Diagnostic model of saliva peptide fingerprint analysis of oral squamous cell carcinoma patients using weak cation exchange magnetic beads

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Synopsis
Saliva diagnostics utilizing nanotechnology and molecular technologies to detect oral squamous cell carcinoma (OSCC) has become an attractive field of study. However, no specific methods have been established. To refine the diagnostic power of saliva peptide fingerprints for the early detection of OSCC, we screened the expression spectrum of salivary peptides in 40 T1 stage OSCC patients (and healthy controls) using MALDI-TOF-MS combined with magnetic beads. Fifty proteins showed significantly different expression levels in the OSCC samples ($P < 0.05$). Potential biomarkers were also predicted. The novel diagnostic proteomic model with $m/z$ peaks of 1285.6 Da and 1432.2 Da are of certain value for early diagnosis of OSCC.

Key words: early diagnosis, histatin-3, matrix-assisted laser-desorption ionization–time-of-flight–mass spectrometry (MALDI-TOF-MS), oral squamous cell carcinoma (OSCC), peptides, saliva.

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INTRODUCTION

Oral cancer, especially oral squamous cell carcinoma (OSCC), is a high-impact disease in the oral cavity. OSCC accounts for $\sim90\%$ of malignant oral lesions and is widely recognized as the most frequently occurring malignant tumour of oral structures. Each year, approximately 500000 new cases are diagnosed worldwide, with a 5-year survival rate of only 50% [1]. In the early stages of OSCC, the tumour responds well to combination therapy, as evidenced by a 5-year survival of 80% in these patients. However, the response to treatment is much lower in advanced OSCC [2]. Thus, there is a need to investigate the molecular mechanisms involved, to identify potential therapeutic targets as well as to discover biomarkers for the early detection of OSCC and subsequent monitoring of its progression.

Saliva has gained notable attention as a diagnostic fluid because it is easy to collect and process, minimally invasive and associated with low costs [3]. It contains a large array of proteins, which may be useful for novel approaches to prognosis, clinical diagnosis and monitoring and management of disease. Comprehensive analysis of the human saliva proteome may contribute to the understanding of pathophysiology and provide a foundation for the recognition of potential biomarkers of human disease [4–6].

MALDI-TOF-MS is a powerful technique that can be used to analyse proteins from saliva [7]. It can detect low-molecular-mass peptides with adequate resolution and sensitivity, making it a useful tool for peptide pattern profiling. In addition, beads with peptide libraries, mesoporous silica particles [8] or a magnetic core [9], such as weak cation-exchanger magnetic beads, may be utilized for selective enrichment of low-molecular-mass peptides before MS analysis. Magnetic beads constructed on nanomaterial are a promising material among the various types of separation beads. This kit based on weak cation exchange (WCX) principle. Proteins and peptides in samples are captured by specific adsorption of magnetic beads in low salt and low pH solution and released in high salt solution, so as to capture proteins and peptides in the serum. The proteins and peptides can be analysed by MALDI-TOF-MS. Using a combination of magnetic beads...

Abbreviations: aPRP, acidic proline-rich protein; bPRP, basic proline-rich protein; gPRP, glycosylated proline-rich protein; OSCC, oral squamous cell carcinoma; TLR, toll-like receptor; WCX, weak cation exchange; WS, whole saliva.

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and MALDI-TOF MS enables efficient and sensitive detection of peptides that are specific for certain conditions; indeed, we used this technique to successfully identify serum peptide profiles in pilot studies [10,11]. This procedure, which involves weak cation-exchanging magnetic beads for sample separation, MALDI-TOF MS for peptide profile detection and a database for construction of condition-specific peptidome models, is a powerful tool that enables early detection, diagnosis and determination of the prognosis of various diseases [12].

In the present study, we investigated differences in the salivary peptide (1–10 kDa) profiles of T1 stage OSCC patients and healthy subjects by MALDI-TOF MS using a magnetic bead-based peptidome analysis of saliva samples. We aimed to identify a panel of specific biomarkers for differential expression.

MATERIALS AND METHODS

Ethics statement
The present study was approved by the Peking University Biomedical Ethics Committee. Adult subjects and parents of paediatric subjects signed an informed consent form before the start of research.

