Salivary proteome profiling of oral squamous cell carcinoma in a Hungarian population

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Running title: Proteomic analysis of salivary biomarkers in OSCC

Abbreviations
OSCC – oral squamous cell carcinoma
ELISA – enzyme-linked immunosorbent assay
MS – mass spectrometry
UPLC – Ultra-Performance Liquid Chromatography

Keywords: OSCC, proteomic analysis, ELISA, biomarker

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Abstract
Oral squamous cell carcinoma (OSCC) is the 7th most common malignancy and the 9th most frequent cause of cancer death in Europe. Within Europe, Hungary has one of the highest rates of OSCC incidence and mortality. Thus, there is an urgent need to improve early detection. Saliva, as a readily available body fluid, became an increasingly important substance for the detection of biomarkers for many diseases. Different research groups have identified salivary biomarkers specific for OSCC for different countries. In this study, saliva samples of Hungarian OSCC patients were studied in order to discover disease- and perhaps region-specific biomarkers. LC/MS/MS analysis on a linear ion trap-Orbitrap mass spectrometer was used for qualitative and quantitative salivary protein profiling. More than 500 proteins were identified from saliva by shotgun proteomics. The up- and downregulated proteins in the saliva of patients with OSCC highlighted the importance of protein-protein interaction networks involving the immune system and proteolysis in disease development. Two potential biomarkers from our shotgun analysis and a third candidate reported earlier by a Taiwanese group were further examined by ELISA on a larger reference set of samples. Resistin, a biomarker reported in Taiwan but not validated in our study, highlights the necessity of application of standardized analysis methods in different ethnic or geographical populations in order to identify biomarkers with sufficient specificity and sensitivity.

Introduction
The oral cavity is the most frequent site of head and neck cancers, developing predominantly as oral cavity squamous cell carcinomas (OSCC) in the upper aerodigestive epithelium [1,2]. The three major recognized risk factors of OSCC are tobacco, and alcohol-consumption and poor oral hygiene [3–5]. OSCC mortality rates reflect the different consumption patterns of alcohol and tobacco in European countries [6]. Annually more than 300,000 new patients are diagnosed with OSCC worldwide. The disease is associated with poor prognosis and high mortality mainly due to late diagnosis because of the lack of reliable early diagnostic markers [7]. Mortality rate from OSCC is about 10-fold higher for men than for women. However, female OSCC incidence increased dramatically in the last decade. In addition, a rising tendency was observed in younger patient cohorts [8]. In contrast to other European countries where the mortality rates of OSCC started to decline, unfavorable incidence and mortality figures remained exceedingly high in Hungary since the 1970s’ representing a major public health challenge [9].

Development of cancer diagnostic tools with sufficiently high sensitivity and specificity is required to enable early detection of OSCC [10]. Recent treatment strategies of OSCC patients are based on traditional stage-predicting indices and histological grading [11]. Unfortunately, these predictors are relatively subjective and unreliable because tumors with same staging and grading may respond to therapy differently. Thus, improving the diagnostic methods is required. A potential way of improving our diagnostic tools is to perform in-depth salivary analyses to discover and to assess biochemical and immunological markers in the saliva for early oral cancer diagnosis [12-13]. Biomarkers identified in the last decades in biological fluids can be linked to carcinogenesis and may serve as prognostic factors and saliva is a new clinical biomarker source that can be easily collected by non-invasive means [14-18]. Since there is direct contact between saliva and the oral lesion(s) disease-related concentration changes of saliva ingredients may provide as good or better clues than serum samples [19]. More than 3700 salivary proteins have been identified by several research groups [20,21]. Many proteins were declared potential salivary biomarkers of OSCC in different countries [22–24]. In this
study, we present a two-stage approach for the discovery of candidate OSCC-specific salivary biomarkers in the Hungarian population. LC/MS/MS analysis using ultra-Performance Liquid Chromatography (UPLC) coupled to a linear ion trap-Orbitrap hybrid tandem mass spectrometer was applied for qualitative and quantitative salivary protein profiling. Selected proteins, based on the shotgun analysis of a few randomly selected samples, were further investigated by ELISA on a reference set of samples.

Materials and Methods

Patients and saliva collection

Donor enrollment, sample collection and processing were conformed to the principles of the Helsinki Declaration. Ethical approval was obtained from the University of Debrecen Ethics Committee (No. 3385-2011) and all subjects provided written informed consent. Clinical examinations were performed by dental surgeons from the Faculty of Dentistry, University of Debrecen. Adult patients (>18 yrs) with histology-proven OSCC were recruited into the study. Saliva samples were collected before starting any anti-tumor therapy. Age-matched controls (MCTL) were consecutive patients and young controls (YCTL) were medical students admitted to the Faculty of Dentistry for regular dental checkup. Exclusion criteria included children (≤18 yrs), pregnancy and breast feeding, diabetes mellitus, human papillomavirus infection, human immunodeficiency virus infection, autoimmune and immunodeficiency disorders and cancer other than OSCC.

