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Elucidating role of salivary proteins in denture stomatitis using a proteomic approach†

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Abstract

Denture stomatitis (DS) is the most common oral pathology among denture wearers, affecting over one-third of this group. DS is usually associated with *C. albicans*. However, unlike other oral candidiasis, most DS patients have intact host immunity. The presence of a denture alone is usually sufficient for DS. Saliva and its protein contents can theoretically predispose some denture wearers to DS and others resistant toward DS. Here we proposed for the first time to define salivary proteomic profiles of denture wearers with and without DS. SELDI-TOF/MS analysis suggests that there is a proteomic differentiation among control, localized and generalized DS. Based on initial SELDI-TOF/MS profiling, we further used reversed phase liquid chromatography, MALDI-TOF/MS, and LC-MS/MS to characterize the salivary proteins associated with DS. Nineteen proteins based on SELDI-TOF/MS profiling were found including cystatin-SN, statherin, kininogen-1, desmocollin-2, carbonic anhydrase-6, peptidyl-prolyl *cis–trans* isomerase A like peptides, cystatin C, and several immunoglobulin fragments. The proteomic content gives evidence of the interaction between host tissue, saliva, and candida. Further examination in larger populations of these proteins may help to gain a better understanding of DS pathological processes and improve DS treatments.

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Statement of author contributions

SB1, GFR, SS, JC, ZL, SB2 and SO contributed the design of the study. SB1, GFR, SS, WCB and JC conceived and carried out mass spectrometry analysis and data analysis. SKA and MBB recruited subjects, collected samples, and data analysis. SB1, MBB, CRM, CFP, WCB and SS wrote the manuscript. All authors had final approval of the submitted and published versions.

Introduction

Denture stomatitis (DS) refers to an inflammatory reaction of mucosal tissue beneath the denture. DS affects one-third to one-half of all denture wearers and, therefore, is the most common oral pathological lesion in denture-wearing patients.[1–5] The etiology of DS is complex and there are controversial theories of how DS may be developed. There are recognized etiological factors for DS including the presence of a denture, candidal organism (in denture, saliva, or mucosal tissues), traumatic factors (often related to ill-fitting dentures), inflammatory/immune response of the host, and oral environment (*e.g.* saliva, other micro-organisms, *etc.*).[1,6,7] DS clinical pathology was described by Newton in 1962 who classified DS into types I, II, and III depending on the extent of clinical pathology.[8]

DS only occurs in denture wearers and removal of the denture eliminates the condition.[1–3] The roles of other factors in DS development are not clear. DS is often associated with candidal infection; however unlike other forms of oral candidiasis, most DS patients have an intact systemic immunity. In another difference, the presence of candida in mucosal tissues does not correspond to the clinical severity of DS.[2] The porosity of acrylic denture base allows for biofilm formation and retention of microorganisms (such as candida) both on the denture surface and within the denture.[2,9] The presence of candida organisms in dentures is directly associated with the extent of the mucosal tissue inflammation.[7] The fitting of dentures and irritation caused by denture movement often intensify DS.[1–3,6]

Clinically some denture patients present with recurrent or non-responsive DS while others almost never develop DS suggesting that host factors play a role in DS development. One unique feature of DS is the characteristic clinical inflammation of mucosal tissues underneath the denture. Note also that DS is very common in maxillary dentures, regardless of how well-fitted the denture is, while DS is much less abundant in mandibular dentures.[1,6,7] DS appears, therefore, to be patient-specific and a reaction to site-specific inflammatory responses. Besides inflammatory host responses, the quantity of saliva and salivary contents is important in all forms of oral candidiasis, including DS. Lack of saliva, or xerostomia, is highly prevalent in-populations as a result of aging, medication side effects, and other therapies, and further predisposes patients to DS.[10,11]

Certain salivary proteins have been shown to play a role as biomarkers and antifungal proteins for oral candidiasis. For example, anti-p24 antibodies were increased in HIV+ patients with oral candidiasis.[12] Increased salivary expression of Th1 and Th2 cytokines, including IFN-gamma and IL-4, was shown to reduce candidal colonization in mice.[13] Low levels of salivary histatin-5, a protein shown to have anti-candidal activity *in vitro*, are correlated to increased candidal infection in HIV+ patients.[14–16] While there are several studies investigating the role of salivary proteins in other forms of oral candidiasis, the role of salivary proteins in DS that occurs in the healthy denture wearing population is not well-understood.

In this study, we compared the proteomic profiling of edentulous patients with and without DS using SELDI-TOF/MS to see if we could differentiate DS and non-DS denture wearing subjects with salivary proteomics. Furthermore, we identified DS-associated salivary proteins using MALDI-TOF/MS and LC-MS/MS based on SELDI-TOF/MS profiling. The purpose of parallel platform utilization was to allow screening of the biomarkers from samples in each individual subject without identification using the SELDI-TOF platform, and at the same time, to allow identification of biomarkers using pooled samples from control and DS II groups (the group with the majority of biomarkers with highest expression compared to control and DS III). Using a combination of mass spectrometry- based

proteomic analyses, we provide novel proteomic insight into the role of salivary proteins in DS.