Patients and saliva collection
All patients were enrolled in the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology between April 2013 and August 2014. Samples of unstimulated whole saliva (WS) were collected from 40 OSCC patients (19 males, 21 females; aged 58.5 ± 14.06) and 23 healthy controls for comparative analysis. The 40 OSCC patients were diagnosed according to the results of oral pathology tests and had not taken any prescription medication to prevent changes in the flow rates of saliva. Patients with other medical disorders and complicated medication requirements were excluded from this study. Detailed clinical and serological characteristics of OSCC patients are shown in Table 1. A total of 23 WS samples were collected from healthy controls with a mean age of 54.47 ± 11.83 years. Unstimulated WS samples were collected between 9 and 10 a.m. Patients were asked to refrain from eating, drinking, smoking and conducting oral hygiene procedures for at least 1 h before saliva collection. Before collection, the subject was instructed to rinse orally with water and then rest for 5 min with his/her eyes open and head tilted slightly forward. The WS was collected over a period of 15 min or more with a paper cup on ice and then centrifuged at 2600 g for 15 min at 4 °C. Then the supernatant was removed and immediately stored at −80 °C in 200 μl of aliquots for further analysis. Prior to proportional peptide mass fingerprint analysis, the deep-frozen samples were quickly thawed via brief immersion into hot water to maintain the integrity of proteins.

| Subject   | Diagnosis          | Age (years) | Sex |
|-----------|--------------------|-------------|-----|
| (a)       |                    |             |     |
| Patient 1 | OSCC               | 64          | M   |
| Patient 2 | OSCC               | 58          | M   |
| Patient 3 | OSCC               | 65          | F   |
| Patient 4 | OSCC               | 81          | F   |
| Patient 5 | OSCC               | 51          | M   |
| Patient 6 | OSCC               | 63          | M   |
| Patient 7 | OSCC               | 60          | M   |
| Patient 8 | OSCC               | 36          | F   |
| Patient 9 | OSCC               | 61          | M   |
| Patient 10| OSCC               | 76          | F   |
| Patient 11| OSCC               | 59          | M   |
| Patient 12| OSCC               | 65          | M   |
| Patient 13| OSCC               | 41          | F   |
| Patient 14| OSCC               | 62          | F   |
| Patient 15| OSCC               | 72          | F   |
| Patient 16| OSCC               | 53          | M   |
| Patient 17| OSCC               | 46          | M   |
| Patient 18| OSCC               | 51          | F   |
| Patient 19| OSCC               | 60          | M   |
| Patient 20| OSCC               | 68          | F   |
| Patient 21| OSCC               | 32          | M   |
| Patient 22| OSCC               | 65          | F   |
| Patient 23| OSCC               | 62          | M   |
| Patient 24| OSCC               | 34          | F   |
| Patient 25| OSCC               | 48          | M   |
| Patient 26| OSCC               | 62          | F   |
| Patient 27| OSCC               | 59          | F   |
| Patient 28| OSCC               | 40          | F   |
| Patient 29| OSCC               | 56          | F   |
| Patient 30| OSCC               | 64          | M   |
| Patient 31| OSCC               | 70          | F   |
| Patient 32| OSCC               | 41          | M   |
| Patient 33| OSCC               | 25          | F   |
| Patient 34| OSCC               | 51          | F   |
| Patient 35| OSCC               | 76          | F   |
| Patient 36| OSCC               | 67          | F   |
| Patient 37| OSCC               | 21          | F   |
| Patient 38| OSCC               | 51          | F   |
| Patient 39| OSCC               | 79          | M   |
| Patient 40| OSCC               | 55          | F   |
| (b)       |                    |             |     |
| Subject 1 | Normal             | 30          | M   |
| Subject 2 | Normal             | 31          | F   |
| Subject 3 | Normal             | 39          | M   |
| Subject 4 | Normal             | 39          | M   |
| Subject 5 | Normal             | 45          | F   |
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| Subject | Diagnosis | Age (years) | Sex |
|---------|-----------|-------------|-----|
| 6       | Normal    | 50          | M   |
| 7       | Normal    | 50          | M   |
| 8       | Normal    | 50          | F   |
| 9       | Normal    | 53          | F   |
| 10      | Normal    | 53          | F   |
| 11      | Normal    | 56          | F   |
| 12      | Normal    | 56          | F   |
| 13      | Normal    | 56          | F   |
| 14      | Normal    | 60          | M   |
| 15      | Normal    | 60          | M   |
| 16      | Normal    | 60          | M   |
| 17      | Normal    | 63          | F   |
| 18      | Normal    | 67          | M   |
| 19      | Normal    | 67          | M   |
| 20      | Normal    | 67          | M   |
| 21      | Normal    | 67          | M   |
| 22      | Normal    | 67          | M   |
| 23      | Normal    | 73          | F   |

**Instruments and reagents**

A weak cation exchange (WCX) magnetic bead kit (Bioyoong SPE-C) and robotic separation device for a 96-well plate format magnetic separator were purchased from Bioyoong (Bioyoong technologies Inc). An LT-2 MALDI-TOF MS (Bioyoong technologies Inc) was used for the MS analysis.

