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Quantitative analysis of differentially expressed saliva proteins in human immunodeficiency virus type 1 (HIV-1) infected individuals

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Abstract

In the present study, we have established a new methodology to analyze saliva proteins from HIV-1-seropositive patients before highly active antiretroviral therapy (HAART) and seronegative controls. A total of 593 and 601 proteins were identified in the pooled saliva samples from 5 HIV-1 subjects and 5 controls, respectively. Forty-one proteins were found to be differentially expressed. Bioinformatic analysis of differentially expressed salivary proteins showed an increase of antimicrobial proteins and decrease of protease inhibitors upon HIV-1 infection. To validate some of these differentially expressed proteins, a high-throughput quantitation method was established to determine concentrations of 10 salivary proteins in 40 individual saliva samples from 20 seropositive patients before HAART and 20 seronegative subjects. This method was based on limited protein separation within the zone of the stacking gel of the 1D SDS PAGE and using isotope-coded synthetic peptides as internal standards. The results demonstrated that a combination of protein profiling and targeted quantitation is an efficient method to identify and validate differentially expressed salivary proteins. Expression levels of members of the calcium-binding S100 protein family and deleted in malignant brain tumors 1 protein (DMBT1) were up-regulated while that of Mucin 5B was down-regulated in HIV-1 seropositive saliva samples, which may provide new perspectives for monitoring HIV-infection and understanding the mechanism of HIV-1 infectivity.

Graphical abstract

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Keywords

HIV-1; Saliva; Quantitation; Limited separation; Proteomics

1. Introduction

Human saliva is regarded as a perfect sample to be explored for health and disease surveillance [1]. It is a biofluid that is readily accessible via a totally safe and noninvasive method. Salivary fluid is produced mainly by the major salivary glands and contains a number of various biomolecules, including proteins and ions transported from serum [2,3]. Salivary proteins are involved in digestion, antimicrobial activity, lubrication and cleaning [4,5]. Changes of the compositions are associated with diseases [6–8]. In the past two decades, salivary diagnostic tools have been developed to monitor diseases [6,9–14] including Acquired Immune Deficiency Syndrome (AIDS) by detecting human immunodeficiency virus (HIV) and HIV antibodies [15–19]. It has been recommended saliva as a safe and effective alternative to serum for HIV antibody testing in surveillance programs [20,21].

Recently, studies have been carried out to comprehensively catalog the salivary proteome with regard to cellular localization, biological processes and molecular functions [22–25]. Patient-based proteomics and genomics have also been performed for the discovery of biomarkers in saliva [7,26]. Furthermore, a multicenter systematic comparison of human saliva and plasma proteomes has provided the useful insights of saliva for exploring potential biomarkers of diseases [27]. It has been noted since 1986 that factors present in human saliva could inhibit HIV infectivity in vitro [28,29]. Malamud et al. [30] detected a decrease in viral infectivity in T cells by incubating HIV-1 with human saliva. Subsequently, proteins and peptides in saliva were found to inhibit HIV-1 infection [31–33]. However, the mechanism of the inhibition of HIV infection by saliva is not fully understood. It has been reported that the compositions or functions of saliva will alter after infection [34]. Some differentially expressed proteins were identified in cerebrospinal fluid and sera for HIV-1 associated dementia [35,36]. However, changes of saliva proteins upon HIV-1 infection have not been profiled and identification of the differentially expressed proteins in HIV-1 seropositive patients is an important step in understanding effects of HIV-infection on human biofluids.

In the present work, we have applied LC–MS/MS based protein profiling to find differentially expressed salivary proteins in HIV-1 seropositive patients before highly active antiretroviral therapy (HAART) and seronegative controls. To validate the differentially expressed proteins, a simple sample preparation method was established to determine concentrations of selected salivary proteins in individual saliva samples. This quantitative method is based on the limited protein separation within the stacking zone of 1D SDS PAGE. After in-gel digestion, isotope-encoded peptides were added as internal standards, followed by LC–MS/MS analysis. With this method, we were able to determine the concentrations of 10 proteins in human whole saliva and identified several proteins that are potential makers for monitoring HIV-infection.