Unstimulated saliva samples were collected from 43 donors between 9 a.m. and 11 a.m. at the Faculty of Dentistry, University of Debrecen (collection between 2013.05.09-2016.02.29.). The test set contained 3 randomly selected samples from patients with OSCC and controls for proteomic analysis, whereas the reference set contained samples from 20 patients with OSCC (mean age: 57 year), 6 young (mean age: 24.5 year) and 11 age-matched CTLs (mean age: 59 year) for biomarker verification. Saliva samples were kept on ice during collection and were filtered using 5 μm pore size Millipore SLSV025LS syringe filters (Merck, Billerica, MA, USA). The filtered saliva was aliquoted and immediately placed to -70°C until further use.

Sample preparation for mass spectrometry

Filtered saliva was dried in speed-vac and redissolved in 25 mM pH 8.5 ammonium bicarbonate buffer. Total protein concentration of salivary samples was measured using the Bradford method [25]. Following denaturation with 8 M urea, all samples were reduced with 10 mM dithiothreitol (Bio-Rad, Hercules, CA, USA) in ammonium bicarbonate buffer. Then samples were alkylated with 20 mM iodoacetamide (Bio-Rad, Hercules, CA, USA) in ammonium bicarbonate buffer and diluted with 25 mM ammonium bicarbonate (Sigma, St. Louis, MO, USA) in order to reduce the urea concentration to 1 M. Each sample was digested by MS grade modified trypsin (ABSciex, Framingham, MA, USA) in 1:25 enzyme to protein ratio (w/w) at 37°C, overnight. The digested samples were dried in speed-vac and redissolved in 0.1% formic acid. The digests were desalted on Pierce C18 Tips (Thermo Scientific, West Palm Beach, FL, USA) and the eluates were dried and stored at -70°C until mass spectrometry analysis.
Mass spectrometry analysis

Tryptic digests representing 2 μg total protein were analyzed by LC-MS/MS using a Waters nanoAcquity UPLC on-line coupled to a linear ion trap-Orbitrap hybrid tandem mass spectrometer (Orbitrap Elite, Thermo Scientific) operating in positive ion mode. After trapping at 3% B (Waters Symmetry C18 180 μm x 20 mm column, 5 μm particle size, 100 Å pore size; flow rate: 10 μl/min), peptides were fractionated using a linear gradient of 3 to 40% B in 100 min (Waters BEH C18 75 μm x 250 mm column, 1.7 μm particle size, 300 Å pore size; solvent A: 0.1% formic acid/water, solvent B: 0.1% formic acid / 5% dimethyl sulfoxide / acetonitrile; flow rate: 400 nl/min). Data acquisition was carried out in a data-dependent fashion, the 10 most abundant, multiply charged ions were selected from each MS survey (m/z: 380-1600, resolution: 60000, acquired in profile mode) for MS/MS analyses. CID analyses were performed in the linear ion trap (normalized collision energy: 35). Dynamic exclusion was enabled (exclusion time: 30 s).

Protein identification

Peak lists generated from the MS/MS data by the ‘PAVA’ software [26] were searched against the human subset of the Uniprot database (downloaded 06/10/2014; 136245 target sequences concatenated with a randomized sequence for each entry) using the ProteinProspector search engine (v.5.10.9.). Search parameters: enzyme: trypsin with maximum 1 missed cleavage site; fixed modification: carbamidomethyl (Cys); variable modifications: acetylation (protein N-terminus), oxidation (Met), pyroglutamic acid formation (N-terminal Gln) allowing up to 2 variable modifications per peptide; mass accuracy: 5 ppm and 0.6 Da for precursor and fragment ions (both monoisotopic), respectively. The following acceptance criteria were applied: score>22 and 15, and E-value<0.01 and 0.05 for protein and peptide identifications, respectively. The false positive rates of the identified proteins and peptides were less than 1%. Relative abundance of individual proteins was estimated by spectral counting: the number of identifications per protein (PSMs) was normalized to the total number of identifications, then these relative spectral counts were compared across the different samples.

Functional analyses were performed in case of proteins with at least three unique peptide identifications. For the calculation of the OSCC/control ratio those proteins were considered which were identified with at least 3 unique peptides in at least two out of three samples in either the control or the OSCC group.

Validation of the candidate biomarkers using ELISA

All saliva samples from patients with OSCC and controls were analyzed in duplicate with quantitative ELISA. The ELISA kit for heparin cofactor 2 (#Cat. number: LS-F13221) was purchased from LifeSpan Biosciences (Seattle, WA, USA), for resistin (#Cat. number: KHP0051) from Thermo Fisher Scientific (West Palm Beach, FL, USA) and for complement c5 (#Cat. number: ab125963) from Abcam (Branford, Connecticut, USA). The concentration of the studied proteins in saliva was measured by sandwich enzyme-linked immunosorbent assay method according to the instruction provided by the vendor of each kit. Absorbance was measured at 450 nm and concentrations were calculated based on the recorded 7-point calibration curves.

