Oral Oncology 86 (2018) 206–215

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Label-free tissue proteomics can classify oral squamous cell carcinoma from healthy tissue in a stage-specific manner

Amy Dickinson[a,b,*,1], Mayank Saraswat[a,d,1], Antti Mäkitie[b,e], Robert Silén[a], Jaana Hagström[d,h], Caj Haglund[f,g], Sakari Joenväärä[a,d], Suvi Silén[b,c]

[a] Transplantation Laboratory, Haartman Institute, University of Helsinki, Haartmaninkatu 3, PO Box 21, 00014, Finland
[b] Department of Otorhinolaryngology – Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[c] Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden
[d] HUSLAB, Helsinki University Hospital, Helsinki 00290, Finland
[e] Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden
[f] Department of Surgery, University of Helsinki and Helsinki, University Hospital, Helsinki, Finland
[g] Research Programs Unit, Translational Cancer Biology, University of Helsinki, Helsinki, Finland
[h] Department of Pathology, University of Helsinki, Finland

ARTICLE INFO

Keywords:
Oral squamous cell carcinoma
Head and neck cancer
Tongue cancer
OPLS-DA
Tissue proteomics
CA1
S100A7
Neutrophil defensin 1
ANP32B
Immunohistochemistry

ABSTRACT

Objectives: No prognostic or predictive biomarkers for oral squamous cell carcinoma (OSCC) exist. We aimed to discover novel proteins, altered in OSCC, to be further investigated as potential biomarkers, and to improve understanding about pathways involved in OSCC.
Materials and Methods: Proteomic signatures of seven paired healthy and OSCC tissue samples were identified using ultra-definition quantitative mass spectrometry, then analysed and compared using Anova, principal component analysis, hierarchical clustering and OPLS-DA modelling. A selection of significant proteins that were also altered in the serum from a previous study (PMID: 28632724) were validated immunohistochemically on an independent cohort (n = 66) to confirm immunopositivity and location within tumour tissue. Ingenuity Pathways Analysis was employed to identify altered pathways.
Results: Of 829 proteins quantified, 257 were significant and 72 were able to classify healthy vs OSCC using OPLS-DA modelling. We identified 19 proteins not previously known to be upregulated in OSCC, including prosaposin and alpha-taxilin. KIAA1217 and NDRG1 were upregulated in stage IVa compared with stage I tumours. Altered pathways included calcium signalling, cellular movement, haematological system development and function, and immune cell trafficking, and involved NF-kB and MAPK networks.
Conclusions: We found a set of proteins reliably separating OSCC tumour from healthy tissue, and multiple proteins differing between stage I and stage IVa OSCC. These potential biomarkers can be studied and validated in larger cohorts.

Abbreviations: ALYREF, THO complex subunit 4; ANP32B, Acidic leucine-rich nuclear phosphoprotein 32 family member B; AUROC, area under the receiver operating characteristic curve; BCA, bicinchoninic acid; CA1, carbonic anhydrase 1; CAIX, carbonic anhydrase IX; CYFRA-1, name of a test for soluble cytokeratin fragments; DEFA1, neutrophil defensin 1; DNAJC8, DnaJ homolog subfamily C member 8; DSS, disease-specific survival; DTT, dithiothreitol; EDTA, ethane-1,2-diyldinitrilotetraacetic acid; FC, mean fold change; H&E, haematoxylin and eosin; HCA, Hierarchical clustering analysis; HNSCC, head and neck squamous cell carcinoma; IAA, iodoacetamide; IHC, immunohistochemistry; IPA, Ingenuity pathway analysis; IP3R, Inositol triphosphate receptor; KIAA1217, sickle tail protein homologue; KRT, keratin; MAPK, mitogen activated protein kinase; MS, mass spectrometry; NAP1L1, nucleosome assembly protein 1-like 1; NDRG1, protein NDRG1; NF-κB, nuclear Factor-κB; nUPLC-UDMSE, Nano Ultra Performance Liquid Chromatography Label Free Ultra-definition mass spectrometry; OPLS-DA, Orthogonal Projections to Latent Structures Discriminant Analysis; OSCC, Oral squamous cell carcinoma; PABPN1, Polyadenylate-binding protein 2; PCA, Principal component analysis; PMCA1, plasma membrane Ca2+ ATPase isoform 1; PPI, protein-protein interactions; PSAP, prosaposin; SCLC, small cell lung cancer; STMN1, stathmin 1; TMA, tissue microarray; TNM, tumour-node-metastasis; TPA, cytokeratin detection test