**Sample application and MALDI-TOF-MS analysis**

The suspension in the WCX magnetic bead kit was mixed by shaking. After eluting and more shaking, the magnetic beads were separated from the protein and the eluted peptide samples were transferred to a clean 0.5 ml of tube for further MS analysis. Then, 5 μl of hydroxy-α-cyano-cinnamic acid (HCCA) substrate solution (0.4 g/l, dissolved in acetone and ethanol) and 0.8–1.2 μl of elution were mixed and 0.8–1.2 μl of this mixture was applied to a metal target plate and dried at room temperature. Finally, the prepared sample was analysed by MALDI-TOF MS. Peptides with molecular masses of 1000–10000 Da were collected and 400 shots of laser energy were used. Peptide mass fingerprints were obtained by accumulating 50 single MS signal scans. The saliva samples collected from each patient were analysed serially three times using MALDI-TOF MS. The mean values of each sample were used for data analysis.

**Statistical analysis**

The t test was used for comparisons between the OSCC and healthy subjects groups. Data were analysed using the BioExplorer statistical package (Bioyoong Technology Inc). A P-value <0.05 was considered statistically significant.

**RESULTS**

The general clinical characteristics of the subjects are presented in Table 1. The entire mass spectra of the extracted peptide samples from 63 subjects in the two groups were generated using the same instrument settings in the range of 1000–10000 Da (Supplementary Figure S1). Most peaks were detected in the range of 1000–3500 Da.

An average of 50 peptide mass peaks was found when the two groups were compared. Next, the peaks among the mass spectra were quantified and compared. Eight of these peptide mass peaks (1285.6, 1731, 1191.4, 1353.9, 1584.6, 1553.5, 1329.9 and 1432.2 Da) were significantly different between OSCC patients and healthy controls (Figures 1, 2 and Table 2). Among them, four peptides (at 1285.6, 1553.5, 1329.9 and 1432.2 Da) were up-regulated and four (at 1731, 1191.4, 1353.9 and 1584.6) were down-regulated in the OSCC patients (Figure 2).

All the eight mass peaks were used to establish the diagnostic model using the radial basis function method. Two peaks (1285.6 and 1432.2 Da) exhibited the most significant difference (P < 0.05, by t test) between the two groups compared with the other combinations of peptides. Thus, we used these two peptides to establish a fitted curve. 2D-cluster plot analysis demonstrated represents the best separating peaks in 2D spaces (Figure 3), whereas 3D view of principal component analysis (PCA) scores plot analysis indicated a well differential distribution of mass peaks between controls and OSCC patients (Figure 4). Columns represent samples; rows are m/z peaks as indicated by the average molecular mass. The shape of the two figures showed the well-separated locations of the samples from the two groups, indicating that the fitting results were satisfactory.

**DISCUSSION**

Detection of oral cancer at an early stage is important for successful clinical therapy [13]. Patients with OSCC often present with advanced-stage disease, which is associated with poorer prognosis. Late-stage OSCC also requires more aggressive therapy,
3D m/z ratio-intensity maps showed the two significantly different peptides at 1285.6, 1432.2 Da, which had a particular trend among the two groups. Green curve, healthy control group; red curve, 7-month group; blue curve, OSCC patients group.

The peak intensities of the two different groups showing an increasing trend in peak intensity at 1285.6, 1432.2, 1353.5 and 1329.9 Da and a decreasing trend at peak 1731, 1191.4, 1353.9, 1584.6 Da. (*P < 0.05; **P < 0.01).

which results in increased functional disability. Conventional diagnostic techniques, including direct inspection and imaging technology such as positron emission tomography-computed tomography, are limited in their ability to detect early stage OSCC and are ineffective for screening high-risk populations [9]. Screening tools are needed that combine high sensitivity and specificity and are sufficiently non-invasive and inexpensive to enable widespread use.

In recent years, interest in saliva for clinical purposes as an alternative to other body fluids, such as blood and urine, has increased. WS is a complex biological fluid due to the many processes involved in its production. In addition to the exocrine components, there are several non-exocrine contributors such as desquamated epithelial cells, intact and partial blood cells, gingival fluid and possibly fluid entering the oral cavity through mucosal seepage. This renders diagnosis of disease by the analysis of saliva both challenging and attractive. Saliva was found to be similar in microbial profile to the soft tissues [14]. This was a significant finding from the study of the OSCC-free population [15]. So the screening test of salivary peptides for OSCC is appealing. MS-based proteomics is a high-throughput method used to analyse salivary proteomics and has been employed in the study of protein/peptide spectra, biological marker spectra, as well as single biological markers for complicated diseases such as cardiovascular and cerebrovascular diseases, OSCC and neuro-degenerative diseases.

To date, more than 2000 peptides have been discovered in the salivary peptidome [16–18]. By mapping the corresponding protein entries, it has been possible to assign those peptides to 695 non-redundant protein species [18]. Since the 1970s, salivary peptides have been grouped into six structurally-related major classes [19], namely, histatins, basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), glycosylated proline-rich proteins (gPRPs), statherin and cystatins [20–22]. Salivary PRPs, as well as bPRPs, aPRPs and gPRPs, are usually identified from the small peptide fraction (< 3 kDa). Some PRPs, along with statherin and...
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**Figure 3** Plots of the two groups generated by combining the 1285.6 and 1432.2 Da proteins

The scatter plots showed a well-fitting curve of two peaks with a significant difference ($P < 0.01$, by $t$ test) in their distribution between healthy controls and OSCC patients.

