International Journal of Peptide Research and Therapeutics
https://doi.org/10.1007/s10989-019-09909-0

Salivary Proteomic Analysis of Betel Nut (*Areca catechu*) Consumers by Mass Spectrometry Revealed Primary Indication of Oral Malignancies

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Accepted: 10 August 2019

Abstract

Areca nut is the fourth most widely used addictive and psychoactive substance consumed by approximately 10% of the world's population. The use of areca nut is estimated to account for up to 50% of oral cancer in the low-income, and middle-income countries. In the present study, the effect of betel nut chewing on saliva proteomics was investigated by using mass spectrometry. Matrix-assisted laser desorption ionization mass spectrometry was used to generate a profile of the peptides in betel nut consumers and control group. We found 13 peptide peaks which were significantly altered ($p < 0.05$) in the betel nut addicts when compared with the control group. These significant peptides signals were corresponding to protein cystatin SN (CST1), cystatin S (CST4), alpha 2 macroglobulin (A2M), complement C3 (C3), apolipoprotein E (APOE), serum albumin (ALB), matrix metalloproteinase-9 (MMP-9), deleted in malignant brain tumor protein 1 (DMBT1), zinc-alpha-2-glycoprotein (ZAG), and protein S100A8. The correlation analysis of significant peptides intensities with the history of betel nut chewing was also performed. The peptides of CST1 and CST4 showed negative correlation, whereas the peptides of the MMP-9, DMBT1, APOE, and C3 showed positive correlation with significant differences. STRING analysis of these proteins revealed that most of these proteins are interacting with each other. The present study identifies a number of proteins in a significantly different abundance in the betel nut consumers group. Some of these proteins are the reported biomarkers of several oral malignancies, which implies that the usage of betel nut could lead to inflammation, and development of oral cancer.

Keywords Areca nut · Salivary proteomics · Mass spectrometry

Introduction

Areca nut (*Areca catechu*) is the seed of *Areca palm*, which grows in the Asia–Pacific region, and Africa. Areca nut is used to prepare a wide variety of chewing products, such as betel quid with tobacco (e.g., paan), gutka, mainpuri, naswar, khaini, mawa, and zarda with different compositions (Bhisey et al. 2004; Gupta and Warnakulasuriya 2002). The areca nut and betel quid products are mixtures of slaked lime wrapped in a betel leaf (Piper betle vine) with added flavorings. The use of areca nut products confers a widespread public health challenge due to insufficient data on different products and practices associated with their use in people of all ages. Areca nut is the fourth most widely consumed addictive and psychoactive substance used by approximately 10% of the world's population after tobacco, alcohol, and caffeine.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10989-019-09909-0) contains supplementary material, which is available to authorized users.

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Published online: 17 August 2019

Areca nut and betel quid have been classified as carcinogenic to human beings by the International Agency for Research on Cancer (IARC) (Gupta and Warnakulasuriya 2002). According to IARC, areca nut and betel quid products cause oral cancer, and cancer of the pharynx and oesophagus (Boucher and Mannan 2002; Oakley et al. 2005; Chu 2002; Park et al. 2002; Boucher and Mannan 2002). The use of areca nut and betel quid products account for up to 50% of oral cancers (11th most common cancer worldwide) in low, and middle-income countries (Dasgupta et al. 2010; Javed et al. 2010; Benjamin 2001), including oral submucous fibrosis, oral leukoplakia, and erythroplakia, and oral lichenoid lesions (Javed et al. 2010). Areca nut affects almost all organs of the body, and is reported to cause obesity, type 2 diabetes, metabolic syndrome, myocardial infarction, hepatotoxicity, asthma, hypothyroidism, and infertility (Lin et al. 2008; Mahmood 1982). There have also been reports of psychological effects from areca nut products, including a sense of euphoria, a warm sensation in the body, heightened alertness, and an increased capacity to work (Shah et al. 2002). The alkaloid arecoline is reported to affect nicotinic acetylcholine receptors, which could explain its dependence-producing effects (Balaram et al. 2002; Merchant et al. 2000).

Pakistan is among the countries where the use of areca nut chewing is a culturally common practice. It was reported in a study carried out in Karachi city that 21% of men and 12% of women used areca and betel nut (Mahmood 1982), 7.3% (both men and women) used pan, 6.7% use chalia, 7.5% used gutka, 14.6% used naswar, and use of betel and chewed tobacco was 20% and 17%, respectively (Bhurgri et al. 2003, 2005). In Pakistan, the incidence rate of oropharyngeal cancer (9.9%) was found to be significantly higher than in other member states of the World Health Organizations Eastern Mediterranean Region. The Urdu speaking communities were found to have higher rate of oropharyngeal cancer (20.4%), followed by Balochis (19.9%), Sindhis (16.8%), Punjabis (11.7%), and Pashtuns (9.6%).