First, the variation coefficient of the parallel measurements was calculated and those data having more than 25 CV % value were excluded from statistical analysis.
Bioinformatics

The cluster analysis was carried out with Cluster 3.0 (http://cluster2.software.informer.com/) using the C Clustering Library version 1.52 and the heat map was created with Java TreeView version 1.1.6r4 [27]. The protein-protein interaction network of salivary proteins was generated using String version 10.5 [28,29] applying default settings and medium stringency. After the generation of networks the enriched GO terms provided by the software were also examined. The statistical analysis of ELISA data was performed using the Mann–Whitney U test and the two sample T-test in order to compare the protein concentrations between groups. Those data were considered significantly different where the p value was p < 0.05.

Results and Discussions

Demographic and clinical characteristics of patients with OSCC

Among the included 17 patients 13 were males and 4 females between the age of 44-73 yrs. In six cases the tumor developed in the tongue (T), in 4 cases in the floor of the mouth (F) and in 3 cases it was detected in the gingival (G) region. In 4 cases the tumor development showed multiple localization, in 2 patients the tumor developed in the tongue and either in the floor of the mouth or the gingival region, while in another 2 patients the tumor development was detected in the tongue, in the floor of the mouth and also in the gingival region. Eight patients were discovered in early tumor development stage (stage I.: 5, stage II.: 3) and 9 patients were diagnosed with advanced tumors (stage III.: 4 and stage IV.: 5). There were six well differentiated (W), 7 moderately differentiated (M) and 4 poorly differentiated (P) OSCC samples (Table 1).

Shotgun proteomic analysis of saliva samples

Three randomly selected samples from patients with OSCC and matched controls respectively, were subjected to shotgun proteomics analysis. More than 500 proteins were identified from salivary samples. For protein quantification spectral counting was used and the ratios of OSCC:CTL protein quantities have been determined. Detailed information of the identified proteins is presented in Supplementary Table 1. The proteins with at least 3 unique sequences and with at least 2 fold change value (OSCC/CTL ratio <0.5 or >2) were subjected to further examination. A cluster analysis was carried out and a heat map was generated to visualize the changes in protein amount in CTL and OSCC samples (Figure 1). Based on cluster analysis the protein levels can discriminate the OSCC group from the CTL group. Proteins were classified as salivary proteins or proteins being present in saliva under normal conditions and as acute phase proteins (Table 2). For protein classification the Uniprot and the Sys-BodyFluid databases were used, the latter contains more than 10,000 proteins of different body fluid proteomes [30]. In addition, some proteins were classified as salivary proteins based on literature data [21, 31–35].

2 proteins, the cytochrome c and mucin-7 were only present in the CTL samples and 6 proteins, the complement factor H (CFH) and C5 (C5), corticosteroid binding globulin (SERPINA6), heparin cofactor 2 (SERPIND1), apolipoprotein E (APOE) and serum paraoxonase/arylesterase 1 (PON1), were only present in the OSCC samples (Table 3).
Functional analysis of salivary proteins

It was observed that the level of apolipoproteins, components of the complement system, proteinases, proteinase inhibitors, components of the coagulation cascade is upregulated indicating a change in proteolysis most probably associated with the interrelated coagulation cascade-complement activation processes. In the same time the level of proteins having role in metabolism and host defense was downregulated showing extensive cancer-related changes (Table 2). For a more detailed functional analysis of the differentially expressed proteins gene ontology (GO) analysis was performed; the Biological Process, Molecular Function and Cellular Localization according to GO (http://www.geneontology.org/) was examined. First, the network of differentially expressed proteins was generated using String version 10.5 [28,29] followed by GO enrichment analysis provided by String. The network of downregulated proteins contained 35 proteins (nodes) and 27 possible protein-protein interactions analyzed at medium stringency (Figure 2A). No biological function was enriched in the down-regulated proteins in this loosely connected network (Figure 2B), however 7 out of 35 downregulated proteins are metabolic enzymes participating mainly in carbohydrate metabolism and 10 proteins out of 35 have a role in defense. The upregulated 45 proteins show a highly interconnected protein-protein interaction network with 400 interactions analyzed at medium stringency (Figure 2C). The enriched functions indicate active regulatory mechanisms implicating the immune system, lipid metabolism, plasminogen activation, antioxidant activity and inhibition of enzymatic activities (Figure 2D). Regarding localization of up- or downregulated proteins, all are mainly extracellular proteins according to GO (Figure 2B and 2D), but a part of the upregulated proteins originate from lipid particles or platelet alpha granules indicating the presence of a possibly cancer-induced complex process involving systemic mechanisms.