2. Materials and methods

2.1. Sample collection

Recruitment of subjects was carried out by the AIDS Clinical Trial Unit (ACTU) at Bellevue Hospital in New York City, following the full IRB approval from NYU and Bellevue. Flyers announcing the study were posted throughout the medical center and at local HIV testing sites. Inclusion criteria required HIV infected subjects (age 18 and above) who were antiretroviral naïve, but ready to begin therapy. HIV uninfected subjects were matched to the HIV+ group in terms of gender, age, and race. Exclusion criteria include the pregnant women, and current antibiotic users. Samples were barcoded, aliquoted and frozen at -80°C . For all subjects (HIV+ and HIV–) there were a full medical history survey and an oral examination. For HIV+ individuals, viral load and CD4/CD8 values were determined at each visit. Whole saliva samples were collected using the previously established protocols [37]. The specimen was centrifuged at $14,000 \times g$ for 5 min to remove unwanted particles (debris or cells). A protease inhibitor cocktail containing sodium orthovanadate and dithiothreitol was added to minimize the degradation of the proteins (Sigma, St Louis, MO). The specimen was then divided into aliquots and stored at -80°C .

2.2. Sample preparation and protein separation

Aliquots from five HIV-1 seropositive patients before HAART and five seronegative subjects were thawed and pooled, respectively, followed by centrifuge at $14,000 \times g$ for 5 min to eliminate particles in suspension. Subsequently, 500 μL of the pooled saliva sample was centrifuged in a speedvac to reduce the volume. After incubation with the LDS sample buffer (Invitrogen, Grand Island, NY) at 100°C for 5 min, the saliva proteins were separated on a 4–20% gradient Tris-Glycine 5-well gel (Invitrogen, Grand Island, NY) and stained with Colloidal Coomassie Blue (Invitrogen, Grand Island, NY).

2.3. In-gel digestion and LC–MS/MS analysis

Each lane was cut into 10 slices and each gel slice was reduced with 10 mM dithiothreitol (Calbiochem, San Diego, CA) and alkylated with 100 mM iodoacetamide (Sigma, St Louis, MO). In gel digestion was then carried out with sequencing grade modified trypsin (Promega, Fitchburg, WI) in 50 mM ammonium bicarbonate at 37°C overnight. The peptides were extracted twice with 1% trifluoroacetic acid in 50% acetonitrile aqueous solution for 30 min. Extracts were then centrifuged in a speedvac to reduce volume to 40 μL .

For LC–MS/MS analysis, each digestion product was separated by a 65 min gradient elution at a flow rate $0.250 \mu\text{L min}^{-1}$ with a Dionex 3000 nano-HPLC system, which was directly interfaced with a Thermo LTQ–Orbitrap mass spectrometer. The analytical column was a home-made fused silica capillary column ($75 \mu\text{m ID}$, 150 mm length ; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 Å, $5 \mu\text{m}$, Varian, Lexington, MA). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The LTQ–Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.0.7 software and there is a single full-scan mass spectrum in the Orbitrap ($400\text{--}1800 m/z$, 30,000 resolution) followed by 6 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy.

2.4. Data processing and relative quantitation of differentially expressed salivary proteins

MS/MS spectra from each LC–MS/MS run were converted from RAW file format to DTA files using BioWorks 3.3.1 (Thermo-Fisher, San Jose, CA). The DTA files were searched against the human IPI database using an in-house Mascot searching algorithm. The following search parameters were used in all of the Mascot searches: maximum of 1 missed trypsin cleavage, cysteine carbamidomethylation as fixed modification, methionine oxidation as the variable modification. The maximum error tolerance was 10 ppm for MS and 0.8 Da for MS/MS. Proteins were designated as “hits” only when the Mascot score was more than 30 and there were at least 2 unique peptides matches. When several proteins matched the same sets of peptides, only the proteins with the greater percentage of coverage were selected. Significance was regarded only when the ratio of spectral counts between two groups were more than 2 or less than 0.5. The differentially expressed proteins were then annotated and analyzed using DAVID Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/home.jsp>) to connect proteins to biological processes.

2.5. Quantitation of differentially expressed salivary proteins with synthetic peptides

Isotope-encoded peptides corresponding to tryptic peptides of selected proteins were synthesized with the standard Fmoc chemistry and purified at the Proteomics Resource Center of Rockefeller University. The isotope-encoded amino acids were used at selected positions in peptide sequences. To quantify the differentially expressed proteins, salivary samples from HIV-1 seropositive patients ($n = 20$) before HAART and HIV-1 seronegative subjects ($n = 20$) were used. The quantitation was performed using the protocol established earlier. Briefly, $250 \mu\text{L}$ of whole saliva from each individual was separated within the stacking zone of the 5-well SDS PAGE gel. The gel was stained by Coomassie Blue, and each lane was cut into 5 bands, followed by reduction, alkylation and in-gel digestion. Subsequently, tryptic products from 5 bands were pooled and spiked with the synthetic isotope-encoded peptides at certain concentrations. Then the pooled digestion products were separated by a 180 min gradient elution on a LTQ–Orbitrap mass spectrometer. The quantitation was carried out using the accurate mass full scan mass spectrometry.

