Pre-analytic saliva processing affect proteomic results and biomarker screening of head and neck squamous carcinoma

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Received October 26, 2006; Accepted December 8, 2006

Abstract. The objective of this study was to assess the effect of pre-analytical processing on proteomic analysis of saliva and to identify salivary biomarkers for potential clinical applications. Saliva samples from five healthy individuals and three head and neck squamous carcinoma (HNSC) patients were initially depleted of major protein constituents. Saliva from healthy subjects was divided and processed by three different methods prior to liquid chromatography and tandem mass spectrometry technique (LC-MS/MS) analysis. The results showed marked differences amongst the methods. The SDS-PAGE separation and in-gel digestion method yielded the highest number of proteins that included the majority of those identified by the other two methods. The in gel-digestion method was used in the LC-MS/MS analysis of saliva from three HNSC patients and the results were compared with those from healthy subjects. Our analysis identified two proteins, α-1-B-glycoprotein and complement factor B proteins, to be present in patients but not in normal specimens. Paradoxically, cystatin S, parotid secretory factor, and poly-4-hydrolase ß-subunit proteins were detected in most normal salivas but not in patient specimens. Subsequent analysis of complement factor B by Western blotting showed strong immunoreactive bands of complement factor B in HNSC patients' and negative or weakly positive in normal saliva samples. We conclude that: 1) initial saliva processing affects protein analysis, 2) in-gel digestion followed by LC-MS/MS detects the most saliva proteins, 3) certain proteins are differentially found in patient and normal salivas and 4) a small set of proteins can be targeted for future validation for clinical investigation.

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Abbreviations: HNSC, head and neck squamous carcinoma; LC-MS/MS, liquid chromatography and tandem mass spectrometry; SAX, strong anion exchange; TFA, trifluoroacetic acid; FPLC, fast-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBST, phosphate buffered saline Tween

Key words: saliva, biomarkers, head and neck, squamous cell carcinoma, proteomics

Introduction

Saliva is a readily accessible secretion that plays an important role in the maintenance of oral homeostasis, tooth integrity, initiation of digestion, and antimicrobial defense (1-3). Saliva is protein rich and may constitute a valuable source of diagnostic and therapeutic markers in head and neck squamous cell carcinoma (HNSC) patients (4-6). The major components of saliva are mucins, proline-rich glycoproteins, amylase, lipase and a variety of antimicrobial proteins and peptides that include agglutinin, cystatins, lisozyme, lactoferrin, immunoglobulins, histatins and defensins (7-17). Numerous less common proteins derived from either active salivary secretion or through plasma filtration have also been identified (18-21). Analysis of these proteins may provide important information in health and diseased states.

It has recently been acknowledged that proteomic analysis reflects the net sum of the metabolic and functional states of cells and secretions from tissues (22). Advances in both technological and bioinformatic fields have led to a plethora of efforts to identify diagnostic, prognostic and therapeutic targets using human secretions and body fluids (4). However, major obstacles remain to complicate and impede the clinical implementation of this technology in human materials. These include sample-related factors such as quality, timing, composition, sample collection and processing, technical limitations and the complexities and the dynamic variations of human proteins (22,23).

The increasing interest in proteomic profiling of saliva has led to several attempts to characterize its proteins (24-29). In these studies, only a limited number of normal specimens with varied collection and processing methods were analyzed. This suggests the need to define implications of saliva collection and evaluate the role of pre-analytical processing factors on the quality and nature of saliva protein analysis. To address these issues a standardized collection protocol and three different processing techniques were used to prepare

saliva specimens prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We then used the best method to analyze saliva from HNSC patients and compared the results to those of the healthy subjects to identify biological markers of potential clinical utility.

Materials and methods

Subjects. Whole saliva specimens were collected according to a standardized protocol from 8 individuals between the hours of 10 a.m. to 4 p.m. who consented to a protocol approved by the M.D. Anderson IRB committee. Of the 8 specimens, 5 were from healthy subjects (2 males and 3 females) and 3 from patients with HNSC, 2 males and 1 female. All saliva from HNSC patients were harvested prior to treatment.

