Mass spectrometry-based evaluation of pathological biomarkers on head neck cancer patients

Thesis of Doctoral (PhD) Dissertation

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List of abbreviations

ALDH3A2 - fatty aldehyde dehydrogenase 3A2
c-MET - c-mesenchymal-epithelial transition
COTL1 - coactosin like f-actin binding protein 1
DNA - deoxyribonucleic acid
DSG1 - desmoglein-1
DYNLL1 - dynein light chain 1, cytoplasmic
EGFR - epidermal growth factor receptor
ESI - electrospray ionization
FFPE - formalin-fixed paraffin-embedded
H&E - hematoxylin and eosin
HGF - hepatocyte growth factor
HNSCC - head neck squamous cell carcinoma
HPV - human papilloma virus
IPA - Ingenuity Pathway Analysis
KRT9 or K1C9 - type I citosceletal keratin 9
LC - liquid chromatography
LHSCC - laryngeal-hypopharyngeal squamous cell carcinoma
MALDI - matrix-assisted laser desorption/ionization
miRNA - micro ribonucleic acid
mRNA - messenger ribonucleic acid
MRM - multiple reaction monitoring
MS - mass spectrometry
m/z - mass-to-charge ratio
NCL - nucleolin
NFE2L2 - nuclear factor erythroid 2-related factor 2
REIMS - rapid evaporative ionization mass spectrometry
RNA - ribonucleic acid
SCC - squamous cell carcinoma
SNRNP200 - small nuclear ribonucleoprotein U5 subunit 200
snRNA - small nuclear ribonucleic acid
SPRR2D - small proline rich protein 2D
TMED2 - transmembrane p24 trafficking protein 2
TNC - tenascin-C
TOF - time-of-flight

I. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignant tumor worldwide. Approximately, 660,000 new cases are diagnosed each year, placing a significant burden on healthcare systems and negatively affecting patients' quality of life. HNSCC includes malignant laryngeal and laryngopharyngeal squamous cell carcinomas (LHSCC). Although diagnostic and therapeutic procedures have undergone significant advances over the recent decades, the overall survival did not improved substantially. One main reason for this is that the disease is often diagnosed only at an advanced stage when treatment options are more limited. On the other hand, the aggressive biological behavior of the tumor also makes successful treatment difficult.

Therefore, early tumor detection is crucial for improving patients' chances of survival. Alongside traditional diagnostic methods (physical examination, imaging procedures, biopsy), there remains a high demand for research of tumor markers. Tumor markers are molecules (e.g. proteins, DNA fragments) that are present in altered quantities in the body when a tumor is present. Ideally, tumor markers allow for the early diagnosis of tumors, as well as monitoring therapeutical response and potential tumor recurrence. Proteins carried by the tumor not only serve as diagnostic tools but can also act as therapeutic targets for biological therapy.

Among proteomic methods, mass spectrometry (MS) has opened new avenues in tumor marker research. MS is an analytical technique used to determine the mass of charged particles. During the process, the sample is ionized, and the ions are separated in an electromagnetic field based on their mass-to-charge ratio (m/z). The spectrum obtained allows for the identification and quantification of the components of the examined material. This method offers the possibility to identify differences in protein composition between phenotypically tumorous and healthy tissues. Therefore, not only the detected proteins but also their quantitative differences can serve as potential tumor markers

II. OBJECTIVES

In this study, our objective was to investigate protein differences within tissues using label-free MS, comparing LHSCC with the surrounding normal phenotypic mucosa, based on formalin-fixed paraffin-embedded (FFPE) tissue sections, in search of potential tumor markers. We aimed to verify our hypothesis that certain proteins are over- or underexpressed in LHSCC.

Additionally, based on the international literature and the Ingenuity Pathway Analysis (IPA) database, we mapped the role of the identified proteins - potentially serving as tumor markers and future therapeutic targets - in signal transduction pathways.

III. MATERIAL AND METHOD

Patients

A total of 16 male patients with histologically confirmed LHSCC were included in our research. The average age of the patients was 61 years (the youngest patient was 45, the oldest was 79). All participants were classified as heavy smokers and reported regular alcohol consumption. The diagnosed tumors were all identified as primary cases. The diagnosis was made through direct laryngoscopy. During direct laryngoscopy, the laryngoscope used for exposure was inserted through the mouth of the patient in a supine, hyperextended neck position, into the laryngeal and hypopharyngeal region. Tumor extent was determined intraoperatively using visualization with an operating microscope or a rigid laryngological endoscope. Diagnostic sampling was performed using laryngomicrosurgical instruments. The tissue samples for analysis were obtained from sections of formalin-fixed paraffin-embedded (FFPE) tissue blocks.

Sample preparation

Deparaffinization was performed by washing the unstained slides with xylol, ethanol 90% (m/m), ethanol 70% (m/m), ethanol 50% (m/m) and 50 mM ammonium-bicarbonate, respectively. Antigen retrieval was performed with 100 mM Tris-HCl buffer (97 °C, 30 min). According to the marked field lining histologically obvious malignant part, the tissue wasmicrodissected with a fine needle and collected to 2ml Eppendorf tube containing SDS lysis buffer. Tumor adjacent normal tissue was also microdissected and each sample was incubated in the lysis buffer (97 °C, 30min). After centrifugation ice cold absolute ethanol was added to the supernatant with 9 volume surplus. Samples were kept at 4 °C overnight for protein precipitation. The pellet was washed twice with absolute ethanol, proteins were solubilized with 8 M urea. Reduction and alkylation were followed by overnight trypsin digestion. The resulting tryptic peptides were cleaned using Pierce C18 spin columns (Thermo Fisher Scientific, Waltham, MA).

