DISCRIMINATION OF NORMAL ORAL MUCOSA FROM ORAL CANCER BY MASS SPECTROMETRY IMAGING OF PROTEINS AND LIPIDS

Katarzyna Bednarczyk[1], Marta Gawin[2], Mykola Chekan[2], Agata Kurczyk[2], Grzegorz Mrukwa[1], Monika Pietrowska[2], Joanna Polanska[1], Piotr Widlak[2]

[1]Faculty of Automation, Electronics and Computer Science, Silesian University of Technology, ul. Akademicka 16, 44-100 Gliwice, Poland

[2]Center for Translational Research and Molecular Biology of Cancer, Maria Skłodowska-Curie Institute – Oncology Center, Gliwice Branch, ul. Wybrzeże Armii Krajowej 15, 44-101 Gliwice, Poland

Introduction

Head and neck squamous cell carcinomas (HNSCC) belong to oral cancers, and their etiology is connected mainly with exposure to tobacco and alcohol. Generally accepted molecular biomarkers to guide management of HNSCC patient are still missing, and determination of molecular factors discriminating between cancerous and normal mucosa for proper delineation of tumor area belongs to critical issues in the field of molecular diagnostics of HNSCC.

Aims

- Direct comparison of the ability of proteins and lipids (i.e. two domains of molecular components of HNSCC) to discriminate cancerous and normal oral mucosa
- Estimation of their potential usefulness as a source of novel hypothetical biomarkers

Experimental

CLINICAL MATERIAL

Tissue material was collected from four patients (3 males and 1 female; aged: 36-59) who underwent surgery due to head and neck squamous cell carcinoma located in tongue. In all cases no neo-adjuvant chemo- or radio-therapy was involved prior surgery.

Case	Stage	Case	Stage
1	T4N2M0	3	T1N0M0
2	T4N2bM0	4	T2N0M0

SAMPLE PREPARATION

- post-operative material: frozen, stored at -80°C
- 10 µm serial sections on ITO-coated glass slides

PEPTIDE IMAGING

- lipid removal:
70% EtOH, 1 min
70% EtOH, 1 min
100% EtOH, 1 min
- vacuum drying: 1 h
- trypsin coating:
20 µg Promega trypsin in 50 mM NH_4HCO_3
- incubation: 37°C, 18 h, humid chamber

LIPID IMAGING

no additional tissue preparation was performed

MATRIX COATING (peptides/lipids)

2,5-DHB 30mg/mL in 50% methanol and 0.2% TFA, ImagePrep standard matrix coating program with doubled phase 5

LC-MS/MS

- Each protein digest was separated into 680 nano-LC fractions
- Up to 10 MS/MS precursors per fraction were fragmented
- MS/MS spectra were searched against NCBInr human database using ProteinScape v.3.1. software

MALDI-MSI measurements

reflectron positive mode
800-4000 m/z (peptides)
300-1200 m/z (lipids)
raster width: 100 µm
400 shots/raster

INSTRUMENTATION

- ImagePrep (Bruker Daltonik)
- ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker)
- EASY-nLC chromatograph (Proxeon) coupled with PROTEINEER fc II fraction collector (Bruker)

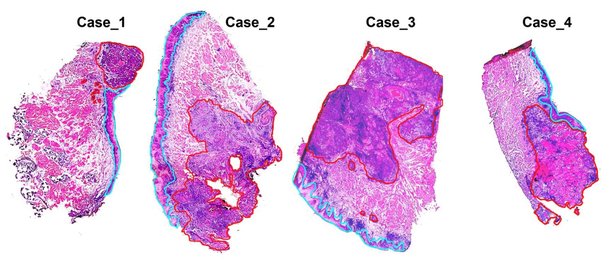
COMPUTATIONAL ANALYSIS

- Coefficient of variation was used to measure molecular components' dispersion.
- Cohen's d value was used to estimate significance of differences in abundance of each molecular component between cancer ROIs and normal epithelium ROIs.
- Divisive iK-means algorithm for spectra clustering was used to determine sub-regions in tissue preparations.
- The logistic regression technique was applied to spectra classification between cancer and (normal) epithelium ROIs.
- Bayesian Information Criterion (BIC) was used for model selection.