In order to get more insights into the changes associated with OSCC a literature search was performed to see which proteins have been associated with oncogenesis. Most of the proteins were already associated with OSCC, and 32 proteins were identified to be present in saliva in this pathological condition.

Complement C4B (C4B), complement factor B (CFB), complement C3 and alpha-1-antitrypsin were shown to be associated with the risk of developing OSCC according to a targeted proteomics study [36]. The level of apolipoproteins A and E, PON1, inter-alpha-trypsin inhibitor heavy chain H1, H2 and H4, kininogen 1, protein AMBP, nucleobinding-2, SERPIN1D1 and SERPINA6 were found to be upregulated in OSCC in shotgun proteomics experiments carried out on saliva samples [23]. The presence of ApoE was related to the increased invasion potential of OSCC [37].

The alpha-1-acid glycoprotein, alpha-1B glycoprotein, alpha amylase, beta-2-glycoprotein, carbonic anhydrase 1, cystatin-SA, hemopexin, phosphoglycerate kinase and serum albumin were identified as potential salivary markers of OSCC [14,16,22].

Some of the proteins found to be differentially expressed in our study such as fibrinogen alpha, beta and gamma chains, haptoglobin, SERPINB5, retinol binding protein 4 and ceruloplasmin, were shown to be plasma markers of OSCC while the presence of integrin alpha-M and fibronectin FN1 was demonstrated in the OSCC tissue [12,38-43].

In case of 36 proteins no association with OSCC was found so far (Table 2). Angiotensinogen and plasminogen themselves were not found to be associated with OSCC but the plasminogen activator system was shown to be a predictive marker for early OSCC and by bioinformatics analysis the angiotensin converting enzymes were associated with malignant epithelial neoplasia characteristic for OSCC [44,45]. In case of 6 proteins not the
protein from our list, but another protein from the same family was already demonstrated to be differentially expressed in OSCC (Table 2). In case of SERPINB5 there are contradictory data; in our study the level of SERPINB5 was found to be elevated in OSCC, however the SERPINB5 and different forms of SERPINS from clade B were found by other groups to be downregulated in OSCC on mRNA level and higher SERPINB5 levels were found to correlate with better prognosis of patients with oral cancer [46,47].

Plasma protease C1 inhibitor (SERPING1), antithrombin III and fibronectin were found to play a role in carcinogenesis but their implication in oral cancer, especially in OSCC has not been demonstrated yet [48,49]. The complement factor H was previously identified in lung adenocarcinoma and cutaneous squamous cell carcinoma, but not in OSCC [50,51] and Apo B100 was found in serum of patients with head and neck squamous cell carcinoma [52]. No data were found on the presence of complement C5 and mucin-7 in cancer, however other components of the complement system and other forms of mucins were all identified in different forms of cancer and in OSCC as well [36, 53].

As for the involvement of cytochrome c, it was shown that the HIF-1alpha-dependent suppression of hypoxia-induced apoptosis in OSCC happens through the inhibition of cytochrome c release [54].

Examination of the level of selected proteins by ELISA

Many of the studies published in the scientific literature are based on shotgun proteomic experiments. Only few of the proteins listed in Table 2 were verified or validated either using SRM-based targeted or antibody-based methods. Considering the proteins present only in OSCC based on our shotgun experiments, the data presented in the literature, and the availability of antibodies, SERPIND1 and C5 were selected for further studies. In order to test the utility of potential biomarkers identified in Asia for a European population, resistin reported to be a potential biomarker for OSCC in Taiwan [23] was also selected.

The concentrations of C5, SERPIND1 and resistin were examined in the saliva of patients with OSCC, age-matched and young controls using quantitative sandwich ELISA kits (Figure 3). In case of C5 the difference was significant but only when young controls and patients with OSCC or young controls and age-matched controls were compared indicating that the level of C5 was age-dependent or it was influenced by other factors. One such factor can be the inflammatory status related to poor oral hygiene often observed in the middle-aged and elderly population in Hungary [55]. This means that despite the differential expression of C5 in the OSCC group, the level of C5 does not discriminate between the target age-matched control and diseased group and hence it cannot be used as a biomarker for OSCC.

In case of resistin and SERPIND1 no significant differences were found between the groups. Resistin was not up- or downregulated according to our shotgun experiments and did not show significant differences in the ELISA experiments either. In case of SERPIND1 one possible explanation of the disagreement between the shotgun proteomics and ELISA data can be that the low number of samples (3 for each group) tested by shotgun proteomics and the high individual variation of the saliva samples collected from the patients may lead to false positive results. This outcome highlights the importance of validation of the shotgun proteomics data on larger patient cohorts to decrease the false positivity of biomarker identifications. In a two-stage experimental approach, starting with a shotgun proteomics experiment, the level of resistin was found to be significantly higher in saliva samples of OSCC patients compared to controls. However, following ELISA assays showed that the median values in the OSCC group were only slightly elevated compared to the control group [23].
same study SERPIND1 was not validated but was shown to be upregulated in the saliva samples of patients with OSCC. In our study a similar experimental setup was applied; in the shotgun experiment the level of SERPIND1 was higher but the level of resistin did not change markedly in the OSCC group and the validation of SERPIND1 and resistin show that none of them turned to be useful potential biomarkers. The fact that resistin was identified as a biomarker for OSCC in Taiwan but not in Hungary gives further evidence for the importance of regional studies highlighted in our previous work [56].