2.6. Statistical analysis

Statistical analysis of the quantitative data was carried out using the SPSS 16.0 for windows. Independent sample *t*-test was applied and both equalities of variances and means were tested. Significance was regarded when *P*-value was less than 0.05.

3. Results

3.1. Identification of salivary proteins separated on 1D SDS PAGE followed by LC–MS/MS

Salivary proteins from 500 µL of pooled saliva samples were separated by 1D SDS PAGE, as shown in Fig. 1A. After in-gel digestion, the tryptic peptides from HIV-1 seropositive patients and seronegative subjects were analyzed by LC–MS/MS, respectively. A total number of 593 proteins from HIV-1 seropositive subjects were identified and 601 proteins from seronegative subjects. The false positive rate for protein identification by Mascot searching was determined by decoy database searching, and was estimated to be 1%. Although the number of proteins identified was a little lower than what have been reported, our results are more reliable since MS measurement was carried out with an LTQ-Orbitrap mass spectrometer with a mass measurement error less than 10 ppm. Earlier studies carried out with an LTQ mass spectrometer usually use 3 Da as the mass measurement error, which greatly increases the false positive rate.

3.2. Identification of salivary proteins differentially expressed in HIV-1 seropositive patients and seronegative subjects by spectral counts

Spectral counts use the number of spectra that have been assigned to a specific protein, to quantify the relative abundance of a protein from two samples. Using 2 folds or more changes as the determinant, we have identified 20 proteins up-regulated and 21 proteins down-regulated in patients with HIV-1 comparing to controls. The differentially expressed proteins are listed in Table 1. Defensins, which are small cysteine-rich peptides, were only identified in HIV-1 seropositive samples.

3.3. Biological processes and molecular functions of salivary proteins differentially expressed in HIV-1 seropositive patients and seronegative subjects

Differentially expressed proteins were analyzed by the Functional Annotation Clustering of DAVID Bioinformatics Resources 6.7. Biological processes of these proteins were displayed in Fig. 2. Protease inhibitors, including Cystatin C, D, S, SA and SN, A2ML1 and MUC5B, were all down-regulated in HIV-1 seropositive patients compared with seronegative controls. Thirteen proteins, S100A7, S100A8, S100A9, S100A12, CLU, DMBT1, IL1RN, LTF, LGALS3BP, LYZ, PLUNC, PGLYRP1 and PRDX2, were involved in defense response, and were up-regulated in HIV-1 seropositive patients, except IL1RN, which has been reported to block induction of HIV replication in vitro [38]. Antimicrobial proteins, such as S100A7, LTF, DMBT1, LYZ and alpha-defensin all showed higher expression levels in HIV-1 seropositive samples. Five enzymes, TPI1, ENO3, LDHAL6B, GAPDH and PGK1 that were associated with glycolysis and gluconeogenesis were down-regulated in HIV-1 seropositive samples. Other differentially expressed proteins were linked to oxidative stress, immune response, calcium ion binding and nucleotide binding.

3.4. Quantitation of 10 selected proteins in 40 saliva samples

Ten peptides from 8 differentially expressed proteins listed in Tables 1 and 2 unchanged proteins, kalikrein 1 (KLK1) and lipocalin1 (LCN1), were synthesized. The isotope-encoded amino acids were used at selected positions in peptide sequences except peptides from CA6 and DMBT1, as marked in Table 2. The leucine residue in the native peptide of LENSLLDHR from CA6 was replaced by a valine residue in the synthetic peptide; the second valine residue in the native peptide of FGQSGPIVLDDVR from DMBT1 was replaced by an isoleucine residue. Masses of two peptides were shifted by 14 Da. The cysteine residue in alpha-defensin peptide, IPACIAGER, was reduced and alkylated before it was spiked into the tryptic peptide samples.