Mass spectrometrical analysis

The MS used for analysis was a Bruker mAXIS II ETD Q-TOF (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 nanoRSLC system (Dionex, Sunnyvale, CA, USA). Samples were injected on an Acclaim PepMap100 C-18 trap column (100 µm× 20 mm, Thermo Scientific, Sunnyvale, CA, USA) online coupled to an ACQUITY UPLC M-Class Peptide BEH C18 column (130 Å, 1.7 µm, 75 µm× 250 mm,Waters, Milford, MA, USA). Peptides were separated at 48 °C with a flow rate of 300 nl/min, 4% solvent B from 0 to 11 min, followed by a 120 min gradient to 50% solvent B. Solvent A consisted of water +0.1% formic acid, while Solvent B was acetonitrile +0.1% formic acid. The injected sample amount was 0.5 µg. Sample ionization was achieved in the positive electrospray ionization mode via a CaptiveSpray nanoBooster ionsource. The capillary voltage was 1300 V, the nanoBooster pressure was 0.2 Bar, drying gas was 150 °C, the flow rate was 3 l/min. External mass calibration was done using the low concentration tuning mix from Agilent technologies via direct infusion. Internal mass calibration was performed via lock mass for each run using sodium formate. The MS spectra were recorded with a fix cycle time (2.5 s) over the mass range of m/z 150–2200 at 3 Hz with a minimal precursor mass of m/z 322. The CID was performed at 16 Hz for abundant precursors and at 4 Hz for ones of low abundance. Singly charged peptides were excluded from analysis, only multiple charged peptides were chosen for fragmentation. Collision energy for precursor signals was set automatically followed by the manufacturer's recommendations based on the isolation m/z, isolation mass range width and ion charge state. Active exclusion of 2 min. After 1 spectra was used except if the intensity of the precursor was elevated threefold.

Protein identification

For protein analysis raw data were recalibrated using the Compass DataAnalysis software 4.3 (Bruker Daltonics, Bremen, Germany). Data were processed by the ProteinScape 3.0 software (Bruker Daltonik GmbH, Bremen, Germany). Proteins were identified by searching against the human Swissprot database (2015_08) using the Mascot search engine version 2.5 (Matrix Science, London, UK) applying the following search parameters: 7 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, 2 missed cleavages, carbamidomethylation of cysteines as fixed modification, deamidation (NQ) and oxidation (M) as variable modifications and proteins were identified using 1% FDR limit. Label-free quantitation (LFQ) was then performed using MaxQuant (software version 1.5.3.30), applying default parameters. MaxQuant analysis

searched only for proteins identified previously by Mascot. Each LC-MS/MS run was aligned using the “match between runs” feature (match time window 0.8 min, alignment time window 15 min).

Statistical and bioinformatical analysis

The data obtained were subjected to discriminant analysis and paired t-test to identify the proteins most characteristic for phenotypically malignant and normal tissues. For bioinformatical analysis, we used the Ingenuity Pathway Analysis software (www.ingenuity.com, Mountain View, California, USA), which we employed to map the roles of the identified proteins within known signal transduction pathways and to explore possible interactions among them.

IV. RESULTS

Using a label-free proteomic method, a total of 1,164 proteins were detected based on at least 2 unique peptides. Through discriminant analysis, we identified 18 proteins characteristic for the tumor and non-tumor groups. With the help of paired t-test, we investigated the presence of any statistically significant differences between the matched tumor-normal sample pairs for these 18 proteins. The p -value was determined as 0.1, considering the low number of matched tumor-normal pairs (n=16).

As a result of these analyses, the following 8 proteins showed significantly higher levels in the tumor tissue:

- tenascin-C (TNC)
- transmembrane p24 trafficking protein 2 (TMED2)
- dynein light chain 1, cytoplasmic (DYNLL1)
- coactosin like f-actin binding protein 1 (COTL1)
- small proline rich protein 2D (SPRR2D)
- nucleolin (NCL)
- small nuclear ribonucleoprotein U5 subunit 200 (SNRNP200)
- fatty aldehyde dehydrogenase 3A2 (ALDH3A2)

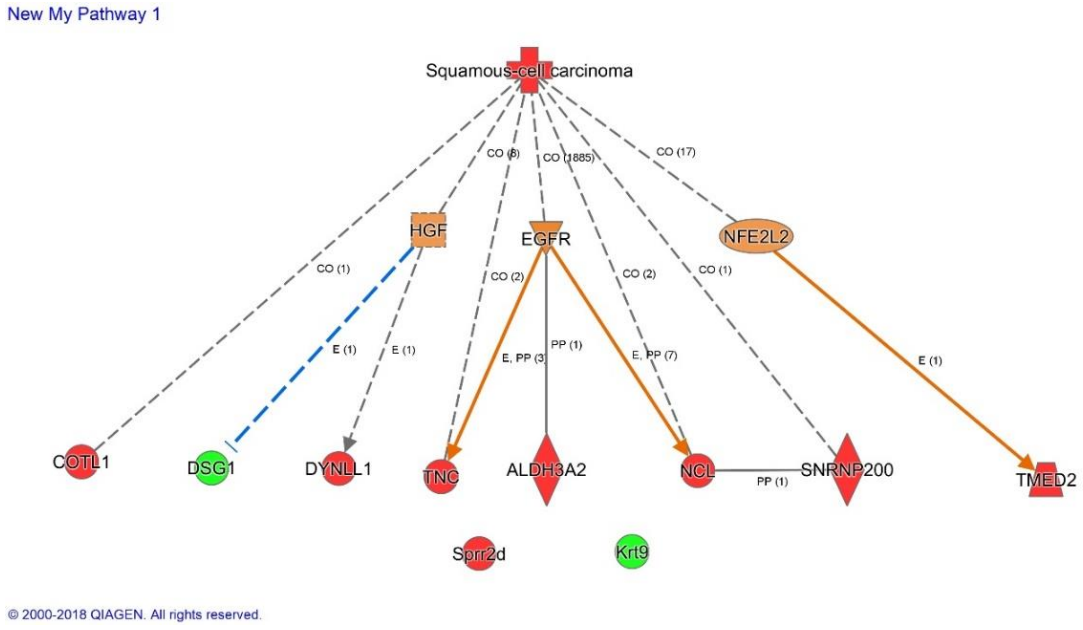
Two proteins were found to be elevated in the phenotypically intact tissue adjacent to the tumor:

- desmoglein-1 (DSG1)
- type I citosceletal keratin 9 (KRT9 or K1C9)

The bioinformatical analysis did not reveal any connections between the identified proteins; however, we mapped 6 interactions involving 6 of the proteins we identified (ALDH3A2, DSG1, DYNLL1, NCL, TMED2 and TNC) and 3 known molecules (epidermal growth factor receptor (EGFR), hepatocyte growth factor (HGF), and nuclear factor erythroid 2-related factor 2 (NFE2L2)). By illustrating the involvement of the proteins we identified in known signaling pathways, we also made an attempt to envision several potential future diagnostic and therapeutic applications.