The poor outcomes for cancer diagnosed at an advanced stage have been the rationale behind research into techniques to detect the disease before symptoms manifest. Screening, and early diagnosis play important roles in the management of oral cancers. Early detection, and treatment of precancerous lesions can substantially reduce cancer-specific morbidity, and mortality. The oral visual examination, vital staining using toluidine blue, brush cytology, and light-based systems are commonly used for early detection of precancerous lesions in oral cancers patients (Carreras-Torras and Gay-Escoda 2015). Mass spectrometry-based proteomics is a powerful approach for the global profiling and characterization of proteins, and to identify new biomarkers for clinical and diagnostic applications. Techniques such as matrix-assisted laser desorption/ionization (MALDI), MALDI TOF/TOF, and LC-MS/MS, followed by protein sequence database search to analyze and map out proteins have been extensively used for biomarker discoveries in ovarian, breast, and prostate cancer (Hudler et al. 2014; Goldblatt and Lee 2010). The use of statistical methodologies for data analysis and creating prediction models is of great value in forecasting malignant potential of tumors. Such strategies will not only help identify signatures between diseased and normal control samples, but can also build prediction models, and therefore, identify a combination of protein biomarkers representing the best sensitivity and specificity for a specific human cancer (Zhang et al. 2004). Human saliva is the plasma ultrafiltrate, and contains proteins, either synthesized in situ in the salivary glands or derived from blood, gingival cervicular fluid, and mucosal transudate. To date, researchers have identified 2340 proteins in the salivary proteome, of which 20–30% are also found in the blood, which is an encouraging indicator of the clinical utility of saliva as a diagnostic fluid. Saliva is also a representative of the body's response to any external agents used particularly on a habitual basis. Human oral fluid is especially attractive for disease diagnosis because: (i) its collection is totally non-invasive as compared to blood for serum/plasma analyses; and (ii) many, if not all, blood components are reflected in oral fluid. The human saliva contains a variety of proteins, growth factors, cytokines, immunoglobulins, and glycoproteins (Spielmann and Wong 2011).

In the present study, we employed a bottom up approach for differential proteomics of the saliva of betel nut chewers in comparison to saliva samples from non-users (control group). MALDI-TOF MS was employed to obtain the peptides profile of both the betel nut chewers saliva, and the control group. These peptides were identified by using nano liquid chromatography mass spectrometry (nano LC-MS/MS). The data was subjected to statistical analysis in order to find the correlation of the peptide intensities with the history of betel nut chewing. The presented data reveals changes in protein abundance due to betel nut consumption, and their potential role in inflammation or protection. The identified proteins hold potential for being biomarker of oral malignancies and other oral disease due to betel nut chewing.

Materials and Methods

Selection of Study Participants and Sample Collection

Before collection of samples, each participating individual went through a verbal screening protocol in order to select only suitable participants for the study, according to the designed selection parameters. In brief, control group included the healthy individuals (n = 28) with mean age

of 29.9 years and no prior history of any type of addiction (i.e. smoking, drinking, and betel nut chewing). In addition, the individuals with any systemic disease, or bacterial or viral infections were excluded from the study. The betel nut chewers (n = 32) had a mean age of 32 years with more than 1 year of betel nut chewing history. All the volunteers were asked to avoid eating for at least 1 h before sample collection. Unstimulated whole saliva (at least 3 mL) was collected in a sterile plastic bottle, and immediately placed in ice. The gender wise distribution of the participants in both groups is provided in electronic supplementary information (ESI Table 1).

Sample Preparation

Unstimulated whole saliva samples were centrifuged at 13,000 rpm for 15 min at 4 °C, and supernatant was collected. Protease inhibitor (MSSAFE Sigma) 2 µL per one mL of saliva was added to deplete protease activity against the salivary proteins. The samples were divided into 400 µL aliquots, and stored at − 80 °C. Protein estimation was performed by using Bradford assay (Bradford 1976).

Tryptic Digestion

For digestion, 100 µg of protein was used, and 80 µL of 1 M NH_4HCO_3 was added to adjust the pH in the range of 8–8.5. For protein denaturation, 20 µL of 40 mM nOGP (n-octyl glucopyranoside), and 50 µL of 45 mM DTT (dithiothreitol) were added. The vials were incubated on a thermomixer (Eppendorf AG, Hamburg, Germany) at 800 rpm at 90 °C for 30 min. After denaturation, the protein solutions were cooled down to room temperature. Alkylation was carried out by using 50 µL of 100 mM iodoacetamide (final concentration 20 mM), followed by 15 min incubation in the dark at room temperature. Subsequently, 100 µL of deionized water was added, followed by the addition of 1 µg trypsin (10 µL of 0.1 µg/µL). Protein solutions were digested on a thermomixer at 600 rpm for 14 h at 37 °C. Finally, tryptic digestion was stopped by adding 60 µL of 2% TFA (pH ≤ 3). All digests were stored at − 20 °C (Mirza et al. 2012).

MALDI TOF MS Analysis

For further analysis, the samples were desalted using C-18 ZipTip. 1 µL of sample was mixed with equal amount of di-hydroxy-benzoic Acid (DHB) (DHB: 10 mg/mL containing 50% ACN in 0.1% TFA) as a matrix. 1 µL of the mixture were spotted on the plate, and 2000 laser shots were accumulated for spectra, using a 337 nm nitrogen laser with a frequency of 50 Hz. MALDI-TOF-TOF (Ultra Flex) was initially calibrated using nine peptides; bradykinin (1–7) (m/z = 904.468 Da), angiotensin I (m/z = 1296.685 Da), angiotensin II (m/z 1046.5418), substance P (m/z = 1347.73540), bombesin (m/z = 1619.82230), renin substrate (m/z = 1758.93261), ACTH Clip (1–17) (m/z = 2093.08630), ACTH Clip (18–39) (m/z = 2465.19830), and somatostatin (m/z = 3147.47100). The MALDI TOF MS data was processed for base correction, noise filtration and peak calibration.

Nano LC-MS/MS Analysis

In order to identify those peptides with differential intensities obtained from MALDI data, the nano LC-MS/MS of the saliva tryptic digest of control samples, and betel nut addicts were performed. Samples were processed on maXis II for nano LC-MS/MS. Following parameters and conditions were used for HPLC settings. A trap column; acclaim PepMap TM nano viper, C-18, 3 µm, 100 Å (0.75 µm × 2.0 cm) was used for concentrated peptide with a flow rate 15 µL/min. Subsequently, it was connected online with a capillary separation column; acclaim PepMap TM RS-LC, C-18, 2 µm, 100 Å (0.75 mm × 15.0 cm), which was used for fractionation. The loading solvent (0.1% TFA in water) along with solvents A (0.1% formic acid in water), and B (0.1% formic acid in acetonitrile/water) with a flow rate: 0.3 µL/min against optimized gradient (40 °C temperature with 214 nm of UV wavelength and injection volume: 10–50 fmol of sample was used). For MS peptide identification, AutoMSMS mode was used in the positive ion mode. The mass range was selected as m/z. 150–2200 Da Otof Control SW version 4.0 was used to control MS.