The image of saliva proteins separated in the zone of the stacking gel of the 1D SDS PAGE was shown in Fig. 1B. In general, the limited separation can be completed in less than half an hour. After in-gel digestion, 10 standard peptides were spiked into tryptic peptide products from each individual sample, followed by LC-MS/MS analysis. Quantitation of selected proteins was manually performed by using the accurate mass full scan mass spectrometry. Fig. 3 showed quantitation of S100A8 with synthetic standard peptide, which was up-regulated in HIV-1 seropositive individuals compared to that in seronegative controls. Based on the intensity ratio for each pair of peptides, concentrations of proteins in whole saliva were calculated for each individual sample. The mass of each protein was obtained from the Uniprot webpage (<http://www.uniprot.org/>).

The concentrations of 10 proteins in whole saliva of HIV-1 seropositive patients and seronegative subjects were listed in Table 3. There were significant differences of 7 proteins between HIV-1 seropositive patients and seronegative controls, which have been found to be differentially expressed by protein profiling as shown in Table 1. No significant difference of CA6 was found in the two groups ($P = 0.132$), nor were KLK1 and LCN1. Compared with those in seronegative controls, S100A7, S100A8, S100A9, alpha-defensin and DMBT1 were all up-regulated in the HIV-1 seropositive samples ($P < 0.05$), while MUC5B were down-regulated ($P < 0.05$). In summary, the results were consistent with those obtained in pooled samples by spectral counts.

4. Discussion

Saliva is an important biofluid that has been used for diagnosis and disease monitoring. The saliva protein components were less complex than those of plasma and serum, allowing saliva to be processed directly without depletion of high abundance proteins. In the present work, we have profiled saliva proteins from HIV-1 seropositive patients and seronegative subjects. Forty-one salivary proteins were found to be differentially expressed in HIV-1 seropositive patients before the highly active antiretroviral therapy and seronegative controls by spectral counts. In order to determine effects of HIV-infection on saliva proteome, HIV-seronegative subjects were matched to HIV+ subjects in terms of gender, age, and race. It is expected that HIV infection is the main factor to define the difference of saliva proteins between HIV-seropositive subjects and seronegative controls. Biological processes and molecular functions of 41 proteins were analyzed by DAVID Bioinformatics Resources. As expected, expressions of antimicrobial proteins, such as S100A8, S100A9, alpha-defensin

and DMBT1, were all up-regulated in HIV-1 seropositive patients compared with those in seronegative subjects. S100A8 and S100A9 are members of S100 protein family of calcium binding proteins. Both proteins were found to be elevated in serum in association with HIV infection [39,40]. Recently, a dysregulated neutrophil response to S100A8/A9 was implicated as a potential source for immune dysfunction in HIV disease [41]. DMBT1, also called glycoprotein-340 (gp340), has been reported to inhibit HIV-1 infectivity through interaction with viral glycoprotein 120 [42]. Alpha-defensins are small cysteine-rich antimicrobial peptides, which are important components of innate immunity [43]. Alpha-defensin 1, 2, and 3 were all found to suppress HIV-1 replication [44]. All three alpha-defensins have a common tryptic peptide, IPACIAGER and the concentration measured in the present work represents the total alpha-defensin in samples.

Earlier studies have shown that human saliva inhibits HIV-1 infectivity [45]. Mucins have been shown to aggregate HIV particles to reduce viral infectivity [46]. It has been reported that cystatins interfere with the proteolytic process occurring in the virus life cycle by inhibiting viral cysteine proteases [47]. In the present studies, six protease inhibitors including Cystatin C, D, S, SN, SA and MUC5B were all down-regulated in saliva from HIV-1 seropositive individuals as quantified by spectra counts. It has been reported that cystatins, defensins, lactotransferrin, lysozyme and mucins have anti-HIV activities [48]. Lactotransferrin and lysozyme, the anti-viral salivary proteins involved in enzyme inhibitor activity were found to be down-regulated. The lower concentrations of these inhibitors in saliva may contribute to the infectivity of HIV-1.

Although spectra counts have been accepted as a general means for protein quantitation, it suffers from sketchy accuracy and narrow dynamic range. The accuracy of spectra counts is further compromised when it is applied to low abundance proteins. Moreover, it is difficult to compare quantitation results by spectra counts from different laboratories and from different batches of samples. To better quantify the differentially expressed proteins, isotope-coded peptides have been used to determine absolute concentrations of proteins. The other limitation of the current proteomic profiling method is time consuming. It is difficult to profile multiple samples using a combination of 1D SDS PAGE and LC/MS/MS with limited instrumentation time. In order to increase throughput of sample analysis, we developed the limited separation method for saliva protein analysis that was based on separation of proteins in the zone of the stacking gel of 1D SDS PAGE. Separation can be completed in half an hour that eliminates excessive peptide components in saliva. For convenience of digestion, the gel band containing proteins were cut into 5 pieces, digested, and pooled up together as one sample for the following LC-MS/MS analysis and quantitation. Therefore, we are able to quantify multiple proteins from multiple samples within relative short time period.