**Figure 4** 3D view created by PCA analysis

3D view displays of the principal component analysis of peptide profiles using BE software. Blue spots represent control individuals; red spots represent OSCC patients.
histatin-1, appear to actively participate in tooth mineralization. Histatins, especially histatin-3 and histatin-5, which are found in high amounts in saliva, are strongly anti-fungal [23]. The cystatin class comprises five major isoforms (S, C, D, SA and SN), which have strong bactericidal and virucidal properties [24]. Statherin, a multifunctional molecule that possesses a high affinity for calcium phosphate minerals, such as hydroxyapatite, contributes to the maintenance of the appropriate mineral solution. Defensins are a family of low-molecular-mass (3–4 kDa) cationic proteins with antibiotic, anti-fungal and anti-viral properties. They are involved not only in innate immunity against infections but also in adaptive immunity, inflammation and wound repair [25].

In the present study, WCX magnetic beads and the MALDI-MS technique were employed to investigate WS samples from OSCC patients and healthy controls. The components extracted by the WCX magnetic method could be either low-molecular-mass peptides or fragments resulting from proteolytic activity occurring in the WS after secretion into the oral cavity. WCX magnetic beads separate the proteins and/or peptides of different isoelectric points from complex biological fluids with specific anionic ligands. The techniques of MALDI-TOF-MS combined with WCX magnetic beads incorporate both of their advantages [26]; the low cost, the simple purification, could capture more proteomes than other methods especially in the low-molecular-mass range [27]; sensitive, fast and essential for clinical use [28] allowed the identification of comprehensive ‘fingerprints’ of protein profiles within biological fluids and were used to identify biomarkers of various diseases. The effectiveness of this combination of techniques has been confirmed in many saliva-based peptide profile identification studies [29,30]. We examined 40 T1-stage OSCC saliva samples and 23 healthy control samples. Eight m/z peaks were found to be significantly different between the groups. Four of these were up-regulated and four were down-regulated. The mass peaks of 1285.6 and 1432.2 were detectable in all OSCC samples at a high intensity, but seldom in the healthy subjects, suggesting that these represent markers of OSCC and may play a role in the occurrence and development of this disorder. The 1731 and 1353.9 mass peaks were detected in the majority of healthy subjects, but seldom in OSCC patients so it may be others biomarkers to detect OSCC.

Our results also differ from those of Jou et al. [9]. They found three significantly different peaks in OSCC patients and healthy controls. Two of these (m/z = 2919, 4373) were up-regulated and one (m/z = 5592) was down-regulated. The differences between the two studies may be attributable to the different age and sex of the participants involved and/or to the different methods used to extract low-molecular-mass peptides. The 95.7% of participants involved in Jou et al.’s study were men (45 male, two female) with a mean± S.D. age of 50.79±10.20 years for the OSCC patients and 76.7% were men (23 male, seven female) with 44.9±10.1 years for the healthy control subjects, whereas our study used 42.5% men (17 male, 23 female) with a mean± S.D. age of 56.25±14.23 years in OSCC patients and 56.5% men (13 male, 10 female) with 54.74±11.83 years in healthy controls. It is possible that the WS proteome changes with age [30]. The small peptides (<10 kDa) used for MS analysis in our study were extracted from WS samples by WCX magnetic beads, whereas the saliva samples used in Jou et al.’s study were precipitated by using C8-magnetic beads. Nevertheless, the different processing methods could lead to artificial losses and modification of the samples, which could influence the results significantly [31].

The peptide sequence identifications made in the present study have led to interesting speculations. The mass peaks of 1285.6 and 1432.2 were both identified as histatin-3 by matching these peaks to the mass spectrum database of Bioryong Technologies Inc. histatin-3 belong to the histatin family which are a class of peptides named according to their high histidine content [21] that were identified in human saliva approximately 30 years ago [32,33]. Histatin family consist of 12 members found in the saliva secreted by the salivary glands of humans and higher primates, are localized in human oral tissues [34]. Histatin-3, which is 32 residues in length, is encoded for by the histatin-3 precursor (HIS2) gene [35]. Histatin-3 could kill Candida albicans, the most common and the most pathogenic oral Candida species [36,37]. However, histatin-3 are also active against other yeasts and fungi, including Candida glabrata, Candida krusei, Saccharomyces cerevisiae and Cryptococcus neoformans [38,39] and some bacterial species, including Streptococcus mutans, Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans [40,41].