Saliva protocol

Specimen collection. Stimulated whole saliva was collected from healthy volunteers and patients according to the following steps: 1) individuals were abstain from eating, drinking, smoking, or brushing their teeth for at least 60 min prior to collection, 2) to swallow or discard any accumulated saliva, 3) x^0 place a standard piece of paraffin (1.5 g) in the mouth and chewed on at a regular pace and 4) to expectorate saliva periodically into a preweighed disposable plastic cup with a preservative to inhibit enzymatic activity for a period of 5 min. The cup was then reweighed and the flow rate was determined gravimetrically. The volume and physical characteristics of the specimen were recorded. Whole saliva (~5 ml) was obtained from each individual.

Specimen processing. Each saliva sample was divided into 1-ml aliquots and placed into bar coded cryotubes. The specimens were immediately frozen (-80˚C) until used. Samples were thawed and centrifuged at 14,000 x g at 4˚C for 10 min to remove suspended particulates. A preliminary LC-MS/MS analysis of normal salivas showed that amylase, albumin, immunoglobulins, transferrin and haptoglobin to be the major protein components that may mask the analysis of uncommon and low level proteins. To deplete samples of these proteins we used multiple affinity columns (Agilent, Palo Alto, CA). Saliva supernatant (200 μl) was diluted to 450 μl with buffer A (Agilent) and subjected to the affinity columns. The volume of saliva used for the depletion process was determined by comparing the level of albumin in serum and saliva using Western blotting with anti-albumin antibody (data not shown). The flow through fractions were collected and dialyzed against water overnight (molecular weight cut-off: 3.5 kDa). Saliva proteins and peptides <3.5 kDa were not included in the present study. The samples were then concentrated with vacuum centrifuge and the volume was reconstituted to 50 μl with water. Each saliva specimen was divided and analyzed using three different procedures as illustrated in Fig. 1.

Post-depletion processing

Method 1 (direct analysis). A 5-μl aliquot of depleted saliva was digested overnight with 10 μl of 0.5 μg/50 μl modified trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate and concentrated by a vacuum centrifuge. Specimens were reconstituted with 5 μl of 5 mM ammonium bicarbonate containing 0.1% trifluoroacetic acid (TFA) and directly analyzed by LC-MS/MS.

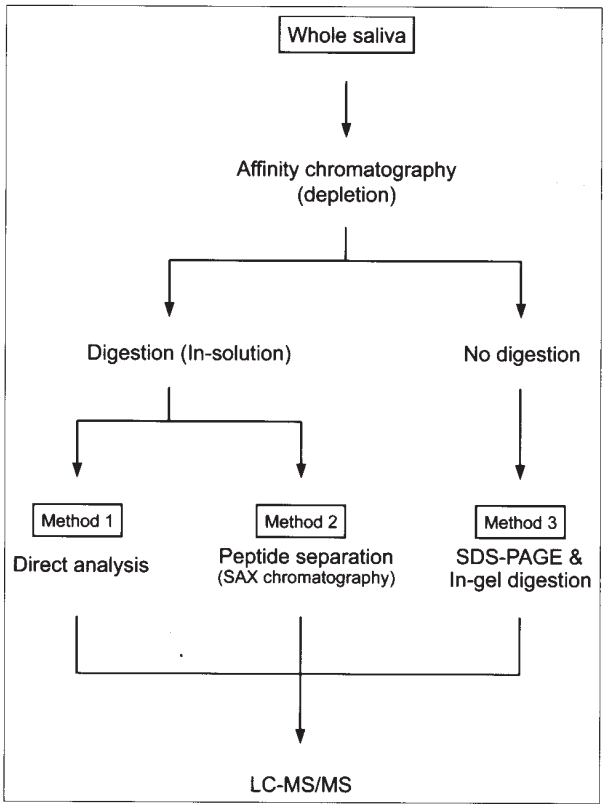

Figure 1. Schematic diagram of saliva protein analysis using three different procedures.

Method 2 [strong anion exchange (SAX) chromatography]. In the second approach, specimens were fractionated using SAX chromatography. A 5-μl aliquot of depleted saliva were digested by trypsin and separated by an FPLC system (Bio-Rad, Hercules, CA) using a SAX column (5x50 mm, Vydac, Hesperia, CA). The peptides were eluted by a linear gradient of NaCl (0-1 M). Thirteen elutes were collected and desalted using C-18 96-well solid-phase extraction disk plates (Empore, 3M, St. Paul, MN). Peptides were eluted with aqueous 70% ACN containing 0.1% formic acid. The eluted peptide fractions were concentrated with a vacuum centrifuge. Sample volume was reconstituted with 5 μl of 5 mM ammonium bicarbonate containing 0.1% TFA and all fractions of samples were individually analyzed by LC-MS/MS.