A talált fehérjék között IPA-val azonosított interakciók

(CO: korreláció; PP: fehérje-fehérje kötés; E: expresszió)



V. DISCUSSION

Despite the ongoing technical advancements in the field of molecular diagnostics, tumor marker research still presents significant challenges. In recent years, interest has gradually shifted towards proteomics, which emphasizes the observation that proteins produced by the tumor and their post-translational modifications are at least as important as the tumor genetics.

The examination of FFPE tissue samples is less common in studies related to HNSCC. Among the publications dealing with proteomic analysis of FFPE tissues, there are only scant references pertaining to HNSCC. Initially, proteomic analysis of solid tumors could only be performed on rapid frozen tissue samples, which poses logistical challenges in terms of time and space. To overcome this, and due to the readily available nature of FFPE tissue samples, the demand for their analysis has significantly increased, and our research group also decided to focus on examining these samples.

During our research, we found that the identified proteins influence the tumor microenvironment in different ways. Proteins that were significantly overexpressed in the tumor (TNC, TMED2, DYNLL1, COTL1, NCL, SPRR2D, SNRNP200, ALDH3A2) promote tumor cell proliferation, migration and invasion. Conversely, two proteins that were overexpressed in phenotypically normal tissue (DSG1, KRT9) play a role in stabilizing the tissue microenvironment and cell adhesion.

Proteins overexpressed in HNSCC, including LHSCC, can be viewed as promising therapeutic targets. Inhibiting these proteins could reduce tumor cell proliferation, migration, and tissue invasion, thus opening the way for novel antibody-based therapies in head and neck oncology.

Limitations of the research

No data were found regarding SPRR2D and KRT9 in the IPA database. This is likely due to the lack of information about the relationship between these two proteins and SCCs in the actual database. As the database's content expands, it is expected to reveal new interactions involving these proteins in the future. Although our study initially was based on a relatively modest number of samples, it is important to emphasize that our primary goal was to conduct an exploratory study aimed at examining the differences in protein composition between malignant and phenotypically healthy tissue surrounding the tumor in LHSCC.

Benefit of the research

We consider it important to emphasize that, in contrast to previous studies based on MS, we examined the differences in protein abundance between tissues exclusively on FFPE LHSCC tissue samples using a label-free LC/MS proteomic method. The use of quantitative LC/MS analysis on FFPE samples has long been a subject of debate. Critics argue against the method due to formalin-induced cross-links and by-products. However, thanks to advances in extraction procedures, the reliability and diagnostic accuracy of protein recovery from FFPE tissue samples are comparable to those from freshly frozen tissues. Moreover, the application of LC/MS to FFPE tissues has also been shown to be equivalent to analyses performed on fresh frozen samples. Additionally, label-free proteomic methods have several advantages over labeled techniques. On the one hand, labeling procedures involve sample preparation steps that can lead to protein loss and data loss. On the other hand, some labeling techniques require the presence of specific molecular components (e.g., cysteine-containing peptides).

Beyond our promising initial results, this study also holds clinical relevance. Besides successfully highlighting proteomic differences between LHSCC and neighboring phenotypically intact tissue, we identified proteins that could potentially serve as markers and targets in LHSCC, which had not previously been the focus of attention. Furthermore, the reliability of label-free LC/MS on FFPE samples enables the analysis of existing histopathological archives. Based on our reasoning, the broad inclusion of such tissue archives could significantly contribute to future tumor marker research and would eliminate the need for costly and time-consuming fresh freezing procedures.

Future aims

At the time of our proteomic measurements, monoclonal antibodies suitable for the verification of the identified proteins were unfortunately not widely available. In the future, with the possession of these antibodies, we would have the opportunity to study the intra-tissue distribution of the analyzed proteins, which could potentially aid the better understanding of the interactions between tumor tissue and the surrounding stroma.

VI. CONCLUSION

The discovery of potential biomarkers that are characteristic for HNSCC remains fundamentally important but also represents a significant challenge even today. The continuously improving technical background has contributed to the rapid advancements in proteomics and consequently in MS. With sophisticated instrumentation and well-developed sample preparation procedures, it has become possible to analyze complex compounds and tissue samples. The application of MS naturally extended to tumor marker research as well. Similar to other types of cancers, the identification of proteins characteristic for HNSCC has become increasingly accessible. Recognizing tumor-specific biomarkers is crucial for early detection. These biomarkers are not only useful for screening but also play an essential role in detecting tumor recurrence and evaluating responses to treatment. By identifying proteins characteristic of LHSCC, we aimed to take another step forward in tumor marker research. Additionally, our label-free LC/MS method applied to FFPE tissue samples appears to be a promising approach for investigating potential biomarkers specific to HNSCC.

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APPENDIX **Paired t-test**

(regarding the 18 most characteristic proteins based on discriminant analysis for the matched tumor and phenotypically healthy tissue sample pairs)