Protein Database Searching

The peak list obtained from nano LC-MS/MS was then searched on Protein Scape v.4, using MASCOT search engine. MASCOT parameters in brief are as follows: enzyme: trypsin with 1 missed cleavage site; variable modifications: oxidation (Met), fixed modification: carbamidomethyl (Cys); peptide mass tolerance: 25 ppm, and MS/MS tolerance 0.5 Da was used with a false discovery rate (FDR) of 1%. The peak list (identified peptides with their mass to charge ratios) obtained from nano LC-MS/MS was matched with the peak list obtained from MALDI TOF MS data, and proteins were identified manually on the basis of their mass to charge ratios.

Statistical Data Analysis

Flex analysis (Version 4.0 Bruker) was used for the analysis of matrix-assisted laser desorption ionization time of flight (MALDI-TOF) data. All the statistical analysis was done on SPSS v.21, and peptides with differential intensities among groups were identified. Man Whitney U test was applied for

groups comparison with a p value < 0.05. The differential peptide list, generated from software were searched against obtained nano LC-MS/MS protein data. Protein ID and sequence information were obtained from Protein scape 4. For functional analysis, GO enrichment analysis was done by using STRING version 11.0.

Results and Discussion

MALDI-TOF MS Profiling

Here, we present a bottom up MS-based strategy to profile differential saliva proteomics of the betel nut addicts. The overall workflow of the experiments is presented in Fig. 1. The unstimulated whole saliva of 60 individuals (28 healthy control, and 32 betel nut addicts) was profiled to identify proteins, and peptides expressed in different intensities in both groups. The proteins were extracted, and quantified using Bradford method. The estimated quantity of proteins was in the range of 0.5–1 mg/mL. The MALDI TOF MS profile of the tryptic digests of the saliva samples of both groups was recorded. The MALDI-TOF MS data was processed and normalized for subsequent statistical analysis, which revealed a panel of proteins and peptides, present at significantly different levels between the betel nut and control populations. A total of 574 peptide peaks were detected in both groups. However, out of these, 58 (9.75%) peptide peaks exhibited a detection rate of more than 25% in both betel nut, and in healthy control samples. Due to the non-normal distribution of data, Mann–Whitney U test was performed in order to identify peptides which exhibited significant difference in signal intensities in the two groups. Out of 58 peptides, 13 peptide peaks were found to be significantly altered ($p < 0.05$) in the betel nut addicts, compared to the healthy controls. Table 1 represents the 58 peptides, their signal intensities in betel nut and control groups and their significant differences. Peptides with significant difference at the level of $p < 0.05$ were highlighted with *asterisks*. Out of these 13 significantly different peptides, 10 peptides (at *m/z* 927.5, 960.5, 990.5, 1185.7, 1224.6, 1421.7, 1471.7, 1633.0, 1639.9, and 1731.9 Da) were found to be significantly higher in the betel nut addicts, whereas 3 peptides peaks (*m/z* 2074.1,