Results

Saliva samples were first analyzed on four different Protein Chip arrays as described earlier. Several SELDI-TOF mass spectra of salivary protein profiles demonstrate differences in profiles corresponding to different arrays for each subject. Examples of SELDI-TOF/MS profiles are shown in Fig. 3 and 4. All identified peptide masses are summarized in Table 3. Out of 61 identified masses, only 13 potential masses are down-regulated (Table S1, ESI[†]). All peptide masses but one that are down-regulated are small peptides ($m/z < 2300$). Out of 48 up-regulated masses, 11 are more intense in DS II, for instance, the IMAC30 12 679 Da mass and the CM10 13 264 Da mass (Fig. 3 and 4). The 13 264 Da mass has its highest expression in the DS II samples. The IMAC30 10 621 Da and 12 679 Da masses are up-regulated in both DS groups. Similarly, the Q10 3822 Da mass shows up-regulation in the DS samples, in particular DS II. This 3822 Da mass was later identified (see below). The data analysis reveals a set of peptide masses with lower expression levels in the DS samples. It is possible that an elevated proteolytic activity under DS conditions may contribute to smaller peptide masses. The CM10 35 125 Da mass is most dramatically reduced in the DS II samples (Table 3). The Q10 12 954 Da mass shows a trend for lower expression, especially in the DS III samples. Finally, the same mass at 2260 Da was detected on three Protein Chip arrays; CM10, IMAC30 and Q10 arrays. SELDI-TOF/MS demonstrates several protein/peptide expression unique to the healthy non-DS control, DS II, and DS III subjects. When compared to all three groups, DS II demonstrates the most dramatic changes of the masses (Table 3, Fig. 3 and 4). Fourteen masses have the highest relative expression in DS II. We used ROC/AUC analyses (Table 3) to demonstrate the expression change for each mass. Down-regulated masses have ROC close to 0, while up-regulated ones have ROC close to 1. ROC ranges from 0.175 (~1436 Da mass) to 0.933 (~13 297 and 33 247 Da masses).

Based on SELDI-TOF/MS profiling we further used LC-MS/MS to examine each mass corresponding to SELDI peptide masses. The identities of 19 peptide masses were elucidated (Tables S1 and S4, ESI[†]). We identified four secreted proteins from salivary glands: statherin (STAT), cystatin-SN (CYTN), carbonic anhydrase 6 (CAH6), and peptidyl-prolyl *cis–trans* isomerase A (PPIA) like peptides; a plasma/tissue protein: kininogen-1 (KNG-1); an epithelial protein: desmocollin-2 (DSC2); and several IgG variable region immunoglobulin fragments. Except for one biomarker, KNG-1, which was eluted out from the ProteinChip array, all of the biomarkers were identified based on approximate m/z values from SELDI-TOF. We compared the level of each identified peptide mass between the DS and control groups. Note that all of the identified peptide masses are up-regulated in DS and most of them have the highest relative expression level in DS II. The level of expression for each peptide (Tables 3 and 4, Table S1, ESI[†]) is described below. We, therefore, chose pooled control samples and DS II samples for LC-MS/MS analysis.

The level of STAT ($m/z = 3822.4$) was increased over threefold in patients with DS compared to controls. Similarly, the level of expression of CYTN ($m/z = 7146.5$) was significantly increased in DS patients. The expression of KNG-1 ($m/z = 5227$) was increased over two-fold in patients with DS compared to controls. The salivary level of DSC2 ($m/z = 12608.4$) was four to sixfold higher in the DS patients. PPIAs ($m/z = 13267.3$) were increased over two-fold in DS subjects. Using mucosal transcriptome analysis we found that PPIA gene expression was 1- to 5-fold higher in DS-affected mucosa compared to the

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unaffected tissues in the same DS patient (Table S2, ESI†). CYTC (m/z= 13274.1) level was elevated in DS patients. Multiple proteins were identified as candidate immunoglobulin (Ig) variable region fragments. All these Ig fragments were highly elevated in DS, especially in the DS type II cases which reflects acute localized DS. They are also elevated (however to a lesser degree than type II) in DS III, which presents with more chronic inflammation and exists as a more generalized form of DS. This tentative identification was based on a significant number of high scoring protein hits from the marker-containing fractions for Ig variable regions.

Discussion

To elucidate the role of salivary proteins in DS development, we examined a salivary proteomic analysis of DS. We selected edentulous patients wearing a maxillary denture with DS compared to edentulous control subjects without DS. Our selection criteria used were aimed to eliminate subjects that may be prone to oral candidiasis as a result of other host factors, for example medication, cancer therapy, immunocompromised conditions, *etc.*[17,18] Non-edentulous (dentate) subjects may have DS if they wear a partial denture. However, the presence of teeth can change the dynamic of biomarkers. Several proteins from circulation travel to the oral cavity through the periodontium around the teeth. The expression of serum and inflammatory proteins from the periodontium is dependent on the periodontal health and number of remaining teeth. Moreover, dentate patients with DS often present with periodontal disease or dental caries. This will confound the results. Therefore, we elected to not include dentate patients. While the cross-sectional nature of the study limits our interpretation to a hypothesis-generating study, identified proteins seem to represent a complex etiological feature of DS. In this study, we limited recruitment to healthy patients in order to represent the elderly edentulous denture-wearing population with intact immune systems.[17,18]

SELDI-TOF/MS analyses provide an unbiased proteomic profile for subject.[19–22] It was important to note here that this proteomic profiling corresponds well with clinical and histological diagnosis of DS. More importantly, each protein is differentially expressed among the non-DS, DS II and DS III individuals. DS development is poorly understood. However, our SELDI-TOF/MS analysis suggests that the DS II group seems to have a more robust host response in saliva. This agrees with the clinical feature of DS II that is localized and acute in nature. On the other hand, the SELDI-TOF/MS profiles suggest the less robust but chronic inflammatory responses in the DS III compared to DS II and control groups.