It has been reported that microorganisms, especially Candida species, are closely associated with OSCC [42–46]. Patients with OSCC tend to possess significantly raised concentrations of certain bacteria in their saliva [15,47,48]. Previous studies by various investigators have demonstrated a significant correlation between oral candidiasis and oral squamous carcinoma in a number of studies [49,50]. Rehani et al. [51] identified Candida as a possible factor in the development of OSCC. Marttila et al. [52] found that Candida colonization frequency and density were higher at oral mucosa of OSCC patients than in healthy controls1. Oral microorganisms inevitably up-regulate cytokines and other inflammatory mediators that affect the complex metabolic pathways and may thus be involved in carcinogenesis [46]. It has been suggested that Candida species play a role in oral carcinogenesis by triggering nitrosamine compounds to activate specified oncogenes, thereby initiating oral neoplasia [51,53,54]. Previous studies were also demonstrated that the secreted anti-microbial proteins responsible for combating oral candidiasis include the salivary histatins [55,56]. So it suggests that the high level of histatin-3 in OSCC patients’ saliva our findings were modulated by the raised concentrations of oral candidiasis.

In addition, histatin-3 are also involved in cell proliferation through the regulation of heat shock cognate protein 70 (HSC70) and cyclin-dependent kinase inhibitor 1B (p27Kip1) in oral cells [57] and could also bound to HSC70 inhibits HSC70-mediated activation of toll-like receptor (TLR) 4 signalling activation [58]. TLRs are a family of transmembrane proteins that recognize a variety of endogenous and microbial agents. The TLR 4 could lead to more aggressive, invasive behaviour of OSCCs [59]. It indicated that the histatin-3 may be involved in the progression of OSCC by interacting with TLR 4.
There is agreement that anti-microbial treatment is important pre-, during and post-therapy for oral cancer patients [46,60–62]. Histatin-3 possesses potent anti-fungal and anti-microbial properties and has the advantage over conventional syntheticazole or polyene anti-fungals and anti-microbial of being a naturally occurring compound in man, with no known cross-reactivity with human cells or tissues [63]. These qualities make it an ideal compound for development as an anti-fungal agent in the treatment of fungal infections of the oral cavity [64]. An important consideration in the development potential of histatin as a therapeutic agent would be the determination of the in vivo mechanism, occurrence and significance of resistance to this peptide.

In conclusion, our results suggested mass peaks of 1285.6 and 1432.2 Da which were both identified as histatin-3 in saliva as correlated with OSCC progression. However, the discovered candidate biomarkers need to be extensively validated with wider cases. Clearly, it is challenging to translate candidate biomarkers from proteomic investigations into real-world diagnostic or prognostic applications. Approval of use of histatin-3 as a biomarker to detect early stage of relies on the results of large-scale multicentre clinical trials. We plan to undertake such a study in the future.

AUTHOR CONTRIBUTION

Feng Chen conceived the study and revised the manuscript. Wei-Peng Jiang drafted the manuscript. Li-Xin Xu performed statistical analysis. Xin Peng revised the manuscript. Zhen Wang performed validation experiments. All authors read and approved the final manuscript.

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REFERENCES

1 Parkin, D.M. (2001) Global cancer statistics in the year 2000. Lancet Oncol. 2, 533–543 CrossRef PubMed

2 Funk, G.F., Karnell, L.H., Robinson, R.A., Zhen, W.K., Trask, D.K. and Hoffman, H.T. (2002) Presentation, treatment, and outcome of oral cavity cancer: a National Cancer Data Base report. Head Neck 24, 165–180 CrossRef PubMed

3 Zhang, A., Sun, H., Wang, P., Han, Y. and Wang, X. (2012) Recent and potential developments of biofluid analyses in metabolomics. J. Proteomics 75, 1079–1088 CrossRef PubMed

4 Besson, D., Pavageau, A.H., Valo, I., Bourreau, A., Belanger, A., Eymeret-Morin, C., Moliere, A., Chassevent, A., Boisdron-Cellei, M., Morel, A. et al. (2011) A quantitative proteomic approach of the different stages of colorectal cancer establishes OLFM4 as a new nonmetastatic tumor marker. Mol. Cell. Proteomics 10, M111 009712 CrossRef PubMed

5 Shelburne, S. A., 3rd, Sumby, P., Sitkiewicz, I., Granville, C., DeLeo, F.R. and Musser, J.M. (2005) Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva. Proc. Natl. Acad. Sci. U.S.A. 102, 16037–16042 CrossRef PubMed

6 Lee, K., Rho, B.S., Pi, K., Kim, H.J. and Choi, Y.J. (2011) Proteomic analysis of protein expression in Lactobacillus plantarum in response to alkaline stress. J. Biotechnol. 153, 1–7 CrossRef PubMed

7 Giacomelli, C., Bazzichi, L., Giusti, L., Ciregia, F., Baldini, C., Da Valle, Y., De Feo, F., Sermisi, F., Rossi, A., Bombardieri, S. and Lucacchini, A. (2011) (MALDI-TOF and SELDI-TOF analysis: “tandem” techniques to identify potential biomarker in fibromyalgia). Reumatismo 63, 165–170 CrossRef PubMed