Within 41 differentially expressed proteins identified by spectra counts, we have chosen 10 to perform targeted quantitation using synthetic isotope-coded peptides as internal standards. Results of 40 individual saliva samples from 20 seropositive patients and 20 seronegative subjects showed that S100A7, A8, and A9, DMBT1, alpha-defensin were significantly up-regulated ($P < 0.05$) and Cystatin C and MUC5B down-regulated ($P < 0.05$) in HIV-1 seropositive samples compared with seronegative samples. It is worth mentioning that the

expression level of Cystatin C is significantly down-regulated in HIV-1 seropositive samples based on quantitation by spectra counts (Table 1). However, the difference in Cystatin C concentrations is less significant between HIV-1 seropositive and seronegative samples (Table 3), indicating that measurement accuracy by spectra counts is rather compromised. The value obtained for CA6 in healthy controls was $4.58 \pm 2.09 \mu\text{g mL}^{-1}$ (range: $2.04\text{--}9.19 \mu\text{g mL}^{-1}$) that is close to values reported in literature [49]. However, concentrations of some proteins determined in the present study were lower than those reported values. The concentration of alpha-defensin in human saliva was reported to range from 0.1 to $10 \mu\text{g mL}^{-1}$ [50] and what we detected in the present study was $0.116 \pm 0.137 \mu\text{g mL}^{-1}$ (range: $0.002\text{--}0.403 \mu\text{g mL}^{-1}$) in HIV-1 seropositive samples and $0.043 \pm 0.051 \mu\text{g mL}^{-1}$ (range: $0.000\text{--}0.134 \mu\text{g mL}^{-1}$) in seronegative samples. The statistical analysis of alpha-defensin in each individual sample showed significant variances. In addition, the concentration of Cystatin C in saliva of healthy adults was reported to be $1.8 \mu\text{g mL}^{-1}$ on average (range: $0.36\text{--}4.8 \mu\text{g mL}^{-1}$) [51], which was higher than that was determined in the present study $0.481 \pm 0.176 \mu\text{g mL}^{-1}$ (range: $0.225\text{--}0.988 \mu\text{g mL}^{-1}$). The limitation of small sample size used in the present study is also an obvious concern. Results obtained in the present study need to be validated in the further study with a larger sample size.

In conclusion, we have profiled the differentially expressed salivary proteins in HIV-1 seropositive patients and seronegative subjects by spectral counts and quantified 10 selected proteins from 40 saliva samples with a high throughput method. The quantitative method was based on the limited separation within the stacking zone of 1D SDS PAGE gel combining with targeted proteomics using synthetic peptides as the internal standards. This method adds a new tool for quantitative analysis of saliva proteome. Our results showed an increase of antimicrobial proteins S100A8, A9, DMBT1 and alpha-defensin, and a decrease of enzyme inhibitors, Cystatin C and MUC5B in whole saliva of HIV-1 seropositive patients compared with seronegative subjects, which provides information to understand effects of HIV-1 infection on human saliva proteome.

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References

1. Wong DT. *Expert Rev. Mol. Diagn.* 2006; 6:272.
2. Humphrey SP, Williamson RT. *J. Prosthet. Dent.* 2001; 85:169.
3. Haeckel R, Hanecke P. *Eur. J. Clin. Chem. Clin. Biochem.* 1996; 34:192.
4. Mandel ID. *J. Dent. Res.* 1987; 66:627.
5. Van Nieuw AA, Bolscher J, Veerman E. *Caries Res.* 2004; 38:253.
6. Li Y, John MARS, Zhou X, Kim Y, Sinha U, Jordan RCK, Eisele D, Abemayor E, Elashoff D, Park NH. *Clin. Cancer Res.* 2004; 10:8450.
7. Hu S, Wang J, Meijer J, Jeong S, Xie Y, Yu T, Zhou H, Henry S, Vissink A, Pijpe J. *Arthritis Rheum.* 2007; 56:3600.
8. Aydin S. *J. Biochem. Mol. Biol.* 2007; 40:35.
9. Malamud D. *BMJ.* 1992; 305:208. [PubMed: 1392819]