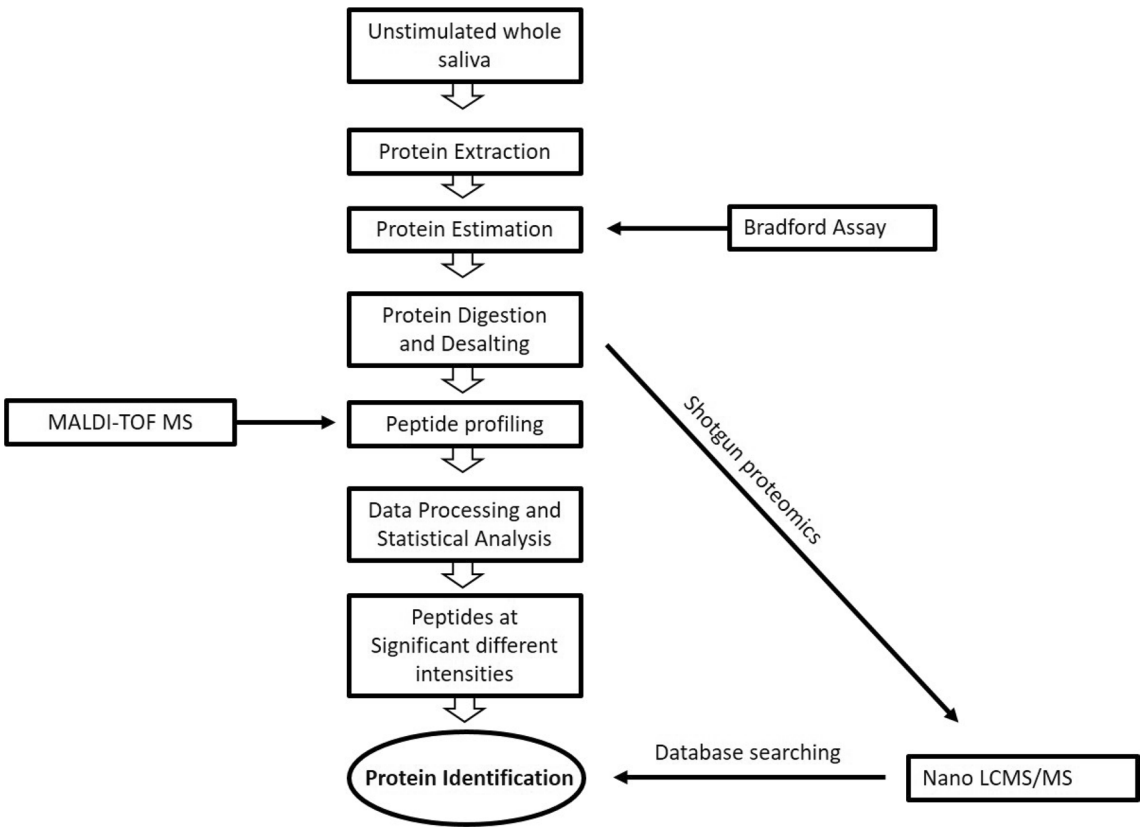


Fig. 1 Outline of the strategy for identification of differential proteomics using mass spectrometry. MALDI-TOF MS was used for profiling of oral fluid samples from 32 betel nut addicts group and 28 control subjects. The obtained data were processed, and statistical analysis was conducted to reveal peptides/proteins at significant difference between the two groups, and the proteins were identified by nano LC-MS/MS

Table 1 MALDI-TOF MS data of the mean intensities of 58 peptides (25% detection rate) in healthy control, and betel nut addicts group

Peptides (*m/z*)	Control mean	Betel nut addicts mean	*p* value
881.6	834.3	2280.9	0.650
896.4	2102.0	4253.5	0.977
918.4	422.3	613.6	0.610
927.5	530.4	3637.8	0.001*
934.4	487.8	496.5	0.838
960.5	408.5	2681.9	0.041*
962.5	523.1	779.8	0.374
990.5	311.8	3018.6	0.002*
1068.5	1876.0	2593.2	0.923
1161.6	1643.6	3630.6	0.343
1185.7	383.0	3562.1	0.004*
1213.6	1537.5	3057.8	0.903
1224.6	503.5	8439.3	0.0002*
1287.6	6328.6	3881.5	0.742
1290.7	2611.5	2125.8	0.606
1292.7	1430.5	710.2	0.004*
1314.6	2225.2	1498.7	0.957
1320.0	2781.0	1713.2	0.743
1342.7	1802.6	1301.5	0.720
1421.7	361.6	5624.6	0.008*
1427.8	1709.2	1693.1	1.000
1449.7	982.1	8911.9	0.172
1467.9	1481.6	1880.6	0.710
1471.7	1854.8	5402.6	0.002*
1490.6	1960.7	1993.8	0.512
1538.7	6523.0	1937.9	0.357
1540.8	1762.3	2582.6	0.529
1605.8	1170.3	8280.9	0.789
1615.8	4215.6	3041.6	0.580
1633.0	821.8	2275.0	0.040*
1639.9	687.8	1595.2	0.004*
1697.9	1276.3	638.8	0.333
1731.9	552.4	6102.8	0.0001*
1797.1	1572.8	1636.0	0.483
1798.9	2825.3	2713.4	0.932
1814.9	3306.6	1975.4	0.672
1819.1	2334.5	544.5	0.244
1835.9	1611.3	1481.3	0.500
1871.0	718.9	1544.0	0.350
1899.1	1941.5	1205.3	0.465
1911.1	709.6	1418.6	0.243
1915.2	440.7	401.4	0.773
1919.1	2991.4	1605.9	0.531
1954.1	2623.5	713.1	0.263
1964.1	1229.7	445.7	0.456
1971.1	1984.8	1123.6	0.908
1976.2	1278.9	701.3	0.696
2045.3	795.8	974.4	0.701
2074.1	1438.3	727.0	0.038*
2089.2	1720.6	774.8	0.315
2099.0	1411.0	345.8	0.005*
2109.1	534.6	359.9	0.161
2126.1	2022.3	814.6	0.364
2271.3	737.3	1738.4	0.331
2303.1	1245.2	3457.9	0.299
2586.4	1668.2	1699.2	0.175

p value indicates the level of significant change in the study groups

*The mean difference is significant at the 0.05 level

2099.0, and 1292.7 Da) were significantly lower in the betel nut addicts as compared to the healthy controls.

Identification of Peptides by Nano LC-MS/MS Analysis

Nano LC-MS/MS analysis was performed in order to identify saliva peptides/proteins displaying significant differences in signal intensities in the MALDI TOF MS analysis. The peptide peaks at *m/z* 2074.1, and 2099.0 Da were identified as the fragments of cystatin SN (CST1). While the peptide peak *m/z* 1292.7 Da was identified as a peptide of Cystatin-S (CST4). CST1 are known protease inhibitors present in saliva, and possess immunomodulatory functions by stimulating bacterial phagocytosis, and promoting endocytosis by macrophages (Ochieng and Chaudhuri 2010). Our study showed a significant decrease in the concentration of CST1 (p value = 0.038, and 0.005) and CST4 (p value = 0.004) in the whole saliva of betel nut addicts as compared to the control (Fig. 2a). It has been reported that during inflammation proceLC-MSsses cystatin level is downregulated in order to stimulate cysteine protease activities in macrophage microenvironment (Chapman Jr et al. 1990).