8 Terracciano, R., Preiano, M., Palladino, G.P., Carpagno, N.G., Barbaro, M.P., Pelaia, G., Savino, R. and Maselli, R. (2011) Peptide profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for non-invasive biomarker discovery of chronic inflammatory lung diseases. Proteomics 11, 3402–3414 CrossRef PubMed

9 Jou, Y.J., Lin, C.D., Lai, C.H., Tang, C.H., Huang, S.H., Tsai, M.H., Chen, S.Y., Kao, J.Y. and Lin, C.W. (2011) Salivary zinc finger protein 510 peptide as a novel biomarker for detection of oral squamous cell carcinoma in early stages. Clin. Chim. Acta 412, 1357–1365 CrossRef PubMed

10 Zhang, J., Zhou, S., Li, R., Cao, T., Zheng, H., Wang, X., Zhou, Y., Du, N., Chen, F. and Lin, J. (2012) Magnetic bead-based salivary peptide profiling for periodontal-orthodontic treatment. Proteome. Sci. 10, 63 CrossRef PubMed

11 Zhang, J., Zhou, S., Zheng, H., Zhou, Y., Chen, F. and Lin, J. (2012) Magnetic bead-based salivary peptide profiling analysis during orthodontic treatment durations. Biochem. Biophys. Res. Commun. 421, 844–849 CrossRef PubMed

12 Wu, Z.Z., Wang, J.G. and Zhang, X.L. (2009) Diagnostic model of salivary protein finger print analysis of patients with gastric cancer. World J. Gastroenterol. 15, 865–870 CrossRef PubMed

13 Sanjay, R.R., Halikkeri, K. and Shivashankara, A.R. (2008) Evaluation of salivary sialic acid, total protein, and total sugar in oral cancer: a preliminary report. Indian J. Dent. Res. 19, 288–291 CrossRef PubMed

14 Liljemark, W.F. and Gibbons, R.J. (1972) Proportional distribution and relative adherence of Streptococcus miteor on various surfaces in the human oral cavity. Infect. Immun. 6, 852–859 PubMed

15 Mager, D.L., Haffajee, A.D., Devlin, P.M., Norris, C.M., Posner, M.R. and Goodson, J.M. (2005) The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J. Transl. Med. 3, 27 CrossRef PubMed

16 Hu, S., Loo, J.A. and Wong, D.T. (2006) Human body fluid proteome analysis. Proteomics 6, 6326–6353 CrossRef PubMed

17 Helmerhorst, E.J., Sun, X., Salih, E. and Oppenheim, F.G. (2008) Identification of Lys-Pro-Gln as a novel cleavage site specificity of salivaa-associated proteases. J. Biol. Chem. 283, 19957–19966 CrossRef PubMed

18 Vitorino, R., Barros, A., Caseiro, A., Domingues, R., Duarte, J. and Amado, F. (2009) Towards defining the whole salivary peptidome. Proteomics Clin. 5, 13
19 Helmerhorst, E.J. and Oppenheim, F.G. (2007) Saliva: a dynamic proteome. J. Dent. Res. 86, 680–693 CrossRef PubMed

20 Schlesinger, D.H., Hay, D.I. and Levine, M.J. (1989) Complete primary structure of statherin, a potent inhibitor of calcium phosphate precipitation, from the saliva of the monkey, Macaca arctoides. Int. J. Pept. Protein. Res. 34, 374–380 CrossRef PubMed

21 Oppenheim, F.G., Xu, T., McMillian, F.M., Levitz, S.M., Diamond, R.D., Offner, G.D. and Troxler, R.F. (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. J. Biol. Chem. 263, 7472–7477 PubMed

22 Hay, D.I., Bernick, A., Schlesinger, D.H., Minaguchi, K., Madapallimattam, G. and Schluckebier, S.K. (1988) The primary structures of six human salivary acute proline-rich proteins (PRP-1, PRP-2, PRP-3, PRP-4, PRP-5 and PRP-6). Biochem. J. 255, 15–21 PubMed

23 Edgerton, M. and Koshiukhova, S.E. (2000) Salivary histatin 5 and its similarities to the other antimicrobial proteins in human saliva. Adv. Dent. Res. 14, 16–21 CrossRef PubMed

24 Amado, F.M., Vitorino, R.M., Domingues, P.M., Lobo, M.J. and Duarte, J.A. (2005) Analysis of the human saliva proteome. Expert Rev. Proteomics 2, 521–539 CrossRef PubMed

25 Chen, H., Xu, Z., Peng, L., Fang, X., Yin, X., Xu, N. and Cen, P. (2006) Recent advances in the research and development of human defensins. Peptides 27, 931–940 CrossRef PubMed

26 Sun, L., Chen, H., Hu, C., Wang, P., Li, Y., Xie, J., Tang, F., Ba, D., Zhang, X. and He, W. (2011) Identity biomarkers of neuropsychiatric systemic lupus erythematosus by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry combined with weak cation magnetic beads. J. Rheumatol. 38, 454–461 CrossRef PubMed