10. Tabak LA. J. Dent. Educ. 2001; 65:1339.
11. Malamud D. J. Am. Dent. Assoc. 2006; 137:286.
12. Wong DT. J. Am. Dent. Assoc. 2006; 137:321.
13. Wong DT, Segal A. Eur. J. Dent. Educ. 2008; 12:29. [PubMed: 18257762]
14. Zhang L, Xiao H, Wong DT. Mol. Diagn. Ther. 2009; 13:259.
15. Hodinka R, Nagashunmugam T, Malamud D. Clin. Diagn. Lab. Immunol. 1998; 5:426.
16. Mortimer PP, Parry JV. Clin. Diagn. Virol. 1994; 2:243.
17. Freel SA, Williams JM, Nelson JAE, Patton LL, Fiscus SA, Swanstrom R, Shugars DC. J. Virol. 2001; 75:4940.
18. Reynolds SJ, Muwonga J. Expert Rev. Mol. Diagn. 2004; 4:591.
19. Balamane M, Winters MA, Dalai SC, Freeman AH, Traves MW, Israelski DM, Klausner DAJD. Open Virol. J. 2010; 4:93.
20. Frerichs RR, Htoon MT, Eskes N, Lwin S. Lancet. 1992; 340:1499.
21. Major CJ, Read SE, Coates RA, Francis A, McLaughlin BJ, Millson M, Shepherd F, Fanning M, Calzavara L, MacFadden D. J. Infect. Dis. 1991; 163:702.
22. Vitorino R, Lobo MJC, Ferrer-Correia AJ, Dubin JR, Tomer KB, Domingues PM, Amado FML. Proteomics. 2004; 4:1115.
23. Guo T, Rudnick PA, Wang W, Lee CS, DeVoe DL, Balgley BM. J. Proteome Res. 2006; 5:1478.
24. Helmerhorst EJ, Oppenheim FG. J. Dent. Res. 2007; 86:693.
25. Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL. J. Proteome Res. 2004; 3:1023.
26. Hu S, Yu T, Xie Y, Yang Y, Li Y, Zhou X, Tsung S, Loo RR, Loo JA, Wong DT. Cancer Genom. Proteom. 2007; 4:64.
27. Yan W, Apweiler R, Balgley BM, Boontheung P, Bundy JL, Cargile BJ, Cole S, Fang X, Gonzalez Begne M, Griffin TJ. Proteom. Clin. Appl. 2009; 3:134.
28. Fultz PN. Lancet. 1986; 1:1215. [PubMed: 2877344]
29. Fox PC, Wolff A, Yeh CK, Atkinson JC, Baum BJ. J. Am. Dent. Assoc. 1988; 116:637.
30. Malamud D, Davis C, Berthold P, Roth E, Friedman H. AIDS Res. Hum. Retroviruses. 1993; 9:637.
31. Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, Orenstein JM. Oral Dis. 1997; 3:S69.
32. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, Kimani J, Lopalco L, Piconi S, Bwayo JJ. J. Immunol. 2000; 165:5176.
33. Quiñones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, Marotta ML, Mirza M, Jiang B, Kiser P. AIDS. 2003; 17:F48.
34. Fox PC. Oral Surg. Oral Med. Oral Pathol. 1992; 73:170.
35. Rozek W, Ricardo-Dukelow M, Holloway S, Gendelman HE, Wojna V, Melendez LM, Ciborowski P. J. Proteome Res. 2007; 6:4199.
36. Wiederin J, Rozek W, Duan F, Ciborowski P. Proteome Sci. 2009; 7:8. [PubMed: 19292902]
37. Navazesh M. Ann. N. Y. Acad. Sci. 2006; 694:77.
38. Poli G, Kinter AL, Fauci AS. Proc. Natl. Acad. Sci. 1994; 91:112.
39. Müller F, Frøland SS, Aukrust P, Fagerhol MK. J. Acquir. Immune Defic. Syndr. 1994; 7:939.
40. Strasser F, Gowland PL, Ruef C. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. 1997; 16:238.
41. Schwartz R, Lu Y, Villines D, Sroussi HY. Biomed. Pharmacother. 2010; 64:575.
42. Wu Z, Van Ryk D, Davis C, Abrams WR, Chaiken I, Magnani J, Malamud D. AIDS Res. Hum. Retroviruses. 2003; 19:209.
43. Ganz T. Nat. Rev. Immunol. 2003; 3:720.
44. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmaso EA, Fu S, Pham T, Mei J, Ho JJ. Science. 2002; 298:1000. [PubMed: 12411698]
45. Shugars DC, Wahl SM. J. Am. Dent. Assoc. 1998; 129:858.