Method 3 (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE, and in-gel digestion). Saliva proteins were fractionated by SDS-PAGE and subjected to in-gel digestion with trypsin of 11-15 molecular weight zones. Aliquot(s) of saliva samples (5 μl) were prepared previously and separated by a gradient gel [4-15% Criterion gel (Bio-Rad)]. After staining of the SDS-PAGE gel with Coomassie brilliant blue, each lane was cut to 11-15 zones. The proteins in each zone were digested with 100-800 ng of modified trypsin overnight in 25 mM ammonium bicarbonate. The extracted peptides were concentrated and desalted with C18 Ziptips (Millipore) and then analyzed by LC-MS/MS.

Table I. The numbers of common and uncommon proteins in the saliva among five healthy individuals per each proteomic method.

	Method 1				Method 2				Method 3			
No.	U	C	M	T[a]	U	C	M	T[a]	U	C	M	T[a]
1	5	4	35	44	4	11	26	41	6	26	32	64
2	0	4	10	14	3	11	7	21	11	26	29	66
3	2	4	8	14	1	11	15	27	6	26	32	64
4	14	4	26	44	26	11	60	97	27	26	82	135
5	13	4	25	42	41	11	66	118	56	26	82	164

[a]U, uncommon protein number (these proteins were identified in only one normal saliva specimen); C, common protein number (these proteins were commonly identified in five normal saliva specimens); M, middle group protein number (these proteins were identified in two-four of five normal saliva specimens); T, total protein number.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Peptides were analyzed by the LC-MS/MS system using an HPLC (LC-packing) with C18 column (Vydac, 0.15x100 mm) to an ion trap (LCQ DecaXP, Thermo, San Jose, CA). The solvent system was 2% ACN (solvent A) and 60% ACN/20% 2-propanol (solvent B); both solvents contained 0.01% TFA. A linear gradient from 2 to 60% solvent B over 90 min was used to elute the peptides. Data-dependent MS/MS acquisition was performed.

Western blotting. Saliva samples (5 μl) prepared as indicated in the Materials and methods were subjected to SDS-PAGE and electroblotted in nitrocellulose membrane, which was preincubated with a blocking buffer (5% skim milk, phosphate buffered saline Tween (PBST); 0.1% Tween-20 in PBS) for at least 1 h at room temperature and incubated with the anti-complement factor B antibody (diluted 1:1000 in blocking buffer, R&D Systems, Minneapolis, MN) overnight at 4˚C. After washing with PBST, the membrane was incubated with anti-goat IgG antibody conjugated with peroxidase for 1 h at room temperature. After washing with PBST, areas reacting with the secondary antibody were detected using ECL Western blot detection reagents (Amersham Pharmacia) and X-ray film.

Database search. The MS/MS data obtained by method 1 were searched using MASCOT (Matrix Science, Boston, MA) against human entries in the NCBI (National Center for Biotechnology Information) database, selecting methionine oxidation as a variable modification and trypsin specificity. The m/z tolerance settings were ±2.0 for parent ions and ±0.8 for fragment ions. Data files were created from all the MS/MS data obtained by methods 2 and 3 using Bioworks Browser and merged to one file, respectively. MASCOT searching was done for each merged file using the same database, modification and parameters to those listed above. All MASCOT searching results were manually verified for their reliability.

Results

Proteins identified in all specimens and proteins unique to individual specimens (uncommon proteins) varied within samples and methods. The number of proteins found in all 5 normal specimens by each method were 4 by method 1, 11 by method 2, and 26 by method 3. A list of saliva proteins from healthy normal specimens are presented in Table I.