Conclusions
Global analysis of salivary samples from patients with OSCC and controls contribute to the better understanding of the disease, including the interaction of tumor cells with their environment and the influence of cancer lesion on salivary protein ecology. Salivary proteins, characterizing patients with OSCC in this study highlighted the importance of networks involving the immune system and proteolysis in this disease. Six proteins were only detected in OSCC samples by proteomic analyses and two of them were further examined using ELISA assay but none of the proteins turned to be a potential biomarker in OSCC in our study group. The fact that resistin was shown to be a possible biomarker in Taiwan but not in our study highlights the importance of regional or population-tailored studies.

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Author contribution statement
IM, EC and CK designed the experiments, IM, JN performed stomatologic examination of patients, BM, ZD carried out the experiments, BM, ZD and EC evaluated the data, BM, ES, EC prepared the figures and tables, EC, BM, ZD wrote the manuscript, JT, KM, CK and IM reviewed the manuscript.

Supplementary information available.

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Figure legends

**Figure 1.** Cluster analysis and heat map of proteins identified in CTL and OSCC groups. The relative peptide count (%), characteristic for each sample is shown.

**Figure 2.** The protein-protein interaction network and functional classification of up- and downregulated proteins in OSCC The network of downregulated (A) and upregulated (C) proteins in OSCC displayed by String 10.4 using default settings and medium stringency. Each node represents a protein and the edges represent protein-protein interactions based on different levels of evidences collected by String. The enrichment table of GO terms calculated by String in case of downregulated (B) and upregulated (D) proteins is shown indicating the number of the proteins belonging to each term and the false discovery rate value calculated by String.

**Figure 3.** Examination of potential salivary biomarkers using ELISA assay The concentration of SERPIND1 (A), resistin (B) and complement C5 (C) proteins in the saliva samples collected from OSSC patients (OSCC), young controls (YCTL) and age-matched controls (MCTL). The p value is indicated, * refers to p<0.05.
Table 1. Demographic and clinical characteristics of OSCC patients. In case of each patient the gender, age tumor localization, TNM classification, stage and stage of differentiation is given. M is for male, F for female. Regarding tumor localization, T is for tongue, G is for gingiva, F is for floor of the mouth. The W is for well differentiated, M is for moderately differentiated and P is for poorly differentiated tumors.

| Patient code | Gender | Age (year) | Tumor localization | TNM classification | Tumor stage | Stage of differentiation |
|--------------|--------|------------|--------------------|--------------------|-------------|--------------------------|
| 1            | M      | 73         | T                  | T2N1M0             | III         | W                       |
| 2            | F      | 69         | G                  | T4N0M0             | IV          | W                       |
| 3            | F      | 67         | F                  | T4N2M0             | IV          | W                       |
| 4            | M      | 52         | T;G;F              | T4N1M0             | IV          | M                       |
| 5            | M      | 57         | T                  | T3N0M0             | III         | W                       |
| 6            | F      | 59         | T                  | T1N0M0             | I           | W                       |
| 7            | M      | 67         | F                  | T1N0M0             | I           | W                       |
| 8            | F      | 50         | T                  | T2N0M0             | II          | M                       |
| 9            | M      | 52         | T;G                | T2N2M0             | IV          | M                       |
| 10           | M      | 48         | T                  | T1N0M0             | I           | M                       |
| 11           | M      | 64         | T                  | T2N0M0             | II          | P                       |
| 12           | M      | 44         | G                  | T4N1M0             | IV          | M                       |
| 13           | M      | 44         | T;F                | T3N0M0             | III         | M                       |
| 14           | M      | 60         | F                  | T2N0M0             | II          | M                       |
| 15           | M      | 49         | T;G;F              | T3N1M0             | III         | P                       |
| 16           | M      | 47         | G                  | T1N0M0             | I           | P                       |
| 17           | M      | 64         | F                  | T1N0M0             | I           | P                       |
Table 2. List of proteins with at least two fold change between OSCC and CTL groups. The Uniprot protein ID, the protein name and function is presented. The representative identification and quantification data, the number (#) of unique peptides, the sequence coverage (% Cov) and the OSCC/CTL ratio are given in each case. Classification indicating salivary (S) or acute phase (A) proteins is presented. The type of sample from patients with OSCC where the protein was identified is also listed. * indicates that not the protein itself, but another close family member of it was already found in OSCC. NI denotes proteins not identified in OSCC yet.