* Corresponding author at: Transplantation Laboratory, Haartman Institute, University of Helsinki, Haartmaninkatu 3, PO Box 21, 00014, Finland.
E-mail addresses: Amy.dickinson@helsinki.fi (A. Dickinson), sarawat.mk@gmail.com (M. Saraswat), antti.makitie@helsinki.fi (A. Mäkitie), robert.silen@iki.fi (R. Silén), jaana.hagstrom@hus.fi (J. Hagström), caj.haglund@helsinki.fi (C. Haglund), sakari.joenvaara@helsinki.fi (S. Joenväärä), suvi.silen@helsinki.fi (S. Silén).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.oraloncology.2018.09.013
Received 6 June 2018; Received in revised form 21 August 2018; Accepted 13 September 2018

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck cancer globally with an annual incidence of around 275 000 [1]. It is more prevalent in men, and prevalence is increasing [2]. In particular, tongue cancer incidence has been rising in various parts of the world in recent years, and especially in patients under 45 [1,3]. Strong risk factors include alcohol and tobacco.

Western 5-year survival rates for oral cavity cancer are around 60% [3–5]. Five-year disease- specific survival (DSS) of OSCC of the mobile tongue decreases as the stage increases. In Finland, 5-year DSS is 87% for stage I carcinomas, but 51% for stage IV. Interestingly, recurrence occurred in 22–34% of patients, slightly varying by stage [6].

However, the TNM classification does not fully account for OSCC patients' outcomes [7]; tumours with the same TNM stage can exhibit different behaviours, treatment responses, and prognoses [8]. Different molecules and pathways have been linked with OSCC cancer behaviours, such as the VEGF-Flt-1 pathway with invasion [9], kallikrein-related peptidase with metastatic capacity [10], and glutathione-peroxidase-I overexpression with poor prognosis [11]. None of these are yet in clinical use.

Nano Ultra-Performance Liquid Chromatography Label-Free Ultra-Definition Mass Spectrometry (nUPLC-UDMS^E) is a highly sensitive form of mass spectrometry (MS), capable of quantifying hundreds-to-thousands of proteins in solution. nUPLC-UDMS^E discovery-driven analysis offers a unique avenue to discover proteins and pathways that are previously not known to be altered in OSCC. Previously, studies using non-pooled paired healthy and OSCC tissue samples for direct comparison with MS have used a gel-based matrix to register the differences between the cases and controls after which protein spots are identified using MS [12–14]. However, shotgun proteomics, as done here, and the electrophoresis approaches have different scopes and limitations. Electrophoresis mainly homes in on critical alterations such as isoform differences, and post-translational modifications, whereas shotgun approaches aim for a broad overview of proteomic changes, including those with smaller abundances, and allows for novel protein discovery. These two approaches can be complementary [15].

The primary aim of this study was to identify and quantify the differences between healthy tissue and OSCC using nUPLC-UDMS^E proteomics. In particular, we aimed to discover novel proteins with significantly different expression in OSCC compared with controls, along with differences in protein expression between high- and low-grade tumours. This will facilitate future research to assess whether these proteins can be used as potential new prognostic or predictive biomarkers, for example predicting different clinical behaviours of OSCCs. Additionally, the discovery of proteins involved can help in identifying important pathways in OSCC pathogenesis and thus potential therapeutic targets.