Among the peptides which showed significantly higher intensities in the betel nut group are signals at *m/z* 1731.96, 1471.7, and 1224.64 Da, which are the peptide fragment of apolipoprotein E (APO E) (p value = 0.0001) (Fig. 2b), complement C3 (C3) (p value = 0.002) (Fig. 2c), and alpha 2 macroglobulin (A2M) (p value = 0.0002), respectively (Fig. 2d). These proteins play an important role in immunity, and are known to be involved in inflammation and body's immune response (Janeway Jr et al. 2001). APO E functions in the regulation of innate immune system (Tavazoie et al. 2018). APOE is mainly involved in the transportation of lipid molecules, and the higher expression of this protein leads to the increase in the cholesterol export that results in lower cholesterol at cellular level. These changes are

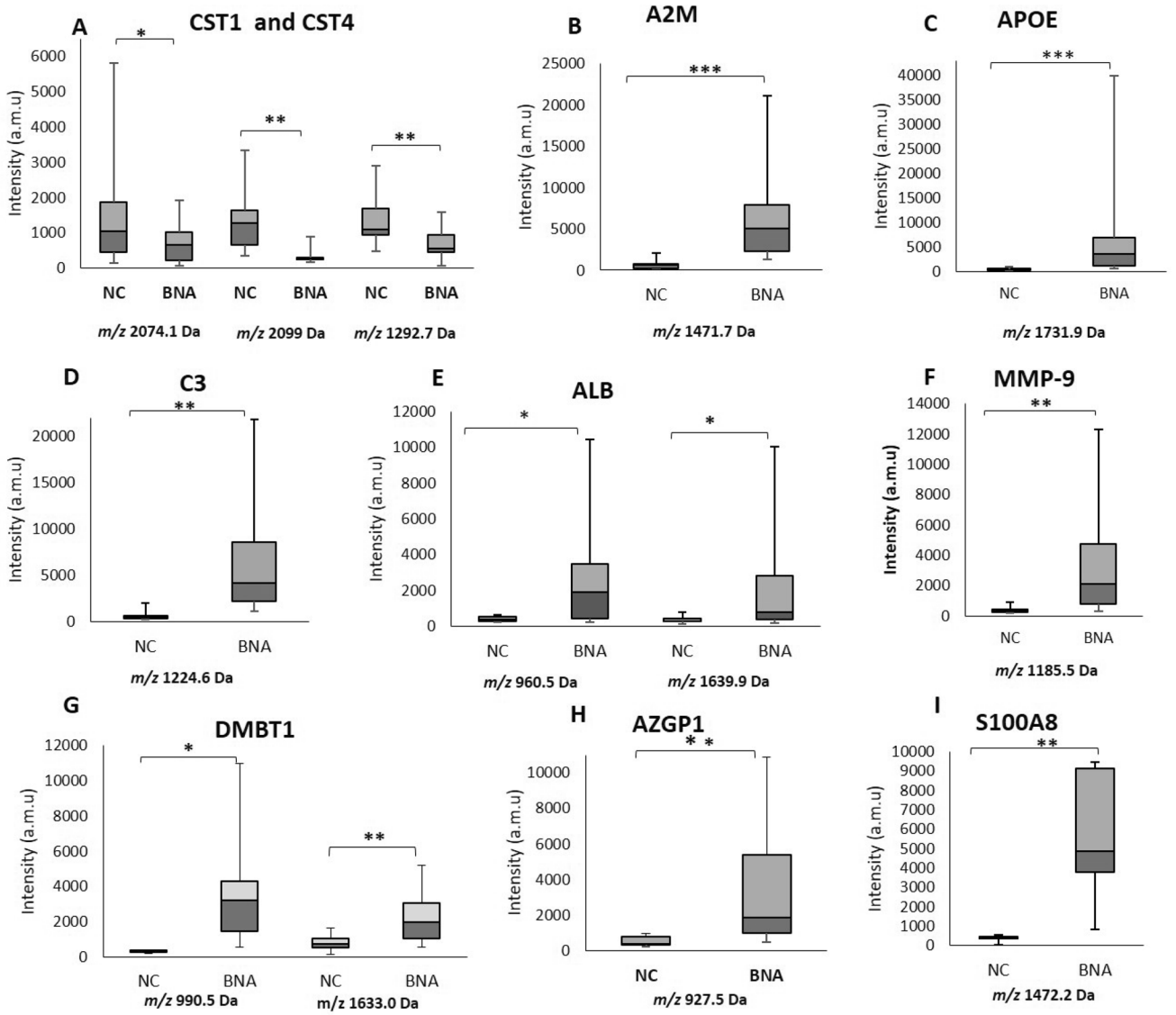


Fig. 2 Box plots of peptides displaying differences in signal intensities in normal control (NC), and betel nut addicts (BNA) group. **a** CYST1 represents cystatin SN, and CYST4 represents cystatin S protein, **b** A2M represents alpha 2 macroglobulin protein, **c** APOE represents apolipoprotein E protein, **d** C3 represents complement C3 protein, **e** ALB represents serum albumin protein, **f** MMP9 represents matrix metalloprotinases-9 protein, **g** DMBT1 represents deleted in malignant tumor 1 protein, **h** AZGP1 represents Zinc-alpha-2-glycoprotein, and **i** S100A8 protein. (*represents significance level at p value < 0.05, **represents significance level at p value < 0.01, ***represents significance level at p value < 0.001)

associated with the elevated intracellular signaling and are known to be involved in the invasion of tumor cells (Jayakar et al. 2017). C3 is reported to be involved in wound healing by the activation of complement system (Holers et al. 1992; Sinno et al. 2013). A2M are glycoproteins, which inhibit the proteinases by hindering the access of large molecular weight proteins to the active site of proteases. They also protect the body against invasive pathogens (Rehman et al. 2013). Two peptides at *m/z* 1639.94 and 960.54 Da, identified as the peptides of serum albumin (ALB), displayed significant increase in signal intensities (p value = 0.004, and 0.041) in the betel nut addict group, as compared to the control (Fig. 2e). The presence of benzopyrene and nitrosamines in betel nut are known to be involved in inducing reactive nitrogen species (ROS). This oxidative stress is overcome by the production of salivary albumin, which serves as an antioxidant in human biological fluid (Johnson 2001).

