for periodontal disease. *Coll Antropol* 2005;**29:**435–439.
6. Veenstra TD, Conrads TP, Hood BL *et al*. Biomarkers: mining the biofluid proteome. *Mol Cell Proteomics* 2005;**4:**409–418.
7. Henskens YM, van den Keijbus PA, Veerman EC *et al*. Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J Periodont Res* 1996;**31:**57–65.
8. Groenink J, Walgreen-Weterings E, Nasmi K *et al*. Salivary lactoferrin and low-M_r mucin MG2 in *Actinobacillus actinomycetemcomitans*-associated periodontitis. *J Clin Periodontol* 1999;**26:**269–275.
9. Wong DT. Salivary diagnostics powered by nanotechnologies, proteomics and genomics. *J Am Dent Assoc* 2006;**137:** 313–321.
10. Ryu OH, Atkinson JC, Hoehn GT, Illei GG, Hart TC. Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass

spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology (Oxford)* 2006;**45:**1077–1086.
11. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003;**422:** 198–207.
12. Yates JR III. Mass spectral analysis in proteomics. *Annu Rev Biophys Biomol Struct* 2004;**33:**297–316.
13. Zhu H, Bilgin M, Snyder M. Proteomics. *Annu Rev Biochem* 2003;**72:**783–812.
14. Sandholm L, Tolo K, Olsen I. Salivary IgG, a parameter of periodontal disease activity? High responders to Actinobacillus actinomycetemcomitans Y4 in juvenile and adult periodontitis. *J Clin Periodontol* 1987;**14:**289–294.
15. Tinoco EM, Beldi MI, Loureiro CA *et al*. Localized juvenile periodontitis and *Actinobacillus actinomycetemcomitans* in a Brazilian population. *Eur J Oral Sci* 1997;**105:**9–14.
16. Haubek D, Ennibi OK, Poulsen S *et al*. Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*. *J Dent Res* 2001;**80:**1580–1583.
17. Johnson V, Johnson BD, Sims TJ *et al*. Effects of treatment on antibody titer to *Porphyromonas gingivalis* in gingival crevicular fluid of patients with rapidly progressive periodontitis. *J Periodontol* 1993; **64:**559–565.
18. Rhodus NL, Cheng B, Myers S *et al*. A comparison of the pro-inflammatory, NF-κB-dependent cytokines: TNF-alpha, IL-1-alpha, IL-6, and IL-8 in differentoral fluids from oral lichen planus patients. *Clin Immunol* 2005;**114:**278–283.
19. Henskens YMC, van der Velden U, Veerman EC *et al*. Protein, album and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis and periodontitis. *J Periodont Res* 1993; **28:**43–48.
20. Califano JV, Schifferle RE, Gunsolley JC *et al*. Antibody reactive with *Porphyromonas gingivalis* serotypes K1-6 in adult and generalized early-onset periodontitis. *J Periodontol* 1999;**70:**730–735.
21. Nygård O, Nilsson L. Translational dynamics. Interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. *Eur J Biochem* 1990;**191:**1–17.
22. Cammarano P, Palm P, Creti R, Ceccarelli E, Sanangelantoni AM, Tiboni O. Early evolutionary relationships among known life forms inferred from elongation factor EF-2/EF-G sequences: phylogenetic coherence and structure of the archaeal domain. *J Mol Evol* 1992; **34:**396–405.
23. Kivela J, Parkkila S, Parkkila AK, Leinonen J, Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. *J Physiol* 1999; **520**(Pt 2)**:**315–320.
24. Ball WD, Mirels L, Hand AR. *Psp* and *Smgb*: a model for developmental and functional regulation in the rat major salivary glands. *Biochem Soc Trans* 2003; **31**(Pt 4)**:**777–780.
25. Ghafouri B, Kihlstrom E, Stahlbom B *et al*. PLUNC (palate, lung and nasal epithelial clone) proteins in human nasal lavage. *Biochem Soc Trans* 2003;**31**(Pt 4)**:** 810–814.
26. Geetha C, Venkatesh SG, Bingle L *et al*. Design and validation of anti-inflammatory peptides from human parotid secretory protein. *J Dent Res* 2005;**84:** 149–153.
27. Bingle CD, Bingle L. Characterization of the human *plunc* gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. *Biochim Biophys Acta* 2000;**1493:**363–367.
28. Singh PK, Parsek MR, Greenberg EP *et al*. A component of innate immunity prevents bacterial biofilm development. *Nature* 2002;**417:**552–555.
29. Henskens YM, van der Weijden FA, van den Keijbus PA *et al*. Effect of periodontal treatment on the protein composition of whole and parotid saliva. *J Periodontol* 1996;**67:**205–212.
30. Henskens YM, Veerman EC, Mantel MS *et al*. Cystatins S and C in human whole saliva and in glandular saliva in periodontal health and disease. *J Dent Res* 1994;**73:**1606–1614.
31. DiMartino SJ, Kew RR. Initial characterization of the vitamin D binding protein (Gc-globulin) binding site on the neutrophil plasma membrane: evidence for a chondroitin sulfate proteoglycan. *J Immunol* 1999;**163:**2135–2142.
32. Meng H, Xu L, Li Q, Han J, Zhao Y. Determinants of host susceptibility in aggressive periodontitis. *Periodontology* 2007;**43:**133–159.