27 Li, Y.H., Wang, J., Zheng, X.L., Zhang, Y.L., Li, X., Yu, S., He, X. and Chan, P. (2011) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry combined with magnetic beads for detecting serum protein biomarkers in parkinson’s disease. Eur. Neurol. 65, 105–111 CrossRef PubMed

28 Guo, N., Wen, Q., Li, Z.J., Xu, R.C., Peng, F.F. and Yu, X.Q. (2014) Optimization and evaluation of magnetic bead separation combined with matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) for proteins profiling of periodontal disease effluent. Int. J. Mol. Sci. 15, 1162–1175 CrossRef PubMed

29 Schwamborn, K., Krieg, R.C., Grosse, J., Reulen, N., Weihskirchen, R., Knuechel, R., Jakse, G. and Henkel, C. (2009) Serum proteomic profiling in patients with bladder cancer. Eur. Urol. 56, 989–996 CrossRef PubMed

30 Fleissig, Y., Reichenberg, E., Redlich, M., Zaks, B., Deutsch, O., Afamian, D.J. and Palmon, A. (2010) Comparative proteomic analysis of human oral fluids according to gender and age. Oral Dis. 16, 831–839 CrossRef PubMed

31 Amado, F., Lobo, M.J., Domínguez, R.M., Duarte, J.A. and Vitorino, R. (2010) Salivary peptidomics, Expert Rev. Proteomics 7, 709–721 CrossRef PubMed

32 Bonilla, C.A. (1969) Rapid isolation of basic proteins and polypeptides from salivary gland secretions by adsorption chromatography on polyacrylamide gel. Anal. Biochem. 32, 522–529 CrossRef PubMed

33 Azem, E.A. (1972) Genetic polymorphism of basic proteins from parotid saliva. Science 176, 673–674 CrossRef PubMed

34 Fitzgerald, D.H., Coleman, D.C. and O’Connell, E.D. (2003) Susceptibility of Candida dubliniensis to salivary histatin 3. Antimicrob. Agents Chemother. 47, 70–76 CrossRef

35 Sabatini, L.M. and Azem, E.A. (1989) Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (His1 and His2). Biochem. Biophys. Res. Commun. 160, 495–502 CrossRef PubMed

36 Calderone, R.A. and Fonzi, W.A. (2001) Virulence factors of Candida albicans. Trends Microbiol. 9, 327–335 CrossRef PubMed

37 Xu, X., Ambudkar, I., Yamagishi, H., Swaim, W., Walsh, T.J. and O’Connell, B.C. (1999) Histatin 3-mediated killing of Candida albicans: effect of extracellular salt concentration on binding and internalization. Antimicrob. Agents Chemother. 43, 2256–2262 PubMed

38 Pollock, J.J., Denepitiya, L., MacKay, B.J. and Iacono, V.J. (1984) Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on Candida albicans. Infect. Immun. 44, 702–707 PubMed

39 Rayhan, R., Xu, L., Santarpia, R.P., III, Tylenda, C.A. and Pollock, J.J. (1992) Antifungal activities of salivary histidine-rich polypeptides against Candida albicans and other oral yeast isolates. Oral Microbiol. Immunol. 7, 51–52 CrossRef PubMed

40 MacKay, B.J., Denepitiya, L., Iacono, V.J., Krost, S.B. and Pollock, J.J. (1984) Growth-inhibitory and bactericidal effects of human parotid salivary histidine-rich polypeptides on Streptococcus mutans. Infect Immun. 44, 695–701 PubMed

41 Murakami, Y., Nagata, H., Amano, A., Takagaki, M., Shizukushi, S., Tsunemitsu, A. and Aimoto, S. (1991) Inhibitory effects of human saliva histatins and lysosome on coaggregation between Porphyromonas gingivalis and Streptococcus mutis. Infect. Immun. 59, 3284–3286 PubMed

42 Nagy, K.N., Sonkodi, I., Szőke, I., Nagy, E. and Newman, H.N. (1998) The microflora associated with human oral carcinomas. Oral Oncol. 34, 304–308 CrossRef PubMed

43 Johnson, N.J., Jayasekara, P. and Amarasinghe, A.A. (2011) Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology. Periodontol. 2000. 57, 19–37 CrossRef PubMed

44 Tanaka, T., Tanaka, M. and Tanaka, T. (2011) Oral carcinogenesis and oral cancer chemoprevention: a review. Patholog. Res. 2011 431246

45 Mantovani, A., Garlanda, C. and Allavena, P. (2010) Molecular pathways and targets in cancer-related inflammation. Ann. Med. 42, 161–170 CrossRef PubMed