| Protein ID | Protein Name                                         | # unique peptide | % Cov | OSCC/CTL ratio | Classification | Function                        | Type of OSCC sample |
|------------|------------------------------------------------------|------------------|--------|----------------|----------------|---------------------------------|---------------------|
| O60218     | Aldo-keto reductase family 1 member B10             | 5                | 17     | 0,10           | S              | metabolic enzyme                | saliva* [23]        |
| P02763     | Alpha-1-acid glycoprotein 1                         | 8                | 37     | 3,14           | AS             | immune response, transport      | saliva [16]         |
| P01011     | Alpha-1-antichymotrypsin                            | 12               | 31     | 3,29           | AS             | protease inhibitor              | NI                  |
| P01009     | Alpha-1-antitrypsin                                 | 25               | 62     | 3,70           | S              | protease inhibitor              | saliva [36]         |
| P04217     | Alpha-1B-glycoprotein                               | 12               | 39     | 3,25           | S              | immune response                 | saliva [16]         |
| P02765     | Alpha-2-HS-glycoprotein                             | 7                | 26     | 2,70           | AS             | protease inhibitor, immune response, transport | NI                  |
| P01023     | Alpha-2-macroglobulin                               | 54               | 51     | 2,16           | S              | protease inhibitor              | NI                  |
| P04745     | Alpha-amylase 1                                     | 42               | 83     | 0,21           | S              | metabolic enzyme                | saliva [14]         |
| P01019     | Angiotensinogen                                     | 7                | 18     | 8,50           | AS             | renin-angiotensin system        | NI                  |
| P01008     | Antithrombin-III                                    | 7                | 22     | 2,08           | AS             | protease inhibitor, blood coagulation | NI                  |
| P02647     | Apolipoprotein A-I                                  | 24               | 69     | 2,14           | S              | lipid metabolism               | saliva [23]         |
| P02652     | Apolipoprotein A-II                                 | 7                | 67     | 3,85           | S              | lipid metabolism               | saliva [23]         |
| P06727     | Apolipoprotein A-IV                                 | 5                | 16     | 3,55           | S              | lipid metabolism               | saliva [23]         |
| P04114     | Apolipoprotein B-100                                | 42               | 13     | 8,12           | S              | lipid metabolism               | NI                  |
| P02649     | Apolipoprotein E                                    | 4                | 18     | only in OSCC   | S              | lipid metabolism               | saliva [23]         |
| P17213     | Bactericidal permeability-increasing protein        | 4                | 12     | 0,24           | S              | immune response                | NI                  |
| P02749     | Beta-2-glycoprotein                                 | 12               | 44     | 3,02           | S              | lipid metabolism, blood coagulation | saliva [22]        |
| P61769     | Beta-2-microglobulin                                | 5                | 57     | 0,46           | S              | immune                         | NI                  |
| Prowork | Description | E-value | SwissProt | Function | Source |
|---------|-------------|---------|-----------|----------|--------|
| Q96DR5 | BPI fold-containing family A member 2 | 11 | 41 | 0.49 | S | immune response, defense |
| Q14CN2 | Calcium-activated chloride channel regulator 4 | 8 | 5 | 0.23 | S | transport |
| P27482 | Calmodulin-like protein 3 | 64 | 6 | 0.37 | S | metal binding, chaperone |
| P27797 | Calreticulin | 19 | 4 | 0.37 | S | chaperone |
| P00915 | Carbonic anhydrase 1 | 34 | 6 | 8.55 | S | metabolic enzyme, acid-base balance |
| P00450 | Ceruloplasmin | 37 | 27 | 3.65 | AS | metal binding |
| O00299 | Chloride intracellular channel protein 1 | 22 | 7 | 0.31 | S | transport, cell cycle regulation |
| P01024 | Complement C3 | 61 | 84 | 2.77 | AS | immune response |
| P0C0L5 | Complement C4-B | 25 | 32 | 6.69 | AS | immune response |
| P01031 | Complement C5 | 5 | 7 | only in OSCC | AS | immune response |
| B4E1Z4 | Complement factor B | 22 | 22 | 5.44 | AS | immune response |
| P08603 | Complement factor H | 22 | 21 | only in OSCC | AS | immune response |
| P05156 | Complement factor I | 7 | 3 | 6.42 | AS | immune response |
| P22528 | Cornifin-B | 79 | 6 | 0.45 | S | cornification |
| P08185 | Corticosteroid-binding globulin | 79 | 4 | only in OSCC | AS | protease inhibitor |
| P04080 | Cystatin-B | 86 | 6 | 0.39 | S | protease inhibitor |
| P01034 | Cystatin-C | 43 | 7 | 0.33 | S | protease inhibitor |
| P09228 | Cystatin-SA | 69 | 13 | 0.35 | S | protease inhibitor |
| P99999 | Cytochrome c | 32 | 4 | 0.00 | A | electron transport chain, apoptosis |
| Q02413 | Desmoglein-I | 12 | 8 | 0.40 | S | desmosome component |
| P61916 | Epididymal secretory protein E1 | 33 | 4 | 0.30 | S | lipid metabolism, cholesterol transport |
| Q01469 | Fatty acid-binding protein, epidermal | 79 | 12 | 0.49 | S | lipid metabolism |
| P02671 | Fibrinogen alpha chain | 49 | 11 | 2.67 | AS | blood coagulation |
| P02675 | Fibrinogen beta chain | 49 | 20 | 2.91 | AS | blood |
| P02679 | Fibrinogen gamma chain | 18 | 48 | 2.43 | AS | coagulation | blood coagulation | blood [40] |
| B7ZLE5 | FN1 protein | 24 | 17 | 5.73 | S | cell adhesion | tissue [40] |
| P00738 | Haptoglobin | 29 | 67 | 2.61 | AS | hem binding | blood [38] |
| P69905 | Hemoglobin subunit alpha | 11 | 92 | 3.37 | S | oxygen transport | saliva* [23] |
| P68871 | Hemoglobin subunit beta | 17 | 94 | 4.41 | S | oxygen transport | saliva* [23] |
| P02790 | Hemopexin | 20 | 52 | 2.41 | AS | hem binding | saliva [16, 22] |
| P05546 | Heparin cofactor 2 | 8 | 17 | only in OSCC | A | | blood coagulation | saliva [23] |
| Q9Y6R7 | IgGFc-binding protein | 52 | 17 | 0.49 | S | | immune response | NI |
| P11215 | Integrin alpha-M | 6 | 9 | 2.01 | S | | immune response | tissue [41] |
| P19827 | Inter-alpha-trypsin inhibitor heavy chain H1 | 8 | 14 | 3.76 | S | protease inhibitor | saliva [23] |
| P19823 | Inter-alpha-trypsin inhibitor heavy chain H2 | 10 | 18 | 11.23 | S | protease inhibitor | saliva [23] |
| Q14624 | Inter-alpha-trypsin inhibitor heavy chain H4 | 13 | 22 | 5.33 | S | protease inhibitor | saliva [23] |
| P02538 | Keratin, type II cytoskeletal 6A | 21 | 39 | 0.44 | S | cytoskeleton | NI |
| P01042 | Kininogen-1 | 11 | 18 | 2.89 | S | protease inhibitor, blood coagulation | saliva [23] |
| P61626 | Lysozyme C | 7 | 54 | 0.47 | S | | host defense | NI |
| P40926 | Malate dehydrogenase, mitochondrial | 4 | 17 | 0.37 | S | metabolic enzyme | NI |
| Q8TAX7 | Mucin-7 | 4 | 12 | 0.00 | S | | host defense | NI |
| P80303 | Nucleobindin-2 | 8 | 26 | 0.32 | S | | metal binding | NI |
| Q6UX06 | Olfactomedin-4 | 7 | 20 | 0.47 | S | | cell adhesion | NI |
| P36871 | Phosphoglucomutase-1 | 6 | 13 | 0.08 | S | | metabolic enzyme | NI |
| Q96G03 | Phosphoglucomutase-2 | 5 | 11 | 0.40 | S | | metabolic enzyme | NI |
| P00558 | Phosphoglycerate kinase 1 | 10 | 33 | 0.44 | S | | metabolic enzyme | saliva [22] |
| P36955 | Pigment epithelium-derived factor | 4 | 12 | 7.17 | S | | tumor development, angiogenesis | NI |
| P05155 | Plasma protease C1 inhibitor | 8 | 21 | 5.95 | S | protease inhibitor, blood coagulation | NI |
| P00747 | Plasminogen | 9 | 17 | 5.11 | AS | | blood coagulation | NI |
| P02760 | Protein AMBP | 6 | 23 | 5.41 | S | protease inhibitor, host defense | saliva [23] |
|-------|-------------|---|----|------|---|--------------------------------|-------------|
| O60888 | Protein CutA | 3 | 33 | 0.43 | S | metal binding, enzyme binding | NI          |
| P02753 | Retinol-binding protein 4 | 5 | 24 | 2.54 | S | protease inhibitor, host defense | blood [42] |
| Q9NQ38 | Serine protease inhibitor Kazal-type 5 | 11 | 13 | 0.32 | S | lipid metabolism | NI          |
| P36952 | Serpin B5 | 4 | 14 | 2.63 | S | tumor suppressor | blood [12] |
| P02768 | Serum albumin | 71 | 84 | 2.53 | S | transport | saliva [22] |
| P27169 | Serum paraoxonase/arylesterase 1 | 9 | 37 | only in OSCC | A | detoxification | saliva [23] |
| P35326 | Small proline-rich protein 2A | 6 | 79 | 0.29 | S | cornification | saliva* [23] |
| Q6UWP8 | Suprabasin | 12 | 33 | 0.05 | S | cell proliferation | NI          |
| P62328 | Thymosin beta-4 | 5 | 64 | 0.42 | S | actin binding, cell proliferation | NI          |
| O60235 | Transmembrane protease serine 11D | 3 | 10 | 0.20 | S | protease, host defense | NI          |
| P68363 | Tubulin alpha-1B chain | 4 | 12 | 0.40 | S | microtubule component | saliva* [23] |
| O75083 | WD repeat-containing protein 1 | 7 | 19 | 0.13 | S | cell migration | NI          |
Table 3. Proteins identified only in the OSCC or CTL groups.