It is important to note that several of the candidate SELDI markers were not identified similar to previously suggested by other investigators.[23–25] This is likely due to several factors. First, protein concentration in the original sample may have been high enough for detection, however, dilution and protein loss during separation may have resulted in the protein or corresponding peptides being below the limit of detection for the MS system. Second, the protein biomarker may have been detected, however, native protein cleavage processes may not be characterized or documented in the existing protein databases. Therefore, the molecular weight (M_w) of the detected proteins in the database does not match the M_w determined by SELDI analysis. Third, differences in system ionization and instruments between the SELDI, MALDI and ESI systems may have resulted in detection of the protein in one instrument, but not across all instruments. Fourth, poor LC/MS/MS fragmentation and database annotation can result in collected LC/MS/MS data that do not match database entries. And finally, modified proteins are frequently identified with low probabilities or mis-identified due to limitations of the database search software. The proteins identified using the methodology in this study are candidate protein matched to the

original SELDI M_w signal. The differential expression of these candidate protein biomarkers will need further assessment and validation in the future.

While SELDI-TOF/MS provides an overall picture of salivary proteomes, it does not give protein identities. We therefore further used pooled saliva samples from control and DS II to define the identity of each mass based on the initial SELDI-TOF/MS profiles. We were surprised to observe that identified masses associated with DS in our study are different from those previously identified in other forms of oral candidiasis. It was suspected that the differences may be a result of different etiological factors, especially in regards to the subjects systemic immunity. In summary, we identified three groups of DS-associated masses based on their origins, including salivary gland, mucosal/epithelial, and immune system origins.

Four salivary gland secreted proteins, including STAT, CYTN, CAH6, and PPIA, were identified. We propose that these proteins may enhance DS through their interactions with candida organism. The higher expression of STAT and CYTN in DS patients suggests the role of salivary proteins in biofilm formation which is important in candidal colonization in dentures.[9] Salivary STAT and CYTN are both associated with Sjögren syndrome.[26–28] It has been shown that STAT and CYTN function together in biofilm formation and mineralization in dentate patients.[29–31] The salivary level of CYTN was increased in situation of a bleeding oral cavity.[32] In addition, it was shown that salivary CYTN inhibits lysosomal protease activity of cathepsins.[33] It is therefore plausible that these proteins may facilitate candidal adherence and growth in dentures. CAH6 is an interesting salivary protein that was found to be associated with several diseases including diabetes, periodontitis, dental caries, breast cancer, and Sjögren syndrome.[28,34–38] CAH6 catalyzes the reversible reaction $H_2CO_3 = CO_2 + H_2O$[39] and, therefore, regulates salivary pH. This controlled pH environment may facilitate candidal growth in saliva. Note that instances of depleted CAH6 have been linked to higher risks of dental caries and periodontitis, an outcome which may be a result of favoring bacterial growth (not fungal growth). Note also that CAH6 is one of the most common masses in saliva associated with oral and systemic disease.[37–38,40–42] PPIAs were shown to play an important role in the infection of HIV to host cells.[43,44] The role of PPIA peptides in candidal infection has yet to be understood. Three mucosal/epithelial originated proteins, including KNG-1, DSC-2, and CYTC, were up-regulated in DS patients, providing evidence of tissue damage resulting from DS. KNG-1 is a vasodilator.[45] It is likely that KNG-1 expression is a result of inflammation in the mucosal tissue. Similarly, DSC-2 demonstrates the signs of epithelial disruption.[46] CYTC was thought to be associated with dry mouth, gingivitis and periodontitis.[36,47,48]

It is important to note here that the majority of identified DS-associated proteins in our study are immunoglobulin (Ig) fragments (11 out of 18 proteins). Again these masses express at the highest level in the DS II group. These Igs can be a contribution of the salivary glands, mucosal tissues, or systemic host immune systems. Increased Igs associated with DS are similar to other conditions including breast cancer,[35] dental caries,[30,40] periodontal disease,[37] and Sjögren syndrome.[26–28] While it is unlikely that Igs would be derived from major salivary glands or systemic circulation in DS cases, it is possible that the inflamed palatal salivary glands and palatal mucosa may be the major source of Igs.[49–56] It is important in the future to examine microbial-based immunoglobulin destruction-specific cleavage of Ig protease. The large presence of Igs clearly demonstrates an important role for the B cell-mediated immune response in DS that is often neglected, as it was previously suggested that T cells and macrophages mediate immune response in DS.[2,57,58] In addition, traditional DS treatments focus only on eliminating candidal organisms and physical trauma. In DS patients that are not responsive to these treatments, anti-inflammatory medications may positively enhance other DS treatments.

Conclusion

In conclusion, our proteomic analyses suggest that salivary proteomic profiling has a potential in differentiating non-DS and DS subjects and therefore studying the salivary proteins based on these profiles may provide insight into DS development. The majority of DS-associated proteins are up-regulated in the DS group, especially DS II. Salivary proteins originating from salivary glands may predispose patients to DS through enhancing candidal survival or biofilm formation. Mucosal/epithelial originated proteins may be a result of tissue damage resulting from the DS condition. Igs, the major group of proteins identified, suggest the role of B cell mediated immunity, especially in DS II. Based on this investigation, we concluded that salivary proteomics may be an important research tool for studying DS in a prospective manner in the future.