46 Meurman, J.H. (2010) Oral microbiota and cancer. J. Oral. Microbiol. 2, doi: 10.3402/jom.v2i0.5195 CrossRef

47 Hooper, S.J., Crean, S.J., Fardy, M.J., Lewis, M.A., Spratt, D.A., Wade, W.G. and Wilson, M.J. (2007) A molecular analysis of the bacteria present within oral squamous cell carcinoma. J. Med. Microbiol. 56, 1651–1659 CrossRef PubMed

48 Pushalkar, S., Mane, S.P., Ji, X., Li, Y., Evans, C., Crasta, O.R., Morse, D., Meagher, R., Singh, A. and Saxena, D. (2011) Microbial diversity in saliva of oral squamous cell carcinoma. J. Med. Microbiol. 61, 269–277 CrossRef PubMed

49 Uittamo, J., Siikala, E., Kihovaloa, R., Salaspuro, M. and Rautema, R. (2009) Chronic candidosis and oral cancer in APECED-patients: production of carcinogenic acetaldehyde from glucose and ethanol by Candida albicans. Int. J. Cancer. 124, 754–756 CrossRef PubMed

50 Rosa, D.D., Pasquato, A.C. and Denning, D.W. (2008) Chronic mucocutaneous candidiasis and oesophageal cancer. Med. Mycol. 46, 85–91 CrossRef PubMed

51 Rehni, S., Rao, N.N., Rao, A., Carneilo, S., Ramakrishnaiah, S.H. and Prakash, P.Y. (2011) Spectrophotometric analysis of the expression of secreted aspartyl proteinases from Candida in leukoplakia and oral squamous cell carcinoma. J. Oral. Sci. 53, 421–425 CrossRef PubMed

52 Arbula, J., Uitto, J., Rusanen, P., Lindqvist, C., Salaspuro, M. and Rautema, R. (2013) Acetaldehyde production and microbial colonization in oral squamous cell carcinoma and oral lichenoid disease. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 116, 61–68 CrossRef PubMed

53 Field, E.A., Field, J.K. and Martin, M.V. (1989) Does Candida have a role in oral epithelial neoplasia? J. Med. Vet. Mycol. 27, 277–294 CrossRef
Saliva peptide for diagnosing OSCC

54 Sanjaya, P.R., Gokul, S., Gururaj Patil, B. and Raju, R. (2011) Candida in oral pre-cancer and oral cancer. Med. Hypotheses 77, 1125–1128 CrossRef PubMed

55 Bercier, J.G., Al-Hashimi, I., Haghighat, N., Rees, T.D. and Oppenheim, F.G. (1999) Salivary histatins in patients with recurrent oral candidiasis. J. Oral Pathol. Med. 28, 26–29 CrossRef PubMed

56 Fitzgerald-Hughes, D.H., Coleman, D.C. and O’Connell, B.C. (2007) Differentially expressed proteins in derivatives of Candida albicans displaying a stable histatin 3-resistant phenotype. Antimicrob. Agents Chemother 51, 2793–2800 CrossRef PubMed

57 Imamura, Y., Fujigaki, Y., Oomori, Y., Usui, S. and Wang, P.L. (2009) Cooperation of salivary protein histatin 3 with heat shock cognate protein 70 relative to the G1/S transition in human gingival fibroblasts. J. Biol. Chem. 284, 14316–14325 CrossRef PubMed

58 Imamura, Y. and Wang, P.L. (2014) Salivary histatin 3 inhibits heat shock cognate protein 70-mediated inflammatory cytokine production through toll-like receptors in human gingival fibroblasts. J. Inflamm. 11, 4 CrossRef

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59 Ahmed Haji Omar, A., Korvala, J., Haglund, C., Virolainen, S., Hayry, V., Atula, T., Kontio, R., Riihimäki, J., Phakari, A., Sorsa, T. et al. (2015) Toll-like receptors -4 and -5 in oral and cutaneous squamous cell carcinomas. J. Oral Pathol. Med. 44, 258–265 CrossRef PubMed

60 Nagy, K., Szoke, I., Sonkodi, I., Nagy, E., Mari, A., Szolnoky, G. and Newman, H.N. (2000) Inhibition of microflora associated with oral malignancy. Aust. Dent. J. 36, 32–36

61 Chandy, A., Stulner, C., Bridgeman, A.M. and Smith, A.C. (2002) Maintenance of mouth hygiene in patients with oral cancer in the immediate post-operative period. Curr. Pharm. Des. 47, 170–173

62 Meyer, J.E. and Harder, J. (2007) Antimicrobial peptides in oral cancer. J. Pharm. Pharmacol. 13, 3119–3130

63 Kavanagh, K. and Dowd, S. (2004) Histatins: antimicrobial peptides with therapeutic potential. Expert Opin. Investig. Drugs 56, 285–289

64 Lupetti, A., Danesi, R., van ’t Wout, J.W., van Dissel, J.T., Senesi, S. and Nibbering, Ph. (2002) Antimicrobial peptides: therapeutic potential for the treatment of Candida infections. Expert. Opin. Inv. Drug. 11, 309–318 CrossRef

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