Proteins in individual samples identified by each method are listed in Table II. Four proteins, α-amylase, bactericidal/permeability-increasing protein-like 1, chromosome 6 open reading frame 58, and hypothetical protein L00124220 were identified by all 3 methods. Method 2 identified cystatin SN precursor and prolyl 4-hydroxylase, ß subunit that was not identified by method 3. Method 3 identified 12 additional proteins that included ß-actin, carbonic anhydrase VI, cystatins, α enolase, heat shock 70-kDa protein 1A, kallikrein, S100 calcium binding protein A9, serine (or cysteine) proteinase inhibitor, clade B, member 1, thioredoxin, transcobolamin I precursor and an unnamed protein product.

Saliva analysis of cancer patients identified 34 proteins to be consistently present in all samples (Tables III and IV). Of these, α-1-B-glycoprotein and complement factor B were not identified in any saliva samples from the healthy individuals. Also, α-1-acid glycoprotein 2 precursor (gi|29170378), fibronectin 1 (gi|53791223) and kininogen (gi|386853) were identified in 2 of the 3 cancer specimens, but not in the saliva of the healthy individuals (data not shown). α-fibrinogen precursor, annexin I, ceruloplasmin, fibrinogen-γ, hemopexin, keratin 1, keratin 10, matrix metalloproteinase 9, and proapolipoprotein were found in all cancer samples and were only present in the saliva from 1 or 2 of the 5 healthy subjects (Table IV). We also identified cystatin S and parotid secretory proteins to be present in normal specimens but not in saliva of the cancer patients.

To validate the LC-MS/MS analysis of complement factor B, Western blotting was performed on the saliva from the 5 healthy individuals and 9 cancer subjects including the 3 original cancer patient specimens. Western blot analysis of salivary complement factor B revealed low levels of protein in salivas from healthy subjects. In contrast, higher band intensities were found in specimens from cancer subjects. Fig. 2 shows multiple immunoreactive bands of variable intensities of complement factor B and only one HNSC sample (Patient lane 4) was negative. Western blot analysis identified complement factor B in all three original HNSC salivas (Patient lanes 1, 2 and 9).

Table II. Saliva proteins identified in all five healthy specimens by each processing method.

Protein designation	Accession no.	Number of proteins		
		Method 1	Method 2	Method 3
α-amylase	gil178585	5	5	5
α-enolase	gil2661039	2	4	5
ß-actin	gil4501885	3	4	5
Bactericidal/permeability-increasing protein-like 1	gil15055535	5	5	5
Carbonic anhydrase VI	gil14530767	3	4	5
Chromosome 6 open reading frame 58	gil55665009	5	5	5
Cystatin A	gil6503217	0	3	5
Cystatin B	gil30582919	2	3	5
Cystatin C	gil181387	0	4	5
Cystatin D	gil398711	2	3	5
Cystatin S	gil30366	4	3	5
Cystatin SA	gil359513	1	2	5
Cystatin SN precursor	gil118188	1	5	3
DMBT1	gil4996278	4	5	5
Heat shock 70-kDa protein 1A	gil62089222	1	2	5
Hypothetical protein L00124220	gil21687060	5	5	5
Kallikrein 1	gil20160213	3	4	5
Lactotransferrin	gil18490850	2	5	5
Lysozyme C precursor	gil48428995	4	5	5
Mucin 5B precursor	gil23821885	3	5	5
Neutrophil gelatinase-associated lipocalin	gil300181	3	5	5
Parotid secretory protein	gil16755850	1	3	5
Prolyl 4-hydroxylase, ß subunit	gil48735337	2	5	4
S100 calcium binding protein A9	gil4506773	3	3	5
Serine (or cysteine) proteinase inhibitor, clade B, member 1	gil30582695	0	2	5
Thioredoxin	gil9508997	0	4	5
Transcobalamin I precursor	gil135533	0	1	5
Unnamed protein product	gil34527805	0	3	5

Table III. Categories of saliva proteins in patients with HNSC specimens using method 3.

Subjects	U	C	M	T[a]
HNSC no. 1	33	34	29	96
HNSC no. 2	14	34	29	77
HNSC no. 3	33	34	29	96

[a]U, uncommon (proteins identified in saliva of only one HNSC subject); C, common (proteins identified in all specimens from HNSC subjects); M, intermediate (proteins identified in 2 of 3 HNSC subjects); T, total proteins.