Experimental procedures

Subjects and samples

Ten denture patients diagnosed with DS, using type II (DS II) and III (DS III) Newton classifications (localized and generalized DS),[8] and nine control denture patients without DS were recruited (Fig. 1). All subjects wore a maxillary complete denture and were carefully selected to minimize confounders (Table 1) as part of the larger cohort.[17] The study protocol was approved by the University of North Carolina Office of Human Research Ethics (IRB No. 07-2014). All subjects consented using a written consent form. The clinical diagnosis of DS was complemented by histological assessments (biopsy and swabs of the lesion), and by culture (tissue, denture, and saliva). Similar to our previous saliva sample protocol,[17,18] subjects were asked to refrain from drinking, eating, and practicing any oral hygiene habits 2 hours before sample collection. Prior to collection, subjects will be asked to rinse the mouth with distilled water. This same protocol was used throughout our entire study to standardize the protein biomarkers and to reduce contamination from food, tissue debris and blood. All saliva samples will be collected before lunch time between 9 am and 12 pm to standardize the collection and, more importantly, to minimize the circadian effect. Approximately 4 ml of unstimulated whole saliva sample was collected in a 15 ml Falcon tube. The tube was then centrifuged to remove food and tissue debris. The supernatant will be placed into aliquots of 250 μl. All aliquots will be immediately frozen in liquid nitrogen to avoid enzymatic and bacterial degradation of the protein content. The subject population includes a control (edentulous patients wearing complete dentures with no DS) group (n=9), and a DS group (n = 10); the DS II (n = 6) and the DS III (n = 4) (Table 2). The samples were kept in −80 °C until all samples were transferred to the mass spectrometry facility. Mass spectrometry analyses were completed for all samples simultaneously. The operators were blinded of the group assignment until the data analysis stage. Fig. 2 shows the flowchart of the mass spectrometry analyses.

Mass spectrometry proteomic analysis

Materials, reagents, and denaturation protocol—Materials for SELDI-TOF/MS analysis included Denaturant (9 M urea, 2% CHAPS), Bio-Rad ProteinChip CM10 arrays, Bio-Rad ProteinChip H50 arrays, Bio-Rad ProteinChip IMAC30 arrays, Bio-Rad ProteinChip Q10 arrays, and Bio-Rad SPA matrix. Specific reagents that were used included CM10 low stringency buffer: 0.1 M Na-acetate, pH 4.0; H50 binding buffer: 10% acetonitrile, 0.1% TFA; IMAC30 binding buffer: 0.1 M sodium phosphate, 0.5 M NaCl, pH 7.0, and Q10 binding buffer: 50 mM Tris–Cl, pH 9.0. Each sample was denatured using the following protocol. Saliva samples (40 μl) were denatured by addition of 60 μl urea/CHAPS denaturant solution and incubated on ice for 30 minutes. A pooled reference sample

(consisting of 10 μl aliquots of each individual denatured saliva sample) was used to optimize the volume utilized in the SELDI protocol.

Instrument setting and protocol—Each of the saliva fractions was profiled on four ProteinChip array types: CM10, H50, IMAC30 and Q10. The ProteinChip arrays were equilibrated by two 5 minutes washes with 100 μl of the appropriate binding buffers listed above. A total of 95 μl binding buffer was added to each ProteinChip array, followed by 5 μl of the denatured saliva. The samples were shaken for 1 hour using a MicroMix shaker. The samples were removed and the arrays were washed with 3 × 150 μl aliquots of binding buffer (5 minutes per wash step). The arrays were washed twice with 200 μl water (1 minute per wash) to remove excess salts. The arrays were allowed to air dry. Finally, 2 × 1 μl aliquots of SPA matrix (50% saturation, prepared in 50% acetonitrile, 0.5% TFA) were added to each spot. The arrays were inserted into a ProteinChip (Enterprise) TOF-MS. Quality control features were included as follows. Each sample was profiled in duplicate. In addition, the pooled denatured saliva was profiled in hextuplicate on each array type to assess reproducibility and performance. All samples were subjected to Protein Chip and SELDI-TOF/MS at the same time. The operator was blinded of sample group assignment.

Data acquisition and analysis—The Bio-Rad SELDI ProteinChip reader (Enterprise version) was used to collect spectra. Using the pooled reference samples, optimal laser settings of 1800 nJ for low mass (2–20 K) and 3800 nJ for high mass (10–200 K) spectra were determined. Both sets of spectra were obtained from each spot. SELDI ProteinChip Data Manager software was utilized. Spectra underwent proper baseline fit and subtraction. Spectra profiled on a specific chip type and using identical laser settings, were normalized relative to the total ion current across the spectra. Mass calibration was performed using All-in-1 peptide and All-in-1 protein standards. Univariate analysis was carried out to indicate specific *m*/*z* markers, which demonstrate differential expression across the various groups. Peak clusters were assigned using the Expression Difference Mapping (EDM) feature of ProteinChip Data Manager software.

Each cluster was constructed by detecting individual peaks with signal/noise values >5 and which were present in greater than 20% of the spectra. The peak intensities detected in the duplicate spectra obtained for each sample were averaged, providing the 21 independent samples for statistical (Mann– Whitney) analysis. The Wilcoxon rank sum test or Kruskal–Wallis analysis of variance (ANOVA) was performed for numeric variables. Pearson's chi-square test is used for categorical variables. For each mass, the discriminatory power was further described *via* receiver operating characteristic (ROC) area under the curve (AUC) analysis using SPSS Version 12.0 software (SPSS Inc., Chicago, IL).[18] Initially, the control, DS II and DS III spectra were analyzed as 3 separate groups. These data are presented in the "Split" markers listed in the Combined Markers spreadsheet. In addition, comparison of the control spectra *vs.* the combined DS (II and III) groups was carried out. These markers are listed in the "Merged" marker list on the same spreadsheet.

Saliva sample 2-dimensional LC separation—A pooled patient aliquot was created for each of the healthy and somatitis II sample subsets and used for protein separation and marker identification. Each pooled aliquot was separated on an Agilent 3100 OFFGEL Fractionator. A total of 4 lanes were prepared for each sample; 500 μL of sample was loaded into each lane. A standard protein QC mix was also prepared for each plate utilized. Samples were separated using a 64 kV h^{-1} separation according to the manufacturer's instructions. A total of 24 fractions were collected after separation. These fractions were pooled, to generate 6 fractions for each of the healthy and DS II samples.