		Relative abundance average values	Standard deviation	Standard error	95% confidence interval of the difference		t-value	Degrees of freedom	Significance (p-value)
					lower limit	upper limit			
1.pár	TENA_HUMAN *ép* – TENA_HUMAN *tumoros*	-2348325,00000	1371919,61000	396039,07810	-3220001,13400	-1476648,86600	-5,930	11	0,000
2.pár	TMED2_HUMAN *ép* – TMED2_HUMAN *tumoros*	-150133,33330	85444,86083	24665,80670	-204422,40780	-95844,25882	-6,087	11	0,000
3.pár	DYL1_HUMAN *ép* – DYL1_HUMAN *tumoros*	-183083,33330	326385,56630	94219,39728	-390458,82850	24292,16187	-1,943	11	0,078
4.pár	PSMD3_HUMAN *ép* – PSMD3_HUMAN *tumoros*	-53666,66667	145052,55060	41873,06456	-145828,66040	38495,32703	-1,282	11	0,226
5.pár	DCXR_HUMAN *ép* – DCXR_HUMAN *tumoros*	-35258,33333	123515,69980	35655,91127	-113736,46490	43219,79825	-0,989	11	0,344
6.pár	DSG1_HUMAN *ép* – DSG1_HUMAN *tumoros*	1390166,66700	2209880,78000	637937,63150	-13924,59339	2794257,92700	2,179	11	0,052
7.pár	COTL1_HUMAN *ép* – COTL1_HUMAN *tumoros*	-264333,33330	397229,34370	114670,23430	-516720,81730	-11945,84941	-2,305	11	0,042
8.pár	K1C9_HUMAN *ép* – K1C9_HUMAN *tumoros*	73326666,67000	97159973,93000	28047668,55000	11594164,41000	135059168,90000	2,614	11	0,024
9.pár	OSTF1_HUMAN *ép* – OSTF1_HUMAN *tumoros*	-21475,00000	81984,66736	23666,93488	-73565,57247	30615,57247	-0,907	11	0,384
10.pár	IF6_HUMAN *ép* – IF6_HUMAN *tumoros*	-3266,66667	193252,24370	55787,11746	-126053,28430	119519,95100	-0,059	11	0,954
11.pár	ACOC_HUMAN *ép* – ACOC_HUMAN *tumoros*	8716,66667	91136,93826	26308,96792	-49188,98130	66622,31463	0,331	11	0,747
12.pár	SPR2D_HUMAN *ép* – SPR2D_HUMAN *tumoros*	-103300,00000	134895,60270	38941,00627	-189008,57690	-17591,42307	-2,653	11	0,022
13.pár	NUCL_HUMAN *ép* – NUCL_HUMAN *tumoros*	-1271858,33300	1047332,64900	302338,89340	-1937301,75100	-606414,91570	-4,207	11	0,001
14.pár	TXNL1_HUMAN *ép* – TXNL1_HUMAN *tumoros*	-41316,66667	84232,22050	24315,74759	-94835,26627	12201,93293	-1,699	11	0,117
15.pár	U520_HUMAN *ép* – U520_HUMAN *tumoros*	-691750,00000	768104,41110	221732,64430	-1179780,26000	-203719,74050	-3,120	11	0,010
16.pár	UD17_HUMAN *ép* – UD17_HUMAN *tumoros*	-9275,00000	163882,95780	47308,93491	-113401,26370	94851,26367	-0,196	11	0,848
17.pár	AL3A2_HUMAN *ép* – AL3A2_HUMAN *tumoros*	-86083,33333	144985,55310	41853,72405	-178202,75890	6036,09220	-2,057	11	0,064
18.pár	TBB6_HUMAN *ép* – TBB6_HUMAN *tumoros*	-230333,33330	647338,93520	186870,65420	-641632,87020	180966,20350	-1,233	11	0,243

Red boldface indicates proteins significantly overexpressed in tumor tissue comparing to the adjacent phenotipically normal tissue ($p < 0,1$)

Green boldface indicate proteins with significantly lower abundance in tumor tissue ($p < 0,1$) comparing to the adjaccent phenotipically normal tissue

APPENDIX List of identified proteins

Genes encoding identified proteins	ID number of encoded protein	Mascot points	Molecule weight (kDa)	Sequence coverage (%)	Number of peptides*
TNC	TENA_HUMAN	354	240,7	8,3	13
TMED2	TMED2_HUMAN	59,1	22,7	12,2	3
DYNLL1	DYL1_HUMAN	82,3	8	21,6	5
COTL1	COTL1_HUMAN	72,7	15,9	5,4	3
SPRR2D	SPR2D_HUMAN	89,1	7,9	46,8	3
NCL	NUCL_HUMAN	316,2	76,6	17,9	11
SNRNP200	U520_HUMAN	51,5	244,4	1,3	2
ALDH3A2	AL3A2_HUMAN	64,6	54,8	7,9	3
DSG1	DSG1_HUMAN	175	113,7	6,9	5
KRT9	K1C9_HUMAN	1493,3	62	68,2	37

* the average number of peptides each protein was identified with

Red boldface indicates proteins significantly overexpressed in tumor tissue comparing to the adjacent phenotipically normal tissue ($p < 0,1$)

Green boldface indicate proteins with significantly lower abundance in tumor tissue ($p < 0,1$) comparing to the adjaccent phenotipically normal tissue

PUBLICATION LIST

First author scientific publication underpinning the dissertation:

Burian A, Lujber L, Gerlinger I, Jarai T, Orosz E, Turiak L, Acs A, Hegedus Z, Peter AK, Tornoczki T, Gombos K, Mark L. Label-Free Semiquantitative Liquid Chromatography-Tandem Mass Spectrometry Proteomics Analysis of Laryngeal/Hypopharyngeal Squamous Cell Carcinoma on Formalin-Fixed, Paraffin-Embedded Tissue Samples - a Pilot Study.
(Pathol Oncol Res. 26(4):2801-2807., 2020). **IF: 2,77**

Co-author scientific publications underpinning the dissertation:

Jarai T, Maasz G, Burian A, Bona A, Jambor E, Gerlinger I, Mark L. Mass spectrometry-based salivary proteomics for the discovery of head and neck squamous cell carcinoma.
(Pathol Oncol Res. 623-8., 18 (3), 2012) **IF: 2,06**

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(Oncotarget. 11(28):2702-2717., 2020) **IF: 3,34**

Additional scientific publications:

Burián A, Járai T, Gerlinger I, Csobó D, Lujber L. Minimál invazív video-asszisztált thyreoidectomia (MIVAT) - kezdeti lépések a Pécsi Tudományegyetem Fül-, Orr-, Gégészeti és Fej-, Nyaksebészeti Klinikán
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(Am J Otolaryngol. 46(3):104622, 2025) **IF:1,78**

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SHORT COMMUNICATION

Label-Free Semiquantitative Liquid Chromatography-Tandem Mass Spectrometry Proteomics Analysis of Laryngeal/Hypopharyngeal Squamous Cell Carcinoma on Formalin-Fixed, Paraffin-Embedded Tissue Samples - a Pilot Study

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Abstract

Squamous cell carcinoma (SCC) of the head and neck region is the sixth most frequent malignancy with high mortality rate. Due to its poor prognosis it is considered a growing public health problem worldwide inspite of existing treatment modalities. Thus, early diagnosis of new diseases and recurrences is emerging on one hand, but on the other hand troublesome in the lack of reliable tumor markers in this field. The rapid development of proteomics has opened new perspectives in tumor marker discovery. Liquid chromatography/mass spectrometry (LC/MS) as the gold standard in proteomics enables the semi-quantitative analysis of proteins within various tissues. Abundance differences between tumor and normal tissue also can be interpreted as tumor specific changes. The aim of this study was to identify potential tumor markers of laryngeal/hypopharyngeal SCC by revealing abundance changes between cancerous and the surrounding phenotypically healthy tissue. After separating the phenotypically cancerous and healthy parts of formalin-fixed paraffin-embedded tissues, each sample underwent protein recovery process and tryptic digestion for label-free semi-quantitative LC/MS analysis. Eight proteins showed significantly higher abundance in tumor including tenascin, transmembrane emp24 domain-containing protein 2, cytoplasmic dynein light chain 1, coactosin-like protein, small proline-rich protein 2D, nucleolin, U5 small nuclear RNP 200-kDa helicase and fatty aldehyde dehydrogenase. Desmoglein-1 and keratin type I cytoskeletal 9 were down-regulated in tumor. Using Ingenuity Pathway Analysis we mapped the signaling pathways these proteins play role in regarding other tumors. Based on these findings these proteins may serve as promising biomarkers in the fight against laryngeal/hypopharyngeal SCCs.