Complement factor B (93 kDa) possesses non-glycosylated and glycosylated forms (30-32), and is cleaved by factor D into N-terminal Ba fragment (30 kDa) and C-terminal Bb fragments (63 kDa) (33). Low and high molecular weight bands of complement factor B in Patient lanes 1, 2, and 9 in Fig. 2 represent Bb fragment and intact complement factor B by LC-MS/MS analysis (method 3) of gel bands and corresponds to ~55-75 kDa and 75-100 kDa, respectively. Therefore, large and small bands indicated by arrows in lanes 1, 5, 6, 7, 8 and 9 and normal lane 4 may represent non-glycosylated forms of total complement factor B and its Bb fragment and large and small bands indicated by arrowheads (Patient lanes 2, 3, and 6 and normal lane 5) the corresponding glycosylated forms.

Discussion

Saliva is a complex secretion affected by numerous intrinsic and external factors that influence the rate, content and quality of secreted materials. Therefore, efforts to minimize the effect of these factors and to develop a simplified and standardized practical procedure for saliva analysis are required. We employed a standardized protocol for stimulated whole saliva collection to reduce variability. Although the protocol is not as selective as individual glandular sampling, it is practical for harvesting whole saliva with minimal circadian fluctuations effect. It also provides a copious quantity in convenient and simplified steps for the patients.

Table IV. Individual saliva proteins commonly present in all HNSC specimens and their frequency in normal specimens.

Protein designation	Accession no.	Number of positive specimens in healthy salivas
Aldolase A	gil28614	3
α-2-macroglobulin	gil46812315	3
α-1-B-glycoprotein	gil69990	0
α-enolase	gil2661039	5
α-fibrinogen precursor	gil182424	2
Annexin I	gil442631	2
ß-actin	gil4501885	5
Ceruloplasmin	gil1620909	1
Complement component 3	gil40786791	4
Complement factor B	gil2347133	0
Cystatin B	gil30582919	5
Fibrinogen-γ	gil223170	2
Gelsolin isoform b	gil38044288	3
Glucose phosphate isomerase	gil1336765	3
Heat shock 70-kDa protein 1A	gil62089222	5
Heat shock 70-kDa protein 8 isoform 1	gil123648	4
Hemopexin	gil226337	1
Keratin 1	gil11935049	2
Keratin 10	gil21961605	2
Lactotransferrin	gil18490850	5
Lipocalin 1	gil55958120	4
Matrix metalloproteinase 9	gil22532481	2
Mucin 5B precursor	gil23821885	5
Myeloperoxidase precursor (MPO)	gil129825	3
Neutrophil gelatinase-associated lipocalin	gil300181	5
Peptidylprolyl isomerase A, isoform 1	gil10863927	4
Phosphogluconate dehydrogenase	gil12653201	3
Proapolipoprotein	gil178775	2
S100 calcium binding protein A8	gil30583595	4
S100 calcium binding protein A9	gil56205191	5
Serine (or cysteine) proteinase inhibitor, clade B, member 1	gil30582695	5
Thioredoxin	gil9508997	5
Transaldolase 1	gil14603290	4
Transcobalamin I precursor	gil135533	3