Each of the OFFGEL saliva fractions and a single selected QC OFFGEL fraction were then separated by LC. Solvent A was 95/5/0.1 H_2O/ACN/FA, solvent B was 5/95/0.85 H_2O/ACN/FA. Each OFFGEL fraction was pre-loaded onto a PROTO 300 C4 5 μm particle size (20 × 2.1 mm) trap column offline using a syringe pump, and the bound material was washed with at least 1 ml 100% A. The trap column was then placed in-line in the LC system and the proteins were separated on a PROTO 300 C4 5 μm particle size (150 × 2.1 mm) column using an Agilent 2100 LC system coupled to an Akta fraction collector. A linear LC gradient 0.2 μL min^{-1} of 0–10 min at 5% B, 12 min to 10% B, 50 min to 80% B, 55 min to 100% B, hold for 10 min at 100% B was used for protein separation. Fractions were collected each minute throughout the gradient.

Collected fractions were concentrated to dryness in a vacuum centrifuge and the proteins were re-solubilized in 10 μL 75/25/0.1 ACN/H_2O/FA. Concentrated samples were layered 1 : 1 with a MALDI matrix (20 mg ml^{-1} sinapinic acid in 75/25/0.1 ACN/H_2O/FA) and deposited on a MALDI target plate. Samples were analyzed on an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer. Two spectra were acquired for each sample, from m/z 800–20 000 and 2000–100 000. Mass spectra were screened using an automated in-house program for masses that corresponded to the candidate markers.

Protein identification—LC fractions containing the m/z markers of interest were trypsin digested and analyzed by LC/MS/MS for protein identification. Fractions were concentrated to dryness in a vacuum centrifuge and reconstituted in 50 μL 50 mM ammonium bicarbonate. Fractions for digestion were reduced and alkylated, and digested overnight with trypsin. Fractions were concentrated prior to LC/MS using a vacuum centrifuge. Processed fractions were analyzed by LC/MS/MS for protein ID. A total of 10 μL of each processed sample was injected and separated on a Waters nanoAQCUITY UPLC coupled to an Applied Biosystems 4000 QTRAP system. A Waters nanoACQUITY BEH 1.7 μm particle size (100 μm × 10 cm) column was used for the protein separation. Mass spectra results were submitted to Mascot (Matrix Sciences) for a database search against the SwissProt (March 2009) and NCBI (December 2009) Human database. Search results were compared against the intact marker M_w identified in the sample fractions. Candidate protein identifications were highlighted that matched or could account for the intact M_w detected by SELDI and MALDI MS analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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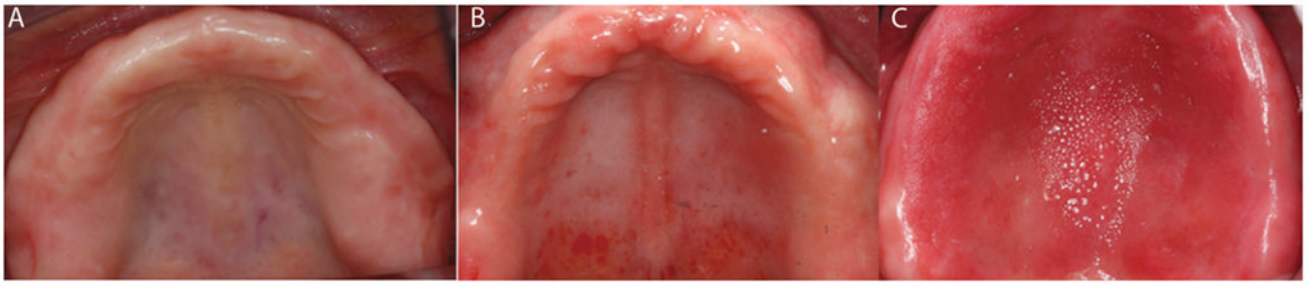

Fig. 1.
Clinical photographs of (A) a control subject, (B) Newton classification II stomatitis, and (C) Newton classification III stomatitis.

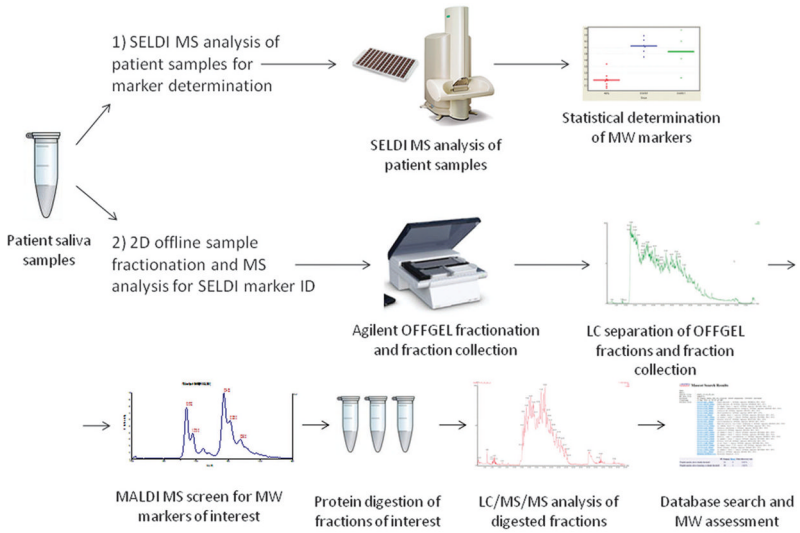

Fig. 2.