Keywords Biomarker discovery · Laryngeal cancer · LC/MS · Squamous cell carcinoma · Proteomics

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Introduction

Head neck squamous cell carcinoma (HNSCC) is the 6th most frequent malignancy [1] related with nicotine and alcohol abuse [2]. Its incidence is three-fold higher in males. Due to the aspecific symptoms most HNSCCs are diagnosed with advanced stage indicating growing public health problem worldwide The low 5-year overall survival rate has not changed significantly in the past decade despite surgical and oncological innovations [3].

Thus, early diagnosis and better understanding of tumor behavior is essential. HNSCCs probably produce several proteins that could be utilized as tumormarkers. In possession of reliable markers not only screening and early recognition, but early detection of recurrence could be achieved. In addition, some markers may serve as promising targets for biological therapies.

Due to rapid proteomics development, a new way has opened in biomarker discovery. Beside evaluating epigenetic modifications, on-tissue protein distribution investigation and protein imaging also became available [4]. As potential biomarkers are mostly unknown, labeling can not be used in discovery contrary to label-free semiquantitative methods. Without labeling, intensity of distinct peptides appropriately follows their abundance changes. Nevertheless, label-free methods are more cost-efficient. LC/MS possesses these advantages. Its high throughput feature also makes it powerful in protein discovery. Initially, proteomic analysis of solid tumors with mass spectrometry (MS) was available only on fresh frozen samples. Therefore, growing demand for analysis of formalin fixed paraffin embedded (FFPE) tissue samples appeared. Development of tissue preparation protocols enabled the elimination of formaldehyde induced protein cross-linkings disturbing MS. Overcoming this formerly limiting factor, an invaluable perspective has opened in retrograd analysis due to broad histological sample archives used in daily clinical practice.

The present study aims to investigate protein abundance differences between laryngeal/hypopharyngeal squamous cell carcinoma (LHSCC) and phenotypically normal tissue on FFPE samples to identify potential tumor markers. Furthermore, based on the literature and Ingenuity Pathway Analysis (IPA), we aim to map the pathways these proteins take part in other malignant tumors in order to evaluate them as possible candidates for target therapy of HNSCCs in the future.

Material and Methods

Patients

Sixteen consenting patients (16 males, median age 61 yrs., range from 45 yrs. to 79 yrs) diagnosed with LHSCC were enrolled. Heavy smoking and regular alcohol consumption were reported in all cases. All tumors were advanced primary cases (stage III-IV.A). Exclusion criteria included non-SCC histology, HPV positivity, previous oncological treatment and recurrence or second primary tumor.

Method

Clinical examination included biopsy from all tumors for diagnosis prior to treatment. Biopsy samples were fixed in formalin followed by paraffin-embedding, sectioning and hematoxylin-eosin (HE) staining. Histological examination confirmed HNSCC in all cases.

For research purposes, all corresponding paraffin-embedded tissue blocks were retrieved and further sections (15 μm thickness) were made from original blocks and placed to conventional histological plate without staining. In addition, another HE stained section (7 μm thickness) was made from blocks with one obvious malignant field marked by the pathologist on the contralateral side of the plate. Based on this marking, identical part of each plate containing unstained paraffin-fixed section was drawn around in the same way. Deparaffinization was performed by washing the unstained slides with xylol, ethanol 90% (m/m), ethanol 70% (m/m), ethanol 50% (m/m) and 50 mM ammonium-bicarbonate, respectively. Antigen retrieval was performed with 100 mM Tris-HCl buffer (97 °C, 30 min). According to the marked field lining histologically obvious malignant part, the tissue was microdissected with a fine needle and collected to 2 ml Eppendorf tube containing SDS lysis buffer. Tumor adjacent normal tissue was also microdissected and each sample was incubated in the lysis buffer (97 °C, 30 min). After centrifugation ice cold absolute ethanol was added to the supernatant with 9 volume surplus. Samples were kept at 4 °C overnight for protein precipitation. The pellet was washed twice with absolute ethanol, proteins were solubilized with 8 M urea. Reduction and alkylation were followed by overnight trypsin digestion. The resulting tryptic peptides were cleaned using Pierce C18 spin columns (Thermo Fisher Scientific, Waltham, MA). The MS used for analysis was a Bruker mAXIS II ETD Q-TOF (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 nanoRSLC system (Dionex, Sunnyvale, CA, USA). Samples were injected on an Acclaim PepMap100 C-18 trap column (100 μm × 20 mm, Thermo Scientific, Sunnyvale, CA, USA) online coupled to an ACQUITY UPLC M-Class Peptide BEH C18 column (130 Å, 1.7 μm, 75 μm × 250 mm, Waters, Milford, MA, USA). Peptides were separated at 48 °C with a flow rate of 300 nl/min, 4% solvent B from 0 to 11 min, followed by a 120 min gradient to 50% solvent B. Solvent A consisted of water +0.1% formic acid, while Solvent B was acetonitrile +0.1% formic acid. The injected sample amount was 0.5 μg. Sample ionization was achieved in the positive electrospray ionization mode via a CaptiveSpray nanoBooster ionsource. The capillary voltage was 1300 V, the nanoBooster pressure was 0.2 Bar, drying gas was 150 °C, the flow rate was 3 l/min. External mass calibration was done using the low concentration tuning mix