| Protein ID  | Protein name                  | Gene name | Function                              | Presence     | Reference to previous studies                                                                 |
|------------|-------------------------------|-----------|---------------------------------------|--------------|------------------------------------------------------------------------------------------------|
| P02649     | Apolipoprotein E              | APOE      | lipid metabolism                      | only OSCC    | identified in saliva of patients with OSCC [23]                                               |
| P01031     | Complement C5                 | C5        | innate immune response, complement component | only OSCC    | not identified in cancer yet                                                                   |
| P08603     | Complement factor H           | CFH       | innate immune response, complement component | only OSCC    | identified in other forms of cancer but not in OSCC [50,51]                                   |
| P08185     | Corticosteroid-binding globulin | SERPINA6  | protease inhibitor                     | only OSCC    | identified in saliva of patients with OSCC [23]                                               |
| P05546     | Heparin cofactor 2            | SERPIND1  | blood coagulation                     | only OSCC    | identified in saliva of patients with OSCC [23]                                               |
| P27169     | Serum paraoxonase/arylesterase 1 | PON1     | detoxification                         | only OSCC    | identified in saliva of patients with OSCC [23]                                               |
| P99999     | Cytochrome c                  | CYCS      | electron transport chain, its release from mitochondria initiates apoptosis | only Ctrl    | its release was inhibited in OSCC [54]                                                        |
| Q8TAX7     | Mucin 7                       | MUC7      | antibacterial activity, host defense   | only Ctrl    | not identified in cancer yet                                                                   |