Flow diagram showing (top) SELDI-TOF/MS analysis screening for masses associated with DS in denture patients by mass (*m*/*z*), and (bottom) combination of liquid chromatography, MALDI-TOF/MS and LC-MS/MS in the identification process for masses correlated with *m*/*z* information from SELDI-TOF/MS.

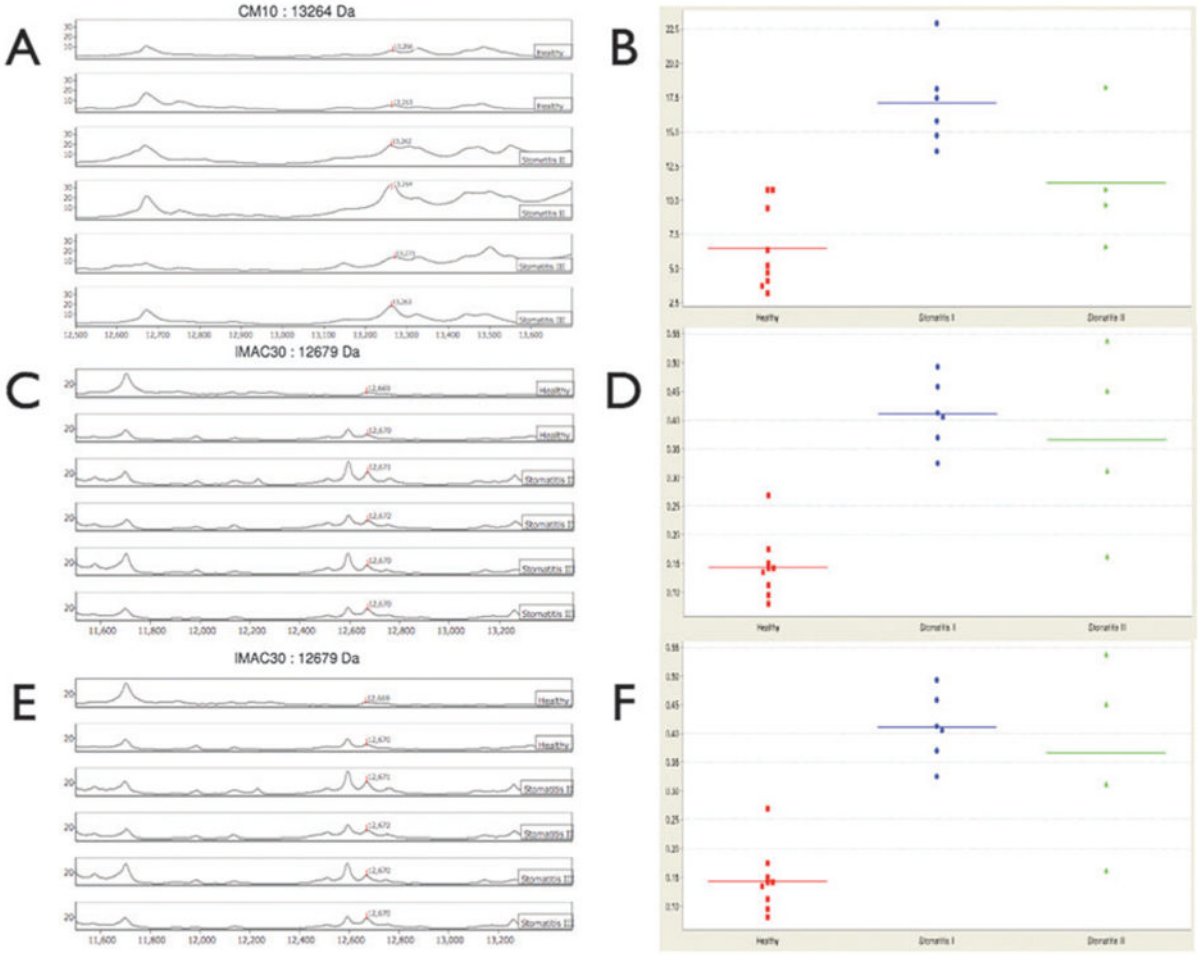

Fig. 3.

Examples of SELDI-TOF profilings for healthy control, DS II and III samples; (A) mass~2260 Da (Q10 array); (B) mass~2260 Da (Q10 array); and (C) mass ~12 670 Da (IMAC30 array).