from Agilent technologies via direct infusion. Internal mass calibration was performed via lock mass for each run using sodium formate. The MS spectra were recorded with a fix cycle time (2.5 s) over the mass range of m/z 150–2200 at 3 Hz with a minimal precursor mass of m/z 322. The CID was performed at 16 Hz for abundant precursors and at 4 Hz for ones of low abundance. Singly charged peptides were excluded from analysis, only multiple charged peptides were chosen for fragmentation. Collision energy for precursor signals was set automatically followed by the manufacturer's recommendations based on the isolation m/z, isolation mass range width and ion charge state. Active exclusion of 2 min. After 1 spectra was used except if the intensity of the precursor was elevated threefold. For protein analysis raw data were recalibrated using the Compass DataAnalysis software 4.3 (Bruker Daltonics, Bremen, Germany). Data were processed by the ProteinScape 3.0 software (Bruker Daltonik GmbH, Bremen, Germany). Proteins were identified by searching against the human Swissprot database (2015_08) using the Mascot search engine version 2.5 (Matrix Science, London, UK) applying the following search parameters: 7 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, 2 missed cleavages, carbamidomethylation of cysteines as fixed modification, deamidation (NQ) and oxidation (M) as variable modifications and proteins were identified using 1% FDR limit. Label-free quantitation (LFQ) was then performed using MaxQuant (software version 1.5.3.30), applying default parameters. MaxQuant analysis searched only for proteins identified previously by Mascot. Each LC-MS/MS run was aligned using the "match between runs" feature (match time window 0.8 min, alignment time window 15 min). The acquired data underwent discriminant analysis and paired sample t-test searching for proteins most characteristically describing tumor and normal tissue. Bioinformatic analysis were performed by IPA software (Ingenuity Databases, Mountain View, CA, USA) to place identified proteins in known signalling pathways.

Results

1164 proteins were identified with at least 2 unique peptides among the samples. Discriminant analysis revealed 18 proteins describing either the tumor or normal tissue group. Paired sample t-test were used to test for statistical significance between each pair regarding these most descriptive 18 proteins (Table 1). The p value was determined 0,1 considering the low number of sample pairs of coherent tumor and normal tissue ($n = 16$). Thus, 8 proteins showed significantly higher density in tumor including tenascin (TNC), transmembrane emp24 domain-containing protein 2 (TMED2), dynein light chain 1, cytoplasmic (DYNLL1), coactosin-like protein (COTL1), small proline-rich protein 2D (SPRR2D), nucleolin (NCL), U5 small nuclear RNP 200-kDa helicase (SNRNP200) and fatty aldehyde dehydrogenase (ALDH3A2). Two proteins, desmoglein-1 (DSG1) and keratin type I cytoskeletal 9 (KRT9) had significantly higher density in the adjacent phenotipically normal tissue. Data and physiological roles of identified proteins are demonstrated in Table 2.

Discussion

Despite rapid development of molecular diagnostics, tumormarker discovery is still challenging. In the past few years the interest of tumor research has gradually turned to proteomics highlighting the observation that secreted proteins are as important as tumor genetics. MS coupled with LC is considered as the „gold standard" quantitative method in tumor protein research. Due to the continuously evolving technical background, growing effort on investigation of HNSCC proteome has also appeared. Initially, studies targeted in vitro cell lines. Analysis of solid tumor tissues opened new ways in tumormarker discovery, as these include the surrounding microenvironment also contributing to the malignant nature of HNSCCs [5]. MS-based protein analysis had been previously possible only on fresh frozen tissue samples with the neccessity of organization steps including planned cryosection and coordination of sample preparation. These drawbacks can be overhelmed by FFPE tissues utilizing deparaffination process [6]. Among existing papers reporting proteomic analysis on FFPE samples, only a few reports address HNSCC [7]. Discovering possible tumor markers can be achieved by finding proteins exclusively expressed by tumor tissue, but protein abundance differences between normal and tumor tissue can also be interpreted as a possible tumor marker. Our aim was to explore protein abundance differences between phenotypically normal and tumor tissue on FFPE laryngeal-hypopharyngeal tumor samples. To our knowledge no LC/MS based proteomical studies exist exclusively investigating LHSCC on FFPE samples.

We found eight and two proteins with significantly higher and lower abundance in LHSCC, respectively, compared to adjacent normal tissue (Table 2).

We foremost found TNC, DYNLL1, COTL1, SPRR2D, SNRNP200, TMED2 and ALDH3A2 abundant in LHSCC by LC/MS. Similar to our findings, one LC/MS study also found NCL levels elevated in LHSCC [8].

We first found DSG1 down-regulated in LHSCC with LC/MS, albeit rather isoform switch among desmogleins seems to be determining in tumor invasivity.

We identified K1C9 down-regulated. One possible explanation for down-regulation is the dedifferentiation. The other probable hypothesis in our opinion is the lack of detectable tryptic peptides of cytokeratins in peritumoral microenvironment due to non-tryptic digestion during invasion.

IPA found no connection between proteins, but we uncovered existing interactions being considered as possible targets for future therapies in LHSCCs (Fig. 1).

Table 1 Paired sample t-test (for the most descriptive 18 proteins of corresponding tumor-normal tissue samples)