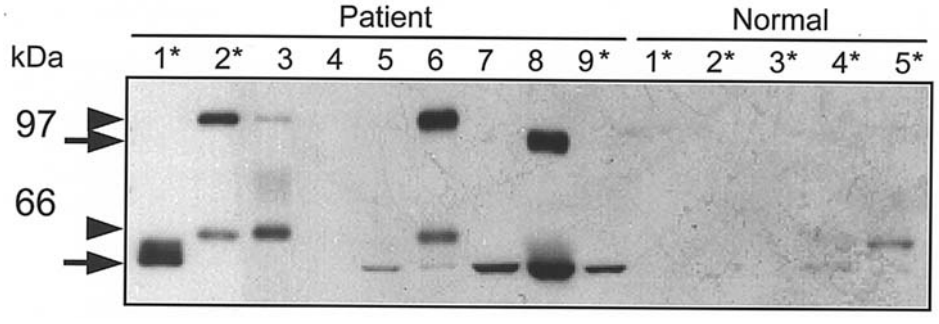

Figure 2. Western blotting of complement factor B in the saliva from normal individuals and head and neck squamous carcinoma subjects. Depleted saliva samples (5 μl) were loaded to a polyacrylamide gel (8%), electroblotted, and probed with anti-complement factor B antibody. Asterisks indicate the saliva samples analyzed by LC-MS/MS. Complement factor B (93 kDa) is cleaved by factor D into N-terminal Ba fragment (30 kDa) and C-terminal Bb fragments (63 kDa) (33) and possesses non-glycosylated and glycosylated forms (30-32). Therefore, two bands indicated by arrows (patient lanes 1, 5, 6, 7, 8 and 9 and normal lane 4) are non-glycosylated forms of intact complement factor B and Bb fragment, respectively, while two bands indicated by arrowheads (patient lanes 2, 3 and 6 and normal lane 5) are glycosylated forms of intact complement factor B and Bb fragment, respectively.

Our analysis of salivas by three different processing methods identified a set of shared and distinctive proteins among samples from healthy individuals. SDS-PAGE and in-gel digestion yielded the highest number of proteins which included the majority of proteins identified by the other two methods. However, the SAX chromatography processing method identified several unique proteins that were not detected by the other two methods. Our data suggest(s) that multiple processing methods may be needed to account for the wide range of saliva proteins. Our results also show that only four saliva proteins were common in all specimens by the three methods. A previous saliva study using a two-dimensional (2D)-LC technique reported 102 proteins in their sample (28). The same method in our study yielded 118 saliva proteins. In contrast, Hu *et al* (25) and Xie *et al* (29) reported 309 and 437 uncommon proteins using molecular weight fractionation and peptide separation by free flow electrophoresis and LC-MS/MS, respectively. Interestingly, 15 and 27 of the proteins reported in these studies were among the 28 proteins found in all of our normal specimens (25,29).

Our results of salivas from cancer patients identified 34 proteins to be present in all three specimens. The majority of these proteins were also found in normal salivas processed by the method except the α-1-B-glycoprotein and complement factor B. None of these proteins have previously been reported in other studies of normal saliva (24-26,28,29). However, our Western blotting detected low levels of complement factor B in saliva of 2 of the 5 normal salivas. This finding can be attributed to differences in the sensitivity between these methods and underscore the importance of validating proteomic analysis using established and standardized techniques. Similar validation of the α-1-B-glycoprotein by Western analysis was precluded by the lack of antibody specific for this marker.

Complement factor B is a component of the alternative complement pathway and its expression is considered to be restricted to hepatocytes, monocytes, and fibroblasts (34). This factor has been reported to be elevated in several tumors and thought to be induced by cytokines including interleukins and interferons (30-32,35,36-38). Complement factor B (93 kDa) is cleaved by factor D into N-terminal Ba (30 kDa) and C-terminal Bb (63 kDa) fragments (33). The latter fragment was found to induce apoptosis, most likely through the effect on host immune response by complement factor B-derived fragment Bb in leukemia studies (39). Support for such immune-mediated effects are reported by Andoh *et al* where complement factor B has been identified in the saliva of healthy individuals by immunoblotting (40). α-1-B-glycoprotein is a member of the immunoglobulin super family and is a constant component of serum (41). Although no biological function of α-1-B-glycoprotein is known, it has been shown recently to form a complex with cysteine-rich secretory protein-3 that may play a possible role in the innate immune system (42).

We also identified α-1-acid glycoprotein 2 precursor, fibronectin 1 and kininogen proteins in saliva from 2 of the 3 HNSC patients, but not in saliva of the healthy individuals. Similarly, two proteins identified in our normal saliva specimens (cystatin S and parotid secretory protein) were not found in any of the saliva samples from HNSC patients. Both proteins have also been identified in previous studies of normal saliva proteins (25,27-29). These and other proteins can be targeted for future validation as potential markers in clinical setting. Moreover, further efforts should be directed to unravel the functional role and regulation of these proteins in HNSC tumorigenesis. This information is necessary to determine the ultimate diagnostic, prognostic and therapeutic values of these proteins.

Our data showed that: 1) collection and processing of saliva plays a major role in proteomic analysis, 2) saliva is a convenient source of unique protein biomarkers of potential clinical value and 3) certain saliva proteins are differentially identified between HNSC patients and healthy individuals.

Acknowledgments

This work was supported in part by the Kenneth D. Müller Professorship and a National Cancer Institute Specialized Program of Research Excellence grant in Head and Neck Cancer (SPORE).

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