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RESEARCH

Mass Spectrometry-Based Salivary Proteomics for the Discovery of Head and Neck Squamous Cell Carcinoma

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Abstract The 5-year survival rates for cases of head and neck squamous cell carcinoma (HNSCC) are only some 60%, mainly because 20%–40% of the patients develop a local relapse in the same or an adjacent anatomic region, even when the surgical margins are histologically tumour-free. Tumours are often discovered in an advanced stage because of the lack of specific symptoms and the diagnostic difficulties. The more advanced the stage of the tumour, the more invasive the diagnostic and treatment interventions needed. An early molecular diagnosis is therefore of vital importance in order to increase the survival rate. The aim of this study was to develop an efficient rapid and sensitive mass spectrometric method for the detection of differentially expressed proteins as tumour-specific biomarkers in saliva from HNSCC patients. Whole saliva samples were collected from patients with HNSCC and from healthy subjects. The proteins were profiled by using SDS PAGE, MALDI TOF/TOF mass spectrometry and the Mascot database search engine. Several potential tumour markers were identified, including annexin A1, beta- and gamma-actin, cytokeratin 4 and 13, zinc finger proteins and P53 pathway proteins. All of these proteins play a proven role in tumour genesis, and have not been detected previously in saliva. Salivary proteomics is a non-invasive specific method for cancer diagnosis and follow-up treatment. It provides facilities for the readily reproducible and reliable detection of tumours in early stages.

Keywords Biomarker discovery · MALDI TOF MS · Saliva · Tumor

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Introduction

Squamous cell carcinomas of the head and neck region (HNSCCs) are the 6th most common malignancies worldwide [1], affecting around half million new patients yearly. In spite of the improvement made in diagnostic and therapeutic tools, the survival rate has not changed over the past 30 years and is constantly around 60% [2]. One of the main reasons is the developement of recurrences in 20–40% of the cases, even though the excision is carried out in histologically proven intact regions. The survival rate is worsened by lateness of the diagnosis in patients rehospitalized with developed tumours. In such a stage, radical excision combined with radio- or possibly chemotherapy is the only possible treatment, which does not increase the quality of life for every patient.

The data of the World Cancer Registry Report demonstrate that the incidence of the head and neck cancers has increased 3-10-fold in a generation. This increase was explained in terms of the spreading of smoking and alcohol consumption. Planocellular carcinoma of the head and neck occurs mostly in those over the age of 40, but in the past few years even younger subjects are being affected in greater numbers. HNSCC is relatively rare in women: the ratio between males and females is around 10:1 [3–6].

The mortality rate of HNSCC has changed significantly during the past 50 years: it has doubled in Europe. While the mortality rate of malignancies in Hungary increased up to 2.8-fold between 1948 and 2000, that of head and neck cancers increased around 6-fold. The data demonstrate that the most dynamic increase in mortality rate in Hungary is caused by head and neck cancers, their proportion being the highest in Europe. The highest rates of mortality from laryngeal cancers in Europe are reported from Hungary, Poland, Slovakia and Romania. The IARC data indicate that about 60% of oral malignancies in males and 40% of those in females in Europe are caused by smoking. Smoking alone and a regular alcohol intake alone increase the incidence of these cancers 2–3-fold, but coexistence of these two aetiological factors leads to a 15-fold increase in incidence. A genetic predisposition and personal mutative sensitivity also play a role. HPV infection is also a further possible factor since HPV 16 and 18 are detected in 70% of cervical tumours [7–13].

An early diagnosis is of major importance in the fight against malignancies. However, head and neck cancers can present a poor symptomatology for a long period, and they may cause non-specific symptoms, such as dysphagia, a lump sensation, huskiness, a stinging sensation and rarely pain. Neck metastasis is an important prognostic factor, but even in the evenet of clinically N0 neck, the possibility of occult metastasis is around 30–40% [14, 15]. If the diagnosis is made early and there are no metastases, surgery or radiotherapy generally leads to very good long-term results, but most of the patients do not belong in this group. When the diagnosis is made later, the radical excision is followed by radiotherapy or radio-chemotherapy, with a resultant worsened quality of life. In spite of these interventions, the rate of recurrence is relatively high and the 5-year survival rate is some 50–60%.

The exploding development of proteomics in recent decades has led to the use of saliva as a new diagnostic tool. Proteomics furnishes high accuracy in the identification of proteins, and determination of changes in their levels is therefore a promising approach for early detection. It has been estimated that approximately 20% of the cellular proteins are secreted. This would allow the identification of novel HNSCC biomarkers via the analysis of tissue "secretomes" that can be detected in saliva as in tumour proximal fluid or serum. For HNSCCs, saliva seems an ideal source. Human saliva can easily be collected in relatively large quantities by a non-invasive method [16–19], this, together with methodological improvements allowing high throughput, makes it a medium of potential value for early detection, possibly in everyday clinical use [20–25]. A population-based screening program for oral cancer has not yet been achieved, though "opportunistic screening" has been suggested [26–28].