Our data showed a peptide peak at *m/z* 1185.7 Da, which belong to the matrix metaloproteinase-9 with a sequence of (R. QLAEEYLYR.Y) in both betel nut and control groups. The peptide peak of this protein showed a significant increase (p value = 0.004) in betel nut addict's saliva

(Fig. 1f). The MMP-9 is among MMP type IV collagen digestive enzymes that is involved in tumor invasion in oral squamous cell carcinoma, and has been known to be part of cancer pathogenesis (Chang et al. 2013). Betel nut contains aercoline that is a nicotinic acid-based alkaloid, known to stimulate the secretion of growth factors, enzymes, and other molecules associated with the progression of sub mucous fibrosis, and pre-cancerous conditions (Peisker et al. 2017; Uehara et al. 2017). The regular intake of betel nut damages the inner lining, and dental pulp in mouth, and eventually triggers the increase expression of several proteins, including MMP-9 (Tsai et al. 2005). Another important protein DMBT1 (deleted in malignant brain tumors 1 protein), which is also known as salivary agglutinin (SAG), is involved in salivary defense. It interacts with different mucosal proteins and pathogens as a pattern recognition molecule which contains different proteins, and carbohydrates binding motifs (Ligtenberg et al. 2010). DMBT1 is also involved in the activation of complement cascade during inflammation process (Ligtenberg et al. 2007). The higher expression of this protein indicates that inflammatory response is being undertaken on the inner lining of the mouth. Our data shows a significant increase (p value = 0.002, and 0.04) in signal intensity of the peptides (at m/z 990.5 and 1633.0 Da) of DMBT1 in the betel nut group as compared to the control group (Fig. 2g). These findings suggest that increased concentration of DMBT1 protein may be providing defense against pathogens, and may facilitates the inflammatory response. The peptide at m/z 927.5 Da, which is a fragment of zinc-alpha-2-glycoprotein (ZAG), was also observed in significant abundance (p value = 0.001) (Fig. 2h). ZAG is a soluble protein that belongs to the macroglobulin family, and is synthesized in normal tissues including both white and brown adipose tissues. It is normally present in most body fluids, including breast milk, blood, plasma, sweat, saliva, and urine (Heawchaiyaphum et al. 2018).

The peptide peak of m/z 1421.7 Da was found to be a fragment of protein S100-A8 with a sequence of K.LLETECPQYIR.K. This peptide of S100A8 protein showed significant increase (p value = 0.008) in signal intensities in the betel nut group (Fig. 2i). S100A8 is a calcium binding protein which belongs to the family of S100 proteins. S100A8 is also known as calgranulin, which forms a heterodimer with S100-A9. Protein S100-A8 is mainly secreted by macrophages, and neutrophils during inflammatory response (Perera et al. 2010). Their expressions act as a danger signal to activate the immune system (Foell et al. (2007). S100A8 is also known to upregulate the level of MMP-9 at inflammatory site (Van lent et al. 2008). The increased expression of these proteins indicates that the substances of betel nut are changing the proteomics of saliva, and also supporting the hosting of the bacteria, and other microorganism. Which results in a number of consequences, including activation of cell proliferation, inhibition

Table 2 Identification of 13 peptides which revealed significant difference in signal intensities in MALDI-TOF MS profiling

Peptide identified (m/z)	Protein ID	Protein name	Gene name	Abundance	Function	Sequence
2074.1	P01037	Cystatin-SN	CST1	Low	Protease inhibitor	R.IIPGGIYNADLNDEWVQR.A
2099.0						K.KQLCSFEIYEVPWENR.R
1292.7	P01036	Cystatin-S	CST4	Low	Protease inhibitor	R.ALHFAISEYNK.A
1224.6	P01023	Alpha-2-macroglobulin	A2M	High	Calcium-dependent protein binding	K.YDVENCLANK.V
1731.9	P02649	Apolipoprotein E	APOE	High	Cholesterol metabolism, lipid metabolism, Transport	K.SELEEQLTPVAEETR.A
1471.7	P01024	Complement C3	C3	High	Immunity, inflammatory response	K.GLEVTITAR.F
1639.9	P02768	Serum albumin	ALB	High	Antioxidant activity source, chaperone binding, copper ion binding, DNA binding	K.DVFLGMFLYEYAR.R
960.5						K.FQNALLVR.Y
1185.7	P14780	Matrix metalloproteinase-9	MMP9	High	Collagen degradation	R.QLAEEYLYR.Y
1633.0	Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	High	Antiviral defense, host-virus interaction, transport	K.VDVVLGPIQLQTPPR.R
990.5						R.GRVEVLYR.G
927.5	P25311	Zinc-alpha-2-glycoprotein	AZGP1	High	Lipid metabolism, transport	K.IDVHWTR.A
1421.7	P05109	Protein S100-A8	S100A8	High	Antimicrobial, calcium ion binding, Toll-like receptor 4 binding,	K.LLETECPQYIR.K

of apoptosis, induction of chronic inflammation, promotion of cellular invasion, and production of carcinogens (Perera et al. 2016). Among the peptide peaks which exhibit non-significant change in signal intensities in betel nut and control group, were also examined for protein identification, and presented in electronic supplementary file (ESI Table 2).