Materials and methods

Briefly, tissues of primary OSCC of the mobile tongue, and paired healthy tongue epithelium were taken preoperatively and immediately frozen. At the time of analysis, the tissues were thawed and lysed, and protein extraction was performed. The proteins were trypsin digested, and the tryptic peptide mixture was analysed using mass spectrometry (nUPLC-UDMS^E), to identify proteins differing between OSCC and paired healthy epithelium. Statistical classification and separation techniques were performed to ensure clear differences between OSCC and healthy tissue. Pathway analysis using two methods were performed to assess altered pathways within OSCC to gain greater understanding. We additionally performed a comparison between stage I and stage IVa OSCC. Selected proteins that have previously been found to be altered in serum of OSCC [16,17] and that we also found to be altered in the tissue were validated with immunohistochemistry (IHC). A tissue microarray block of 66 patients with oral tongue OSCC was used for this IHC analysis. Patient and tumour information is in Supplementary Table 1. Detailed information about the materials and methods are found in Supplementary File 1. The workflow can be visualised in Fig. 1.

Ethical approval was granted by the institutional Research Ethics Committee at the Helsinki University Hospital (Dnro: 64/13/03/02/2014). All patients provided informed written consent to participate in the study.

Results

Proteomics of OSCC and healthy tongue tissue

Of 829 proteins with two or more unique peptides that were quantified, 257 proteins were statistically significant (Supplementary Table 2).

The majority of proteins were present in both the cancer and control tissue in differing amounts, but one protein (alpha-taxilin) was absent

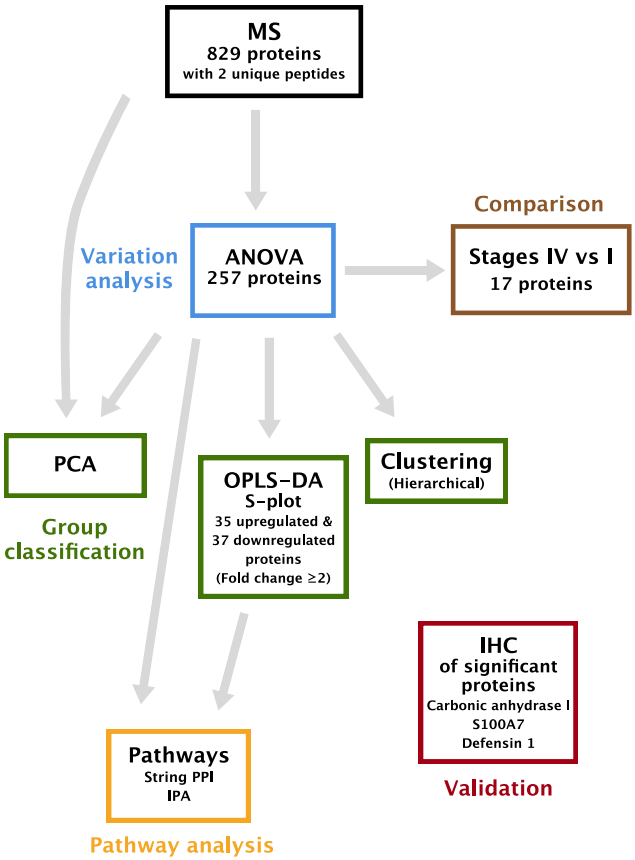

Fig. 1. Overview of methods employed, and results obtained. Mass spectrometry using nUPLC-UDMS^E (Nano Ultra Performance Liquid Chromatography Label Free Ultra-definition mass spectrometry was performed on OSCC of the tongue and paired healthy controls. Anova analysis was performed on these, with a p-value cut-off of 0.05. For group classification, PCA, OPLS-DA and Hierarchical Clustering Analysis were used, and significant proteins were identified using OPLS-DA analysis. Using String PPI and IPA, pathways altered in OSCC were identified. Furthermore, stage IVa vs stage I OSCC protein expressions were compared. Validation was performed on selected proteins, to confirm their presence is consistent in tumour samples, and to identify which cells within the tumour express these proteins. These proteins are known to be significantly altered in OSCC serum compared to controls in a previous study [16]. Key: ANOVA - Analysis of Variance; IHC – immunohistochemistry; IPA - Ingenuity Pathways Analysis; MS - mass spectrometry; OPLS-DA - Orthogonal Projections to Latent Structures Discriminant Analysis; PCA - Principal Component Analysis; String PPI - Protein-protein Interaction (via String).