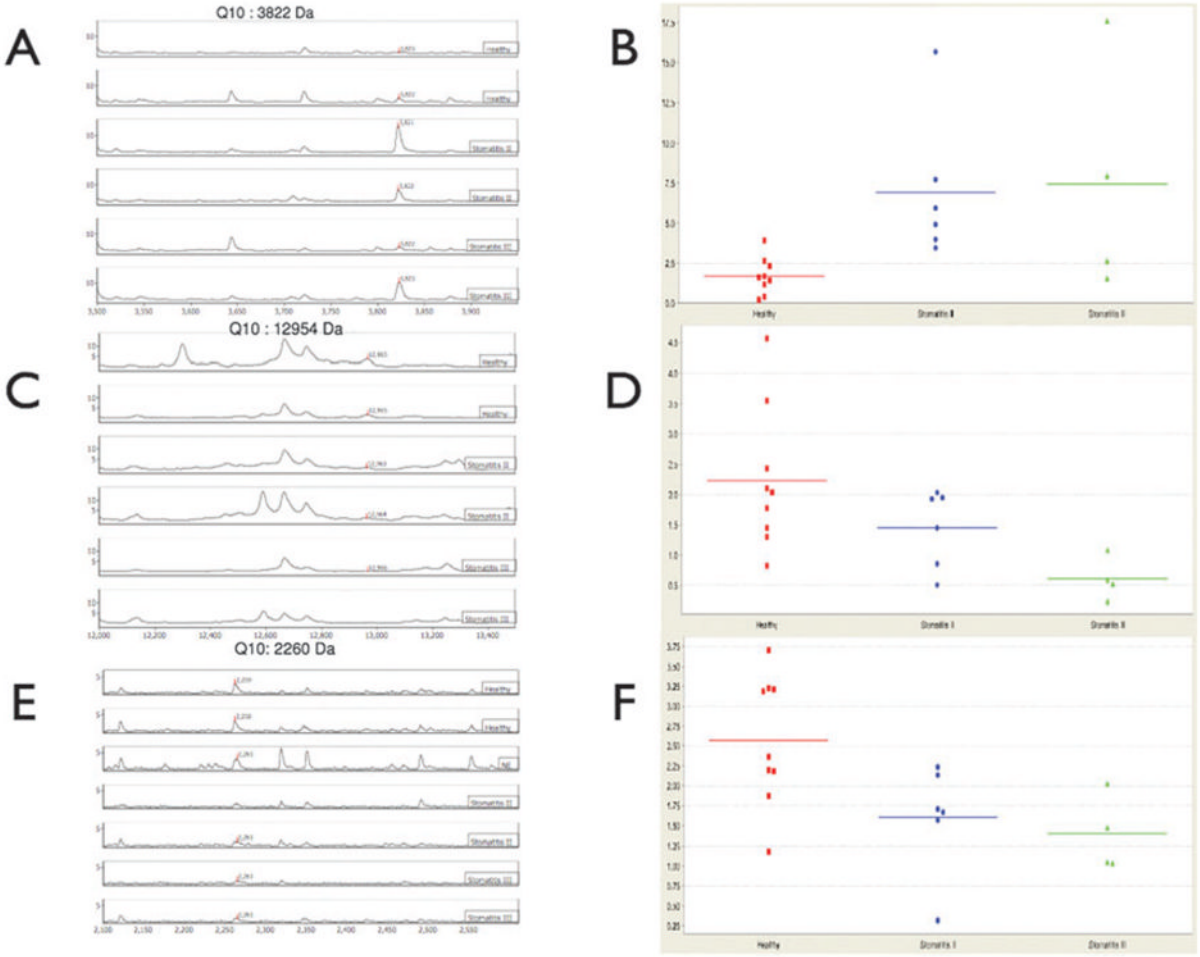

Fig. 4.

Differential fold expression of 4masses identified by SELDI-TOF/MS in four different Protein Chip arrays; (A) H50 array with 5283 kDa mass, (B) Q10 array with 5227 kDa mass, (C) IMAC30 array with 12 679 kDa mass, and (D) CM10 array with 13 274 kDa mass.

Table 1

Inclusion and exclusion criteria

Inclusion criteria		Exclusion criteria	
1	At least 45 years of age	**1**	Less than 45 years of age
2	Male or females without menses for 12 consecutive months or who have had a complete hysterectomy	**2**	Have chronic disease with oral manifestations other than denture/ mucosal stomatitis
3	Wear complete maxillary denture (overdentures, implant or tooth retained dentures acceptable) without daily use of denture adhesives	**3**	Have gross oral pathology
4	Must have read, understood and signed an informed consent form	**4**	Have overt denture abrasion associated with symptoms
5	Must understand and be willing to comply with all study procedures and restrictions	**5**	Participants with clinically significant organic diseases, including impaired renal function, bleeding disorder, or any condition requiring antibiotic pre-medication for dental visits
6	Must be in good general health; diabetics included	**6**	Participants with active infectious diseases such as hepatitis, HIV or tuberculosis
7	Must have Type II or Type III DS for the denture stomatitis group	**7**	Participants who are immunosuppressed because of medications or condition
8	Must have no signs of DS for the control group	**8**	Participants who have used antibiotics or antifungals for any medical or dental condition within 1 month prior to screening
		9	Participants using ongoing medications initiated less than 3 months prior to enrollment
		10	Participants with a known or suspected intolerance to local oral anesthesia
		11	Participants who have participated in another clinical study or have taken an investigational drug within 30 days of screening
		12	Participants who have used tobacco products within 6 months of screening
		13	Employees of the sponsor or the investigator or members of their immediate family
		14	Participants who have previously participated in this study
		15	Post-menopausal women on hormone replacement therapy

Table 2

Patient demographic data

	Control (*n* = 9)	Stomatitis (*n* = 10) DS II (*n* = 6)	DS III (*n* = 4)	All stomatitis
Age average (SD)	71.9 (9.74)	70.3 (8.36)	64.3 (9.81)	67.9 (8.99)
Gender (M/F)	1/8	3/3	2/2	5/5
Race (Caucasian/African American/Asian)	7/2/0	5/0/1	1/2/1	6/2/2
Ethnicity (Nonhispanic/Hispanic)	9/0	6/0	4/0	10/0