Correlation Analysis of the Differentially Expressed Proteins

The correlation analysis of the intensities of 13 significant peptides, and the history of betel nut chewing was also carried out. The signal intensities of peptides at *m/z* 1185.7, 1421.7, 1731.96, and 1471.7 Da, which belong to MMP-9, S100A8, APOE, and C3, respectively, showed significant positive correlations with the history of the betel nut chewing (Fig. 3a–d). The positive correlation of MMP-9 (p value = 0.044), S100A8 (p value = 0.021), APOE (p value = 0.001), and C3 (p value = 0.001) with the chewing history of betel nut suggests that the continuous exposure of betel nut substances is significantly upregulating the expressions of these proteins. It was observed that the two peptides at *m/z* 2074.17 and 2099 Da, which belong to CST1 showed negative correlation. Among these, one peptide at *m/z* 2074.17 Da showed significant negative correlation (p value = 0.024) (Fig. 3e). The negative correlation of CST1 with the history of chewing suggests, that the long term exposure of betel nut substances down regulates the cystatin protein, which is the inhibitor of protease enzymes of the caspase cascade. This might lead to the escape of apoptosis, and development of malignancies. This observation is consistent with other studies suggesting down regulation of cystatin in the inflammation process (Chapman Jr et al. 1990). These results indicate that the normal proteomics of saliva is changing over the course of years, and might lead to modifying the expression of proteins at the genomic and sub-genomic levels. The increased usage of betel nut initiates several oral problems that can cause most dangerous and lethal diseases like cancer. Other differential proteins identified in the present study, such as A2M, ALB, DMBT1, were not observed to show any correlation with the history of betel nut chewing.

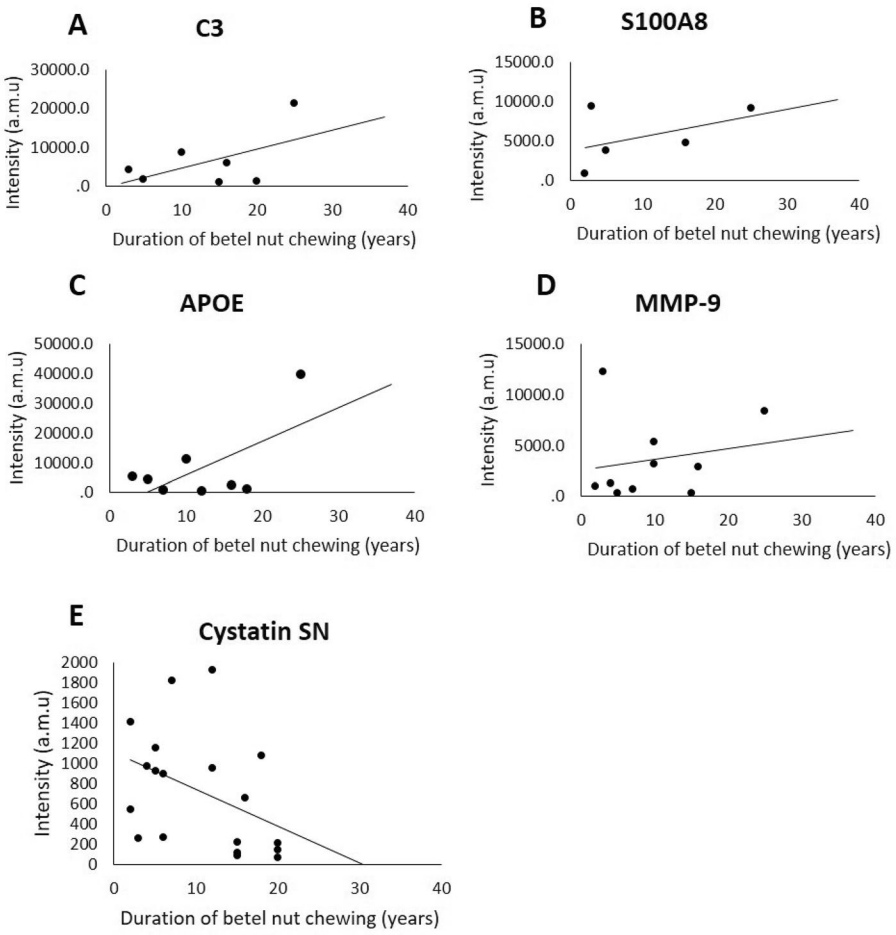
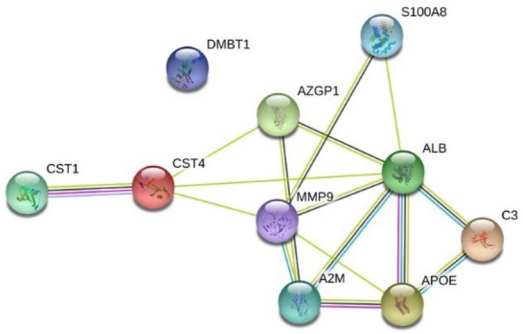


Fig. 3 Correlation analysis between peptide intensities and betel nut chewing history (in years). **a** C3 represents complement C3 protein, **b** S100A8 protein, **c** APOE represents apolipoprotein E protein, **d** MMP9 represents matrix metalloproteinases-9 protein, and **e** CYST1 represents cystatin SN

A



B

Pathway ID	Pathway description	Observed gene count	False discovery rate
1- Biological process			
GO:0030162	Regulation of proteolysis	7	1.25E-05
GO:0010951	Negative regulation of endopeptidase activity	5	1.81E-05
GO:0043086	Negative regulation of catalytic activity	6	0.00012
GO:0001580	Detection of chemical stimulus involved in sensory perception of bitter taste	3	0.00012
GO:0032269	Negative regulation of cellular protein metabolic process	6	0.00028
GO:2000116	Regulation of cysteine-type endopeptidase activity	4	0.00034
GO:0031347	Regulation of defense response	5	0.0006
GO:0043062	Extracellular structure organization	4	0.00079
GO:0050727	Regulation of inflammatory response	4	0.00079
2- Molecular function			
GO:0004866	Endopeptidase inhibitor activity	4	0.00015
GO:0030234	Enzyme regulator activity	5	0.0014
GO:0008289	Lipid binding	4	0.0035
GO:0004869	Cysteine-type endopeptidase inhibitor activity	2	0.0049
GO:0005515	Protein binding	9	0.0049
GO:0048306	Calcium-dependent protein binding	2	0.0049
GO:0016209	Antioxidant activity	2	0.0066
GO:0004252	Serine-type endopeptidase activity	2	0.0285
GO:0005102	Signaling receptor binding	4	0.0315
3- Localization			
GO:0005576	Extracellular region	10	1.25E-07
GO:0005615	Extracellular space	8	2.76E-07
GO:0060205	Cytoplasmic vesicle lumen	5	1.01E-05
GO:0030141	Secretory granule	6	1.85E-05
GO:0044433	Cytoplasmic vesicle part	7	1.85E-05