		Paired Differences					t	df	Significance *p* value (2-tailed)
		Mean value of relative density	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	TENA_HUMAN *Normal* – TENA_HUMAN *Tumor*	−2,348,325,00000	1,371,919,61,000	396,039,07810	−3,220,001,13,400	−1,476,648,86,600	−5930	11	**0,000**
Pair 2	TMED2_HUMAN *Normal* – TMED2_HUMAN *Tumor*	−150,133,33,330	85,444,86,083	24,665,80,670	−204,422,40,780	−95,844,25,882	−6087	11	**0,000**
Pair 3	DYL1_HUMAN *Normal* – DYL1_HUMAN *Tumor*	−183,083,33,330	326,385,56,630	94,219,39,728	−390,458,82,850	24,292,16,187	−1943	11	**0,078**
Pair 4	PSMD3_HUMAN *Normal* – PSMD3_HUMAN *Tumor*	−53,666,66,667	145,052,55,060	41,873,06456	−145,828,66,040	38,495,32,703	−1282	11	0,226
Pair 5	DCXR_HUMAN *Normal* – DCXR_HUMAN *Tumor*	−35,258,33,333	123,515,69,980	35,655,91,127	−113,736,46,490	43,219,79,825	-,989	11	0,344
Pair 6	DSG1_HUMAN *Normal* – DSG1_HUMAN *Tumor*	1,390,166,66,700	2,209,880,78,000	637,937,63,150	−13,924,59,339	2,794,257,92,700	2179	11	*0,052*
Pair 7	COTL1_HUMAN *Normal* – COTL1_HUMAN *Tumor*	−264,333,33,330	397,229,34,370	114,670,23,430	−516,720,81,730	−11,945,84,941	−2305	11	**0,042**
Pair 8	K1C9_HUMAN *Normal* – K1C9_HUMAN *Tumor*	73,326,666,67,000	97,159,973,93,000	28,047,668,55,000	11,594,164,41,000	135,059,168,90,000	2614	11	*0,024*
Pair 9	OSTF1_HUMAN *Normal* – OSTF1_HUMAN *Tumor*	−21,475,00000	81,984,66,736	23,666,93,488	−73,565,57,247	30,615,57,247	-,907	11	0,384
Pair 10	IF6_HUMAN *Normal* – IF6_HUMAN *Tumor*	−3266,66,667	193,252,24,370	55,787,11,746	−126,053,28,430	119,519,95,100	-,059	11	0,954
Pair 11	ACOC_HUMAN *Normal* – ACOC_HUMAN *Tumor*	8716,66,667	91,136,93,826	26,308,96,792	−49,188,98,130	66,622,31,463	,331	11	0,747
Pair 12	SPR2D_HUMAN *Normal* – SPR2D_HUMAN *Tumor*	−103,300,00000	134,895,60,270	38,941,00627	−189,008,57,690	−17,591,42,307	−2653	11	**0,022**
Pair 13	NUCL_HUMAN *Normal* – NUCL_HUMAN *Tumor*	−1,271,858,33,300	1,047,332,64,900	302,338,89,340	−1,937,301,75,100	−606,414,91,570	−4207	11	**0,001**
Pair 14	TXNL1_HUMAN *Normal* – TXNL1_HUMAN *Tumor*	−41,316,66,667	84,232,22,050	24,315,74,759	−94,835,26,627	12,201,93,293	−1699	11	0,117
Pair 15	U520_HUMAN *Normal* – U520_HUMAN *Tumor*	−691,750,00000	768,104,41,110	221,732,64,430	−1,179,780,26,000	−203,719,74,050	−3120	11	**0,010**
Pair 16	UD17_HUMAN *Normal* – UD17_HUMAN *Tumor*	−9275,00000	163,882,95,780	47,308,93,491	−113,401,26,370	94,851,26,367	-,196	11	0,848
Pair 17	AL3A2_HUMAN *Normal* – AL3A2_HUMAN *Tumor*	−86,083,33,333	144,985,55,310	41,853,72,405	−178,202,75,890	6036,09220	−2057	11	**0,064**
Pair 18	TBB6_HUMAN *Normal* – TBB6_HUMAN *Tumor*	−230,333,33,330	647,338,93,520	186,870,65,420	−641,632,87,020	180,966,20,350	−1233	11	0,243

Boldface entries under "Significance *p* value (2-tailed)" indicate proteins significantly overexpressed in tumor tissue comparing to the adjacent phenotipically normal tissue ($p < 0,1$)

Italicized entries under "Significance *p* value (2-tailed)" indicate proteins with significantly lower abundance in tumor tissue ($p < 0,1$) comparing to the adjaccent phenotipically normal tissue

Table 2 Data of identified proteins

Gene symbol	Accession	Role	HNSCC related literature referral	IPA findings	Mascot score	MW (kDa)*	∑ SC (%)**	∑ Peptides***
TNC	TENA_HUMAN	ECM adhesion, angiogenesis, EMT	overexpression as adverse prognostic factor in oral SCC *(mRNA expression analysis)*	activating EGFR due to EGF-like repeats	354	240.7	8.3	13
TMED2	TMED2_HUMAN	trafficking between endoplasmic reticulum and Golgi apparat, cytoskeletal re-arrangement, cell migration	up- and downregulation in oral and hypopharyngeal SCC *(miRNA expression and gene expression analysis, low sample number, n = 5)*	overexpression mediated by NFE2L2 *(a transcription factor that may promote carcinogenesis)*	59.1	22.7	12.2	3
DYNLL1	DYL1_HUMAN	intracellular microtubular vesicle transport, maintenance of cytoskeleton	up-regulation under hypoxic conditions on FaDuDD HNSCC cell line *(LC-MS/MS)*	indirectly facilitating HGF/c-MET pathway	82.3	8	21.6	5
COTL1	COTL1_HUMAN	F-actin binding protein, cellular motility	no reports found	tumor associated protein in chemical-induced SCC model[†]	72.7	15.9	5.4	3
SPRR2D	SPR2D_HUMAN	function in skin barrier, wound healing, quenching of ROS, terminal differentiation marker of stratified squamous epithel	up-regulated in oral SCC and NPC *(RNA analysis)*	no data found	89.1	7.9	46.8	3
NCL	NUCL_HUMAN	co-factor in transcription regulation and RNA transport	overexpressed in laryngeal SCC *(LC-MS/MS on snap frozen tissue samples)*	recruiting EGFR mediated signaling pathways, facilitating EGFR cytoplasmic tail dimerization, direct binding with SNRNP200 playing role in RNA metabolism	316.2	76.6	17.9	11
SNRNP200	U520_HUMAN	DeXH box protein, pre-mRNA splicing	no reports found	gene mutation in human cutaneous SCC, direct binding with NCL acting as nuclear interacting partner	51.5	244.4	1.3	2
ALDH3A2	AL3A2_HUMAN	detoxification of aldehydes originating from lipid peroxidation processes	down-regulated in oral SCC *($^{16}O/^{18}O$ proteomics analysis using ESI-ion trap and MALDI-TOF/TOF MS)*	direct binding with EGFR[‡]	64.6	54.8	7.9	3
DSG1	DSG1_HUMAN	desmosome forming	isoform switch among DSGs in HNSCC *(RNA analysis)*	indirectly down-regulated by HGF in malignant melanoma	175	113.7	6.9	5
KRT9	K1C9_HUMAN	intermediate filament of intracytoplasmatic cytoskeleton	down-regulated in HNSCC lymph node metastasis *(MALDI-Q-TOF MS/MS)*; up-regulated in NPC *(ESI-Q-TOF–MS)*	no data found	1493.3	62	68.2	37

Boldface entries under "Gene symbol" indicate proteins significantly overexpressed in tumor comparing to the adjacent phenotipically normal tissue ($p < 0,1$)