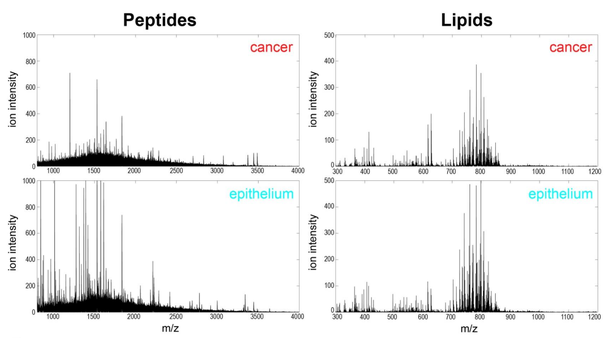
Results

Peptide and lipid domain comprised 2435 and 2108 spectra components, respectively, which represented different molecular species and their isotope envelops.

Tissue regions corresponding to cancer and (normal) epithelium were delineated by an experienced pathologist after molecular image registration, and spectra from these two types of ROIs (regions of interest) were exported for further analyses.

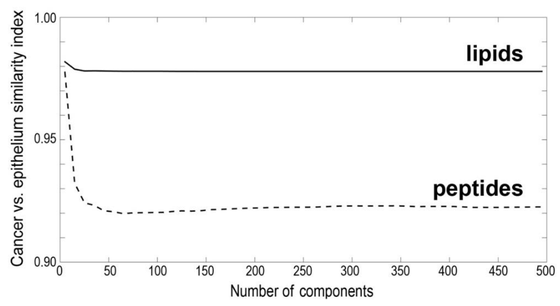


Result of H&E staining of sections (peptide Imaging): cancer and epithelium regions were delineated with red and blue lines, respectively



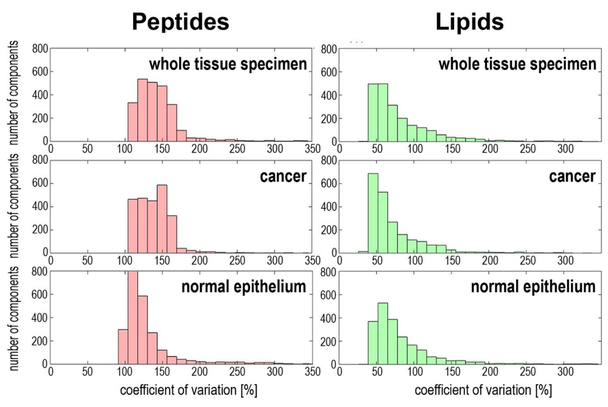
Average spectra registered for cancer ROIs and epithelium ROIs

Average lipid spectra from cancer and epithelium ROIs were more similar than the corresponding peptide spectra – **similarity index** between pairwise analyzed cancer versus epithelium ROIs was estimated in the peptide and lipid domains.

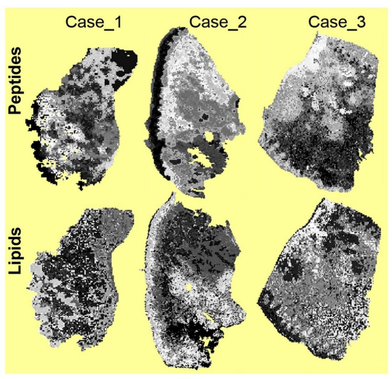


Three tissue specimens (Case_1, Case_2, and Case_3) were used as a training set to establish molecular differences between cancerous and normal epithelium, the fourth one (Case_4) was used only for validation of the obtained cancer classifier.

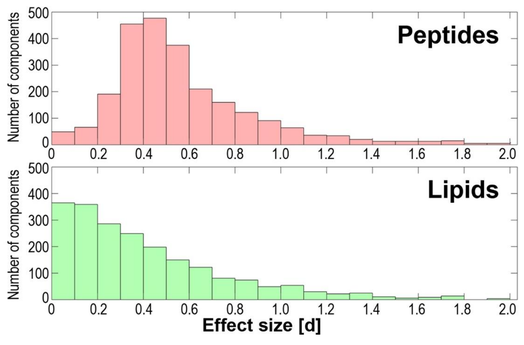
ANALYSIS OF UNIFORMITY OF PEPTIDE AND LIPID COMPONENTS



Uniformity of components estimated by their coefficient of variation in whole tissue specimens or cancer and epithelium regions



Unsupervised segmentation of molecular images; each tissue specimen was processed individually



Estimation of significance of differences between compared ROIs on the basis of the effect size calculated for each component