46. Bergey EJ, Cho MII, Blumberg BM, Hammarskjöld ML, Rekosh D, Epstein LG, Levine MJ. J. Acquir. Immune. Defic. Syndr. 1994; 7:1002.
47. Campo J, Perea MA, Del Romero J, Cano J, Hernando V, Bascones A. Oral Dis. 2006; 12:228.
48. Shugars DC, Sweet SP, Malamud D, Kazmi SH, Page-Shafer K, Challacombe SJ. Oral Dis. 2002; 8:175.
49. Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H. Caries Res. 1999; 33:184.
50. Gardner MS, Rowland MD, Siu AY, Bundy JL, Wagener DK, Stephenson JL Jr. Anal. Chem. 2008; 81:566.
51. Filler G, Bökenkamp A, Hofmann W, Le Bricon T, Martínez-Brú C, Grubb A. Clin. Biochem. 2005; 38:8.

HIGHLIGHTS

- ▶ A high-throughput method for profiling and quantification of the differentially expressed proteins in saliva samples was developed.
- ▶ Identified that DMBT1, S100A7, S100A8, S100A9 and alpha defensin were up-regulated in saliva from HIV-1 seropositive patients.
- ▶ Established analytical strategies are translatable to the clinical setting.

Fig. 1.

(A) 1D SDS PAGE of saliva proteins from 500 μ L pooled whole saliva from 5 seropositive patients and 5 seronegative subjects, respectively. (B) The limited separation of 250 μ L of whole saliva by 1D SDS PAGE from four HIV-1 seropositive subjects.

Fig. 2. Biological processes of the differentially expressed proteins in HIV-1 seropositive patients and seronegative subjects. All the differentially expressed proteins were analyzed using DAVID Bioinformatics Resources showing significant enrichment of proteins involved in antimicrobial and defense response and a decrease of proteins involved in enzyme inhibitor activity.

Fig. 3. Quantitation of the differentially expressed protein S100A8 in whole saliva from HIV-1 seropositive patients and seronegative subjects. (A) MS/MS spectra of the native tryptic peptide from S100A8, GNFHAVYR, and the synthetic isotope-coded peptide with the same sequence. The fragmentation patterns of two peptides match b and y ions of peptides from S100A8. The masses of ion fragments above b4 and y3 have a 3 Da addition in the synthetic peptide in which alanine residue has three ¹³C atoms. (B) Mass spectra of the native peptide (MH²⁺: 482.24) and synthetic standard peptide (MH²⁺: 483.75) from S100A8 in HIV-1 seropositive patients and seronegative subjects. Quantitation of the protein was determined by the intensity ratio of two peptides. The concentration of S100A8 in HIV-1 seropositive patients is higher than that in HIV-1 seronegative subjects ($P < 0.01$).

Table 1

Salivary proteins differentially expressed in HIV-1 seropositive patients and seronegative subjects.

Protein ID	Protein name
Up-regulated	
IPI00022429	Alpha-1-acid glycoprotein 1 (ORM1)
IPI00291262	Clusterin (CLU)
IPI00418512	Deleted in malignant brain tumors 1 protein (DMBT1)
IPI00031547	Desmoglein-3 (DSG3)
IPI00219682	Erythrocyte band 7 integral membrane protein (STOM)
IPI00023673	Galectin-3-binding protein (LGALS3BP)
IPI00026314	Gelsolin (GSN)
IPI00438855	Interleukin-12 receptor beta-2 chain (IL12RB2)
IPI00298860	Lactotransferrin (LTF)
IPI00019038	Lysozyme C (LYZ)
IPI00021085	Peptidoglycan recognition protein (PGLYRP1)
IPI00027350	Peroxiredoxin-2 (PRDX2)
IPI00022974	Prolactin-inducible protein (PIP)
IPI00456158	Proline-rich protein BstNI subfamily 1 (PRB1)
IPI00009856	Protein Plunc (PLUNC)
IPI00218131	Protein S100-A12 (S100A12)
IPI00219806	Protein S100-A7 (S100A7)
IPI00007047	Protein S100-A8 (S100A8)
IPI00027462	Protein S100-A9 (S100A9)
IPI00022432	Transthyretin (TTR)
Down-regulated	
IPI00419215	Alpha-2-macroglobulin-like protein 1 (A2ML1)
IPI00218474	Beta-enolase (ENO3)
IPI00295105	Carbonic anhydrase 6 (CA6)
IPI00032293	Cystatin-C (CST3)
IPI00002851	Cystatin-D (CST5)
IPI00032294	Cystatin-S (CST4)
IPI00013382	Cystatin-SA (CST2)
IPI00305477	Cystatin-SN (CST1)
IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
IPI00000045	Interleukin-1 receptor antagonist protein (IL1RN)
IPI00025023	Lactoperoxidase (LPO)
IPI00016768	L-Lactate dehydrogenase A-like 6B (LDHAL6B)
IPI00027509	Matrix metalloproteinase-9 (MMP9)
IPI00855918	Mucin 5, subtype B, tracheobronchial (MUC5B)
IPI00152154	Mucin-7 (MUC7)
IPI00009123	Nucleobindin-2 (NUCB2)
IPI00219446	Phosphatidylethanolamine-binding protein 1 (PEBP1)