Table 3

Summary of SELDI-TOF/MS analysis. Mass (*m*/*z*), relative intensity for each group, Protein Chips, *p* value and ROC (between healthy and combination of DS I and II)

m/*z*	Healthy	DS I	DS II	Chip	*p* Value[a]	ROC[b]
1436.4[d]	3.5	2.5	2.5	h50	0.04123	0.175
1610.7[d]	3	2.6[e]	—	h50	0.03376	0.250
1965.1[d]	4.5	4	2	q10	0.04892	NaN[c]
2121.6[d]	9	5	5	h50	0.04123	0.250
2257.4[d]	1.4	1.1	0.5	cm10	0.04499	NaN
2259.9[d]	2.7	1.6	1.1	imac30	0.00329	0.117
	2.5	1.7	1.5	q10	0.00705	0.217
2481.5	3	3.5	0.5	cm10	0.04317	NaN
3369.3	1.3	4.6[e]	—	imac30	0.03376	0.733
	5.5	8.5[e]	—	cm10	0.04123	0.733
3822.4	**2**	**7.2**	**7.2**	**q10**	0.00192	0.900
4271.0	1.7	2.4	2.4	h50	0.04123	0.733
4606.5	1	3	2.4	cm10	0.03376	0.700
5219.4	1	2.5	1.4	cm10	0.04715	NaN
5227.4	**4**	**10**	**10**	**q10**	0.04123	0.767
5283.1[d]	0.9	0.6	0.6	h50	0.02749	0.283
6299.0	0.4	0.75	0.53	imac30	0.00705	0.833
7146.5	**0.8**	**0.12**	—	**imac30**	0.04122	0.742
8085.5	0.7	10[e]	—	imac30	0.02749	0.767
8134.2	0.1	0.5	0.5	imac30	0.02224	0.767
9078.0	12	30	35	imac30	0.00705	0.833
9170.8	14	50[f]	40	imac30	0.00705	0.833
9207.5	0.2	1	0.8	imac30	0.00145	0.900
10597.1[d]	34	15	15	q10	0.01431	0.283
10620.9	5	30[f]	15	imac30	0.00082	0.900

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m/z	Healthy	DS I	DS II	Chip	*p* Value[a]	ROC[b]
10656.0	0.1	0.5[f]	0.3	imac30	0.00045	0.900
10807.7	11	24[e]	—	imac30	0.04123	0.708
10827.0	**0.14**	**0.36**	—	**imac30**	0.02749	0.700
12588.8	**2**	**6**	**6**	**q10**	0.02749	0.700
12601.9	**7**	**29**		**imac30**	0.02749	0.767
12608.4	**0.15**	**0.61**	**0.44**	**imac30**	0.00898	0.800
12658.4	**0.2**	**0.4**	—	**cm10**	0.04123	0.742
12673.6	6	22[f]	16	**imac30**	0.00192	0.833
12679.6	**0.15**	**0.4**	**0.35**	**imac30**	0.00045	0.900
12954.6[d]	2.3	1.5	0.6	q10	0.02224	0.250
13260.5	0.1	0.2[f]	0.16	q10	0.00898	0.867
13263.3	**6**	**12**	**7**	**imac30**	0.01431	0.833
13267.3	**2.6**	**6**	**6**	**q10**	0.01789	0.833
13274.1	**7**	**17**	**11**	**cm10**	0.00192	0.933
13296.9	**0.25**	**0.45**	**0.35**	**cm10**	0.02749	0.767
13469.0	**4**	**10**	—	**imac30**	0.01137	0.867
13537.5	**0.1**	**0.5**	**0.3**	**imac30**	0.00192	0.867
13552.9	**6**	**23**	**24**	**imac30**	0.00550	0.867
13645.2	**5**	**15**	**14**	**imac30**	0.01137	0.867
15291.8[d]	**8**	**3**	**3**	**cm10**	0.01789	0.217
16821.1[d]	0.9	0.6	0.6	h50	0.03376	0.317
26924.8	1.7	2.5	2.5	cm10	0.04123	0.742
28031.5	0.7	2.3[e]	—	imac30	0.02224	0.742
33247.5	0.6	1.3	1.9	cm10	0.00191	0.933
35124.9[d]	0.8	0.3[g]	0.6	cm10	0.03855	NaN
41758.0	1.6	0.25	0.25	h50	0.03376	0.642
66360.5	2	4[f]	3	cm10	0.01789	0.808
80430.1	0.5	1	1.5	cm10	0.01430	0.800
94635.0	0.1	0.3	0.4	cm10	0.00145	0.833

m/z	Healthy	DS I	DS II	Chip	*p* Value[a]	ROC[b]
112199.6[d]	0.7	0.2[e]	—	imac30	0.02224	0.208
13296.9	0.25	0.5[e]	0.33	Imac30	0.02749	0.767

[a] *p* Value of 0.05 was used as a cut-off point.

[b] ROC from 0 to 1; closer to 0 referred to downregulation, and closer to 1 referred to upregulation.

[c] These masses appeared in three-group comparison and therefore had no ROC value.

[d] Downregulated masses.

[e] Combined DS I and II due to high variable values.

[f] The highest relative intensity in the DS II group.

[g] The lowest relative intensity in the DS II group. The masses that later got ID are shown in bold.

Table 4

Summary of identified peptide masses

Protein	Mass (Da)
Statherin (STAT)[a]	~3822
Kininogen-1 (KNG1)	~5227
Cystatin-SN (CYTN)	~7146.5
Desmocollin-2 (DSC2)	~12608.4
Carbonic anhydrase 6 (CAH6)	~13 274
Cystatin C (CYTC)	~13 274
Two peptidyl-prolyl *cis–trans* isomerase A (PPIA) like peptides	
gi|239741695	~13267.3
gi|239741695	~13 274
Eleven fragments of immunoglobulins (Ig) and homologous peptides	
Unknown Ig-like peptide	~10 827
KV206	~12585.1
KV206 or KV404	~12588.8
gi|15777209	~12607.8
Unknown Ig variable region	~12 680
HV301	~13 469
HV310	~13 537
HV310	~13537.5
gi|224808232	~13552.9
HV307	~13645.2
gi|1082553	~15 291

[a]The mass was IDed off the Protein Chip.