Materials and Methods

Clinical Sample Collection

A total of 25 consenting head and neck cancer patients (10 females, 15 males) participated in the research; their mean age was 56.48 (range 22 to 78 years). Table 1 shows the detailed parameters on all the investigated HNSCC patients. The HNSCC patients were compared with a control group composed of 25 healthy volunteers (15 smokers, 10 non-smokers), matching in age and gender. In the tumour group, 19 patients had a malignant and 6 a benign tumour in the head and neck region. The subjects with HNSCC were all heavy smokers (at least 1 pack per day). None of the patients had undergone treatment prior to saliva sample collection.

All the subjects were asked to attend without having any drink or food since the night before. Following mouth rinsing with tap water, unstimulated whole saliva samples were collected between 8 a.m. and 10 a.m. from both the patients and the control group, by means of a widely accepted procedure

Table 1 Parameters of all investigated patients

Sex	Age	Diagnosis	Smoker
Tumor group - benign			
M	64	Parotid tumour	Yes
F	66	Ventricular cyst	No
F	57	Parotid tumour	Yes
M	59	Chronic laryngitis	Yes
F	22	Parotid tumour	No
M	49	Pharyngeal papilloma	Yes
Tumor group - malignant			
M	51	Laryngeal and pharyngeal SCC	Yes
M	53	Gingival SCC	Yes
M	49	Pharyngeal SCC	Yes
M	57	Supraglottic SCC	Yes
M	60	Hypopharyngeal SCC	Yes
F	61	Vocal chord SCC	Yes
F	55	Vocal chord SCC	Yes
M	59	Subglottic SCC	Yes
F	51	Supraglottic SCC	Yes
M	55	Cervical SCC (no primary tumor)	Yes
M	48	Cervical SCC (no primary tumor)	Yes
M	52	Hypopharyngeal SCC	Yes
F	69	Vocal chord SCC	Yes
M	63	Vocal chord SCC	Yes
F	60	Oesophageal SCC	Yes
M	65	Vocal chord SCC	Yes
F	57	Vocal chord SCC	Yes
M	78	Parotid tumour	Yes
F	52	Hypopharyngeal SCC	Yes

[29, 30]. Briefly, the participants were asked to rinse their mouth three times with tap water before collection; saliva specimens were taken with 5 ml syringes from the buccal fold int he non-stimulated oral cavity and cooled on ice in Eppendorf tubes (Eppendorf Austria GmbH, Vienna, Austria). This was followed by centrifugation at 2,500 rpm for 12 min at 4°C. The supernatants of the saliva samples were subsequently stored frozen at −80°C until further analysis. The studies were approved by the Ethics Committee on Human and Animal Research at Pécs University in accordance with the Ethical Codex of Human and Animal Experiments (licence no. : 3382/2009).

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

With an Ultra Turrax homogenizer, saliva samples were homogenized in 20 mM Tris/HCl buffer (pH 7.4) containing 3 mM EDTA, 5 mM beta-mercaptoethanol and 1% SDS. After the addition of 1% bromephenol blue, the samples were boiled for 2 min and clarified by centrifugation (8,000 g for 2 min). SDS-PAGE was carried out on 12% gel by the method of Laemmli [31], similarly as described earlier [32]. A low molecular weight calibration kit (Pharmacia) was used for the estimation of molecular weight. Gels were stained with Coomassie brillant blue R-250 and destained with a solution containing 5% (v/v) acetic acid and 16% (v/v) methanol.

Tryptic Digestion and MALDI TOF/TOF Mass Spectrometry (MS)

The spots of interest were excised from the gel with a razorblade, placed in Eppendorf tubes, and destained by washing three times for 10 min in 200 μL of 50% (v/v) acetonitrile, 50 mM NH_4HCO_3 solution. Proteins were then reduced with 20 mM dithiotreitol, 100 mM NH_4HCO_3 and 5%acetonitrile for 1 h at 55°C. The gel pieces were dehydrated at room temperature and covered with 10 μL of modified trypsin (Promega, Madison, WI, USA; sequencing grade) (0.04 mg mL^{-1}) in Tris buffer (2.5 mM, pH 8.5) and left to stand at 37°C overnight. The spots were crushed and the peptides were extracted for 15 min in an ultrasonic bath with 15 μL of an aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v) After extraction, the solution of the peptides was lyophilized and redissolved in water. The aqueous solutions of the lyophilized protein tryptic digests were purified by using ZipTip C_{18} solid-phase extraction (Millipore Kft., Budapest, Hungary) and directly loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by mixing 1.0 μL of each solution with the same volume of a saturated matrix solution prepared fresh every day by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% TFA (1/2, v/v) The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in the reflector mode for MALDI TOF peptide mass fingerprint (PMF) or LIFT mode for post-source decay (PSD) and collision-induced decay (CID) MALDI TOF/TOF with an automated mode using FlexControl 2.4 software. An accelerating voltage of 20 kV was applied for PMF. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH, Berlin, Germany). External calibration was performed in each case with a Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). Peptide masses were acquired in the range m/z 800 to m/z 5,000. Each spectrum was produced by accumulating data from 500 consecutive laser