Fig. 4 STRING analysis (v.11.0) of ten proteins reveals the protein–protein interaction network and functional analysis by using GO enrichment analysis. **a** shows the interaction of protein in which edge represent a protein–protein interaction, and each node represent a protein. **b** shows the functional classification of ten proteins, each term represents the number of genes and indicates the false discovery rate by STRING

Functional Analysis of the Differentially Expressed Proteins

In order to check the interaction and network of ten differentially expressed proteins, we performed STRING version 11.0 (Szklarczyk et al. 2014; Szklarczyk et al. 2016) analysis. It was found that all of differentially present proteins showed direct interaction with each other, except DMBT1 protein (Fig. 4a). For further investigation on their functions, GO (http://geneontology.org/) enrichment analysis with medium stringency was performed. Go enrichment analysis revealed the involvement of ten differentially expressed proteins in the biological process; molecular functions and their localization (Fig. 4b). Many of the proteins that were found to be in high abundance in betel nut addicts are involved in the regulation of proteolysis, negative regulation of endopeptidase activity, negative regulation of catalytic activity, detection of chemical stimulus involved in sensory perception of bitter taste, negative regulation of cellular protein metabolic process, regulation of cysteine-type endopeptidase activity, regulation of defense response, extracellular structure organization, and regulation of inflammatory response. The functions of these proteins mainly involve in binding, regulatory, and antioxidant activities. All of these proteins are found mainly in extracellular region while some of them are also found in the cytoplasmic vesicle lumen, secretory granule, and cytoplasmic vesicle part. To obtain more insights into the salivary proteomics, literature was searched to investigate the association of the ten differentially expressed proteins in oral malignancies. These proteins were reported to be associated with various pathological conditions of mouth. The low abundance of protein cystatin has already been reported in several oral malignancies like chronic periodontitis (Gonçalves et al. 2010). The level of A2M is found to be elevated in oral malignancies such as gingivitis, and periodontitis (Pederson et al. 1995). C3 elevated levels are reported in many cancers, such as oral squamous cell carcinoma (OSCC) (Kawahara et al. 2016; Sivadasan et al. 2015), breast cancer (Dowling et al. 2012), and ovarian cancer (Cho et al. 2014). The upregulation of APOE was also found in the saliva of OSCC patients by shotgun proteomics approach (Wu et al. 2015). The high abundance of ALB is reported in oral malignancies such as oral leukoplakia, and OSCC (Metgud and Patel 2014). The over-expression of MMP-9 protein at the inflammatory site leads to the submucosa fibrosis, and precancerous lesions of the mouth and salivary glands (Ochieng and Chaudhuri 2010), which eventually leads to OSCC (Sundelin

et al. 2005). A lower level of deleted in malignant tumor 1 (DMBT1) protein has also been discovered in various oral malignancies such as oral squamous cell carcinoma (Imai et al. 2005). DMBT1 is also recognized as a tumor suppressor gene in various cancers like gastric, lung, and breast (Imai et al. 2005), and the downregulation of this gene promotes tumor progression. The over-expression of AZGP1 protein is known to be linked with the inhibition of migration of cancer cells, tumor growth, and proliferation. The upregulation of this protein also found in the saliva of head and neck cancer patients (Vidotto et al. 2011), breast cancer (Diez-itza et al. 1993), and prostate cancer (Hale et al. 2001). Several reports suggest that the over expression of AZGP1 is due to smoking, and some cytokines (Bing et al. 2010). An increasing concentration of S100A8 has also been reported in the saliva of periodontitis patients (Haririan et al. 2016; Ramseier et al. 2009), and OSCC (Jou et al. 2014).

Conclusion

Areca nut and betel chewing is widely prevalent in Pakistan, which poses a high risk of oral cancer. Proteomic analysis of human oral fluid (whole saliva) holds promise as a non-invasive method to identify differences in protein compositions of saliva of betel nut addicts and non-users. In the present study, we have developed a straightforward method by first profiling peptides of oral fluids of betel nut chewers and control groups by MALDI-TOF MS, and later on their analysis by nano LC-MS/MS for protein identification. The approach revealed a number of proteins which were upregulated significantly. These proteins have already been reported as biomarkers of several oral malignancies. We have demonstrated that the saliva of betel nut addicts contains signature proteins, which have a potential of being further investigated as early diagnostic biomarkers, especially in patients who developed oral malignancies due to excessive betel nut chewing. These discovered biomarker candidates, once validated, can also be used to monitor oral fluids from patients with oral precancerous lesions and discover oral fluid biomarkers for early oral cancer detection. This approach is practical towards identifying oral fluid biomarkers considering oral fluid is simpler than other body fluids such as serum.

Acknowledgements We would like to greatly acknowledge Dr. Panjwani Center for Molecular Medicine and Drug Research (ICCBS) for supporting this work by providing recurring grant for chemicals and consumables, and for allowing us to work in mass spectrometry core facility (ICCBS).

Author Contributions Authors RS and MRM equally share the first authorship.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All procedures performed in this study involving human participants were approved by the Independent Ethics Committee (IEC), International Center for Chemical and Biological Sciences, University of Karachi.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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