Protein ID	Protein name
IPI00169383	Phosphoglycerate kinase 1 (PGK1)
IPI00300376	Protein-glutamine gamma-glutamyltransferase E (TGM3)
IPI00299729	Transcobalamin-1 (TCN1)
IPI00465028	Triosephosphateisomerase (TPI1)

Table 2

Sequences of synthetic peptides from 10 selected saliva proteins.

Protein	Native peptide	Synthetic peptide	Mass difference (Da)
S100A7	GTNYLADVFEK	GTNYLA [*] DVFEK	4
KLK1	LTEPADTITDAVK	LTEPA [*] DTITDAVK	4
S100A8	GNFHAVYR	GNFHA [*] VYR	3
S100A9	LTWASHEK	LTWA [*] SHEK	4
LCN1	GLSTESILIPR	GLSTESILIP [*] R	6
Alpha-defensin	IPACIAGER	IPA [*] CIAGER	3
CA6	LENSLLDHR	V [*] ENSLLDHR	14
Cystatin C	ALDFAVGEYNK	A [*] LDFAVGEYNK	3
DMBT1	FGQGSGPIVLDDVR	FGQGSGPIVLDDI [*] R	14
MUC5B	TFDGDVFR	TFDG [*] DVFR	2

“*” represents marked amino acids in synthetic peptides were different from native peptides.

Differences of alanine-containing peptides are either 4 Da for having 3 ¹³C and 1 ¹⁵N atoms or 3 Da for having 3 ¹³C atoms in synthetic peptides.

The proline residue in synthetic peptide of LCN1 has 5 ¹³C and 1 ¹⁵N atoms. The glycine residue in synthetic peptide of MUC5B has 2 ¹³C atoms. The leucine residue in the native peptide of CA6 was replaced by valine in the synthetic peptide. The second valine residue in the native peptide of DMBT1 was replaced by isoleucine.

Table 3

Concentrations of ten proteins in whole saliva of HIV-1 seropositive patients and seronegative subjects determined by the accurate mass full scan mass spectrometry.

Protein	Group	<i>n</i>	Concentration ($\mu\text{g mL}^{-1}$) (mean $\pm$ SD)	<i>P</i> -value
S100A7	HIV(−)	20	0.003 $\pm$ 0.008	0.045
	HIV(+)	20	0.025 $\pm$ 0.046	
KLK1	HIV(−)	20	0.422 $\pm$ 0.168	0.343
	HIV(+)	20	0.487 $\pm$ 0.250	
S100A8	HIV(−)	20	0.422 $\pm$ 0.296	0.001
	HIV(+)	20	1.13 $\pm$ 0.795	
S100A9	HIV(−)	20	0.163 $\pm$ 0.250	0.013
	HIV(+)	20	0.829 $\pm$ 1.07	
LCN1	HIV(−)	20	1.08 $\pm$ 0.749	0.627
	HIV(+)	20	1.19 $\pm$ 0.656	
Alpha-defensin	HIV(−)	20	0.043 $\pm$ 0.051	0.035
	HIV(+)	20	0.116 $\pm$ 0.137	
CA6	HIV(−)	20	4.58 $\pm$ 2.09	0.132
	HIV(+)	20	3.50 $\pm$ 2.35	
Cystatin C	HIV(−)	20	0.481 $\pm$ 0.176	0.045
	HIV(+)	20	0.368 $\pm$ 0.164	
DMBT1	HIV(−)	20	12.9 $\pm$ 6.39	0.007
	HIV(+)	20	22.0 $\pm$ 12.8	
MUC5B	HIV(−)	10	15.9 $\pm$ 6.73	0.034
	HIV(+)	10	8.85 $\pm$ 7.04	