Italicized entries under "Gene symbol" indicate proteins with significantly lower abundance in tumor ($p < 0,1$) comparing to the adjaccent phenotipically normal tissue

c-MET: hepatocyte growth factor receptor; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; ECM: extracellular matrix; EMT: endothelial-mesenchymal transition; HGF: hepatocyte growth factor;

HNSCC: head neck cancer squamous cell carcinoma; NFE2L2: nuclear factor, erythroid 2 like 2; NPC: nasopharyngeal carcinoma, ROS: reactive oxygen species; SCC: squamous cell carcinoma

*molecular weight in kDa

**average sequence coverage in percentage

***the average number of peptides each protein was identified with

† without identified participating pathways

‡ with unknown significance

Theoretically, inhibition of TMED2 or its inducer NFE2L2 may suggest a promising tool against HNSCC invasion. NCL can act both as a recruiter and overexpressed protein of EGFR mediated pathways and can facilitate dimerization of EGFR's cytoplasmic tail. Thus NCL overexpression can be both consequence and initiator of EGFR activation. Therefore interfering NCL can also be promising in EGFR-positive HNSCCs, while down-regulation may serve as a marker of anti-EGFR therapy efficacy.

TNC containing EGF-like repeats may serve as targets against EGFR-positive HNSCCs. Inhibiting EGFR results in TNC down-regulation. Considering the diverse correlations between TNC and EGFR, TNC can serve both a potential target in HNSCC and therapeutic response marker in anti-EGFR treatment.

DYNLL1-related cytoskeletal rearrangement and tumor cell migration can be theoretically inhibited by anti-HGF therapy, as DYNLL1 shows indirect interaction with HGF in HGF/c-MET pathway in HNSCC.

IPA found indirect inhibition of DSG1 by HGF in malignant melanoma highlighting that DSG1 down-regulation contributes to cell-cell adhesion disruption easing invasion. Thus inhibition of HGF can exert anti-tumor effect with maintaining cell-to-cell junctions via stabilizing desmosomes by DSG1 overexpression. Considering that EGFR pathway shares common signals with HGF-mediated routes resulting redundancy and frequently moderate therapeutic response to anti-EGFR treatment, combined inhibition of EGFR and c-MET/HGF pathway is emerging. Interfering redundant pathways (p44/p42 MAPK, PI3K/AKT, STAT) may have the desired anti-cancer effect. Until routinely applied anticancer drug combinations are available simultaneously targeting HGF, EGF and NFE2L2 mediated pathways, inhibition of overexpressed DYNLL, TNC, NCL and TMED2 may exert anti-tumor effect on HNSCC beyond their diagnostic role.

IPA also found unclear interactions. COTL-1 is suggested as tumor-associated protein upregulated on mouse carcinogenesis model. SNRNP200 gene mutation was reported in human cutaneous SCC. IPA found EGFR-ALDH3A2 direct binding with unknown significance. NCL-SNRNP200 direct binding demonstrates NCL's place in RNA metabolism and identifies SNRNP200 as a nuclear interacting partner.

Contrary to previous MS studies, abundance differences were determined using label-free LC/MS based proteomics exclusively on FFPE LHSCC samples. Feasibility of quantitative LS/MS methods on FFPE samples had been

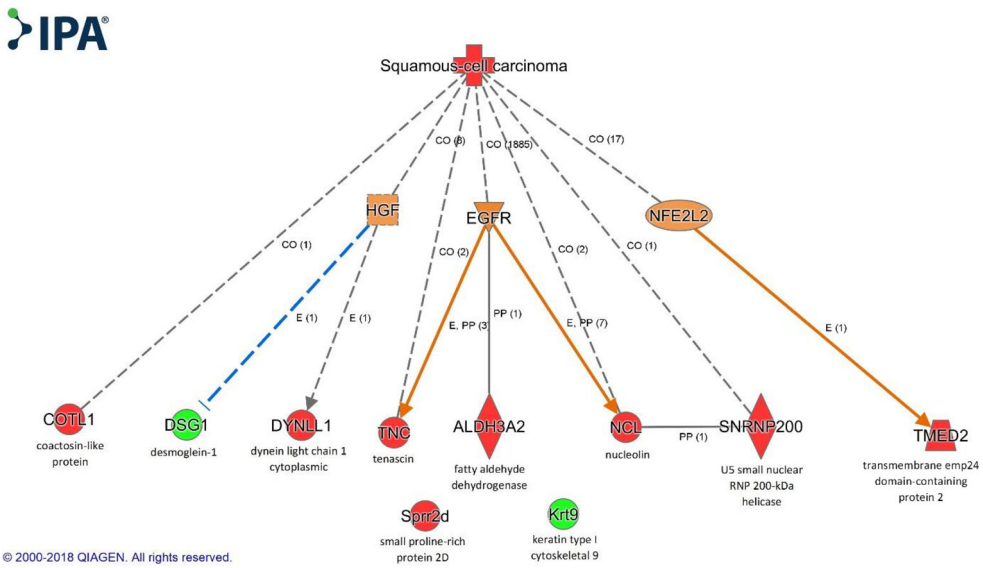

Fig. 1 Note: This data is mandatory. Please provide

CO: correlation; PP: protein-protein binding; E: expression

questionable for a long time due to cross-links and formaldehyde induced adducts [9]. Various extraction innovations made protein recovery from FFPE samples as reliable and diagnostically accurate as from fresh-frozen samples [10]. Labeling has several disadvantages compared to label-free technique: protein loss due to each manipulation step, neccessity of prerequisites (e.g. presence of cysteine-containing peptides) and high costs. These disadvantages also can be bypassed with label-free methods.

Our study also has limitations. Interestingly, IPA did not detect SPRR2D and Krt9. This is probably due to the lack of available IPA data. Continuous amplification of stored data can reveal new interactions. The other drawback of our study is the moderate sample number. It should be noted that our primary aim was to design a pilot study for evaluation of protein abundance differences between LHSCC and adjacent healthy tissue.

Conclusion

Considering our initial favorable results, this study has clinical relevance. Beside highlighting the proteomic difference between LHSCC and adjacent normal tissue, we found possible LHSCC markers/targets that had not been in focus till date. On the other hand, we proposed the potential in involving large histopathological sample archives taking the reliability of label-free LC/MS on FFPE samples into account facilitating protein discovery. Nevertheless, this easy access to HNSCC samples would make fresh frozen sectioning unneccessary offering a cost-efficient and time-saving solution.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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

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