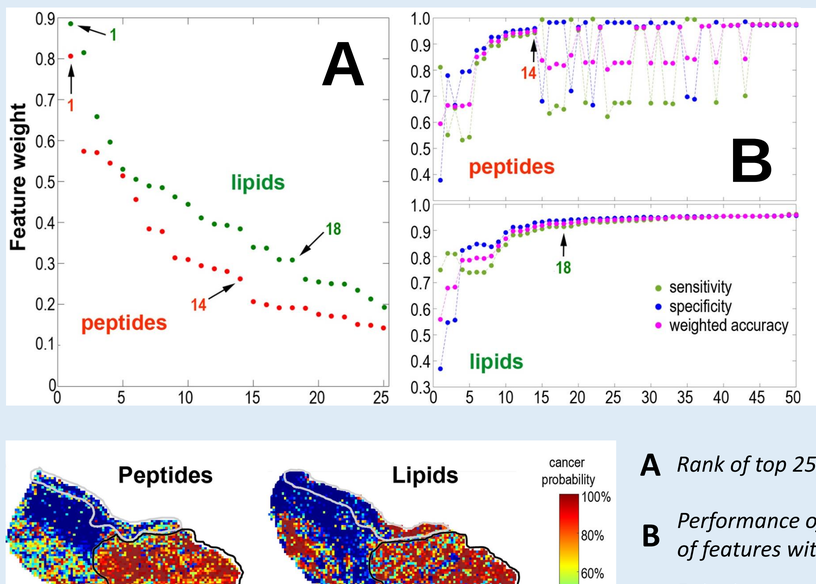
Comparison of the size of clusters obtained during unsupervised image segmentation

Molecular domain	Case_1		Case_2		Case_3	
	Peptides	Lipids	Peptides	Lipids	Peptides	Lipids
Number of clusters	1251	1535	962	1479	1719	1633
Cluster average size, %	0.08	0.07	0.10	0.07	0.06	0.06
Largest cluster size, %	6.88	10.45	4.68	19.93	3.10	10.83

Performance of cancer classifiers built of peptide and lipid components and validated using the independent tissue specimen

Classifier indices	Peptide classifier (14 components)	Lipid classifier (18 components)
sensitivity	78.7 %	56.0 %
specificity	90.7 %	82.4 %
accuracy	89.5 %	79.8 %
weighted accuracy	84.7 %	69.2 %
precision	97.5 %	94.4 %
F-measure	93.9 %	87.9 %

DISCRIMINATION BETWEEN NORMAL AND CANCEROUS EPITHELIUM - CANCER CLASSIFIERS



A *Rank of top 25 components with decreasing weight in the tested classifiers*

B *Performance of classifiers (sensitivity, specificity, and weighted accuracy) built with panels of features with an increased number of components*

C *Pairwise correlation plot for 14 peptide and 18 lipid components selected for the final classifiers (underlined are top components with the counterclockwise decreasing weight of a component); connected are components of at least high effect size correlation (width of the line represents the strength of the correlation)*

D *Results of classification of basic segments (registered spectra) in the validation sample (Case_4); the heat maps illustrate the probability of being classified as < cancer> (grey and black lines delineate expert-determined normal epithelium and cancer, respectively)*

Conclusions

The presented study demonstrates significantly different abundances of a large number of cellular proteins represented by their tryptic peptides imaged by MALDI-MSI between normal and cancerous mucosa.

In contrast, differences between cancerous and normal mucosa were less obvious when corresponding ROIs were compared in respect to the subset of the analyzed lipids.

Nevertheless, imaging of both proteome and lipidome components enabled discrimination of oral cancer and normal epithelium. This indicated that both molecular components of oral epithelium are potential sources of oral cancer biomarkers.

The study was approved by the appropriate Ethical Committee (Maria Skłodowska-Curie Institute, approval number KB/430-17/13 from 12/03/2013), and performed in accordance with European, national and institutional guidelines. This work was founded by the National Science Centre, Poland, Grant 2016/23/B/NZ4/03901 (to P.W.) and Grant 2015/19/B/ST6/01736 (to J.P.), Silesian University of Technology: BKM grant BKM/508/RAU1/2017/t.28 (to K.B.) and the National Centre for Research and Development, Poland, Grant DZP/STRATEGMED2/2554/2014 (to P.W.). The computations were carried out using the GeCONil infrastructure (Grant POIG.02.03.01-24-099).