Based on [http://www.uniprot.org/](http://www.uniprot.org/).
Figure 1.

Figure 2.
A.

B.

| Pathway ID | Pathway description          | Observed gene count | False discovery rate |
|------------|------------------------------|---------------------|----------------------|
| GO:0070062 | Extracellular region part    | 24                  | 3.56e-09             |
| GO:0005576 | Extracellular region         | 26                  | 1.69e-09             |
| GO:0031988 | Extracellular exosome        | 25                  | 1.11e-12             |
| GO:0044421 | Membrane-bounded vesicle     | 25                  | 1.09e-10             |
### Biological process

| Pathway ID   | Pathway description                      | Observed gene count | False discovery rate |
|--------------|------------------------------------------|---------------------|----------------------|
| GO:0002697   | Regulation of immune effector process    | 10                  | 9.91e-07             |
| GO:0031639   | Plasminogen activation                   | 4                   | 9.91e-07             |
| GO:0034370   | Triglyceride-rich lipoprotein particle remodeling | 4                   | 9.91e-07             |
| GO:0034374   | Low-density lipoprotein particle remodeling | 4                   | 9.91e-07             |
| GO:0019216   | Regulation of lipid metabolic process     | 8                   | 9.53e-06             |

### Molecular Function

| Pathway ID   | Pathway description                      | Observed gene count | False discovery rate |
|--------------|------------------------------------------|---------------------|----------------------|
| GO:0005319   | Lipid transporter activity               | 6                   | 9.06e-06             |
| GO:0017127   | Cholesterol transporter activity         | 5                   | 7.23e-08             |
| GO:0016209   | Antioxidant activity                     | 5                   | 6.64e-08             |
| GO:0004857   | Enzyme inhibitor activity                | 19                  | 4.76e-20             |
| GO:0004867   | Serine-type endopeptidase inhibitor activity | 14                  | 4.76e-20             |

### Localization

| Pathway ID   | Pathway description                      | Observed gene count | False discovery rate |
|--------------|------------------------------------------|---------------------|----------------------|
| GO:00700062  | Extracellular exosome                    | 43                  | 9.88e-34             |
| GO:0034364   | High-density lipoprotein particle        | 6                   | 8.65e-10             |
| GO:0005577   | Fibrinogen complex                       | 4                   | 8.3e-08              |
| GO:0005576   | Extracellular region                     | 43                  | 7.68e-26             |
| GO:0031091   | Platelet alpha granule                   | 10                  | 6.88e-15             |
Figure 3.

A. SERM11 concentration (ng/mL)

B. Resistin concentration (ng/mL)

C. CS concentration (ng/mL)