Table 2 Proteomic parameters of the identified salivary proteins in HNSCC patients

No	Protein name	Accession number	Matched peptides	Theoretical MW (Da)	Mascot score	Sequence coverage %
1	Annexin 1	gi: 4502101	12	38690	127	40
2	Cytokeratin 4	gi:194387942	10	56193	95	18
3	Cytokerain 13	gi: 30377	8	45837	77	20
4	Zinc finger protein 28	gi: 56542871	5	77418	54	13
5	Regulator G-protein	gi: 62865654	10	67240	103	22
6	Indoleamine 2,3-dyoxigenase	gi: 215274147	5	45396	60	12
7	Unnamed protein product	gi: 194375299	5	37325	71	18
8	OFD 1 protein	gi: 64654925	12	111675	82	16
9	Chain A, human salivary amylase	gi: 14719766	11	55857	109	31
10	Unnamed protein product	gi: 47077030	7	38266	67	26
11	Keratin type 1 cytoskeletal	gi: 24430192	19	51236	218	41
12	CEP 290 protein	gi: 14250413	7	19223	89	39
13	COL6A3	gi: 219841772	12	278032	67	6

shots. Singly charged monoisotopic peptide masses were searched against MSDB, Swiss-Prot and NCBInr databases by utilizing the MASCOT Server 2.2 search engine (www.matrixscience.com, Matrix Science Ltd., London, UK) and Bruker BioTools 3.0 software (Bruker Daltonics, Bremen, Germany). A maximum of one missed tryptic cleavage was considered, and the mass tolerance for monoisotopic peptide masses was set to 100 ppm. For the proteins not identified by MALDI TOF, we proceeded with PSD and CID MALDI TOF/TOF analysis. Bruker FlexControl 2.4 software (Bruker Daltonics, Bremen, Germany) was used for the control of the instrument and Bruker FlexAnalysis 2.4 software (Bruker Daltonics, Bremen, Germany) for spectrum evaluation.

Results

This work focused on the identification of potential protein biomarkers for HNSCCs from unstimulated whole saliva samples. The potentially HNSCC-regulated proteins were separated and digested with trypsin, and the resulting tryptic peptides were investigated by MALDI TOF/TOF MS for protein identification. The proteins, which were identified in over 85% of the investigated pathological saliva samples, are listed in Table 2. The most abundant well-known proteins that were found in all the saliva samples from the HNSCC patients and the healthy volunteers were salivary amylase, keratins and actins. Additionally, increased levels of annexin 1, zinc finger protein 28, regulator G-protein 3, indoleamine 2,3-dioxygenase, OFD1 protein and CEP290 protein were identified in most of the saliva samples from the malignant cancer patients.

Discussion

In this study we identified several HNSCC-regulated salivary proteins as potential molecular biomarkers. Clinical proteomics is a very young discipline, which provides a tool with which to acquire information about a molecular pathogenesis and to explore biomarkers. Biomarkers can assist in later diagnoses; not only complete proteins, but also their peptides and fragments can be examined.

The great significance of proteomics lies in the fact that signal transmission, regulation, enzymatic activity and structural features encoded by the genome and implemented at the level of the proteins can be directly analysed at the proteome level. Information about expressed and amended proteins can not be obteined only with genomic methods. This is partly due to the more complex structure of the proteome in comparison with the genome. Proteins pass through a process of ripening during the formation and acquisition of functionality. Approximately 300 chemical modifications are known which may occur after protein synthesis in ribosomes. In consequences of these changes, several orders of magnitude more proteins than genes occur in the cell [33].

Over the past few years, proteomics has developed considerably, and we are now able to identify and characterize proteins extracted from cells, tissues and biological fluids, and to determine their relative amounts. This would not be possible without the close cooperation of various disciplines [34, 35].

The early detection of primary and possibly recurrent tumours is the key to improvement of the poor survival rate of HNSCCs [36]. Using a non-invasive method, we have succeeded in identifying a number of low molecular weight peptides from saliva which can serve as biomarkers. Their role in the pathogenesis of head and neck tumours has already been demonstrated, though the exact mechanism is not yet clear. These peptides had previously been isolated only from serum or tumour tissue [37–49].

We believe that this discovery will lead to the widespread use of the method. In contrast with earlier methods of isolation, the saliva test is simple and non-invasive, and could even be used in screening. The biomarkers are expected to facilitate the early detection of both primary and recurrent tumours. We plan a more detailed investigation of these peptides, including their sensitivity and specivicity, together with the further screening of samples, searching for other biomarkers and their tumor-specific breakdown. Our goal is a thorough processing of the patient data, taking into account the tumour localization, the histology, the patient's age and sex, harmful habits (smoking and alcohol consumption) and any drug intake. Further specific examinations are needed regarding the results of various treatments such as surgery, radiotherapy or combined chemoirradiation.

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