Proteomic examination of precancer state- Analyzing saliva samples of diabetic patients

PhD-Thesis

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1. Introduction

Clinical proteomics is a relatively young discipline, but it has developed considerably in the last ten years. Because its easy and non-invasive collection in large quantities-saliva seems to be an ideal diagnostic target. According to this salivary proteomics has proven its suitability as a reliable diagnostic method for HIV, arthritis, but also in early cancer detection over many years.

An early diagnosis of cancerous diseases plays a key role in the fight against malignancies. But in spite of the great emphasis on oral squamous cancer prevention, novel technologies in the diagnostics and therapy, there are still more than 300,000 new cases registered each year. The prognosis is becoming poorer, and the mortality rate is increased 3-4-fold in the last four decade. This is in accordance with the fact, that oral squamous cell carcinoma can present a poor symptomatology over a long period, and the occurrence of non-specific symptoms, such as dysphagia, or pain in oral cavity are not emerging problems for the patients. The lateness of diagnosis of this disease results in worsening of the survival rate. The IARC data indicate that about 60% of oral malignancies develop in males and 40% in females in Europe. The main predisponating factor in the development of cancer is smoking, and alcohol-consumption. The coexistence of these two etiological factors leads to a 15-fold incidence. Genetic conditions as well as personal sensitivity are also contributing factors.

The correlation between type-2 diabetes and oral diseases were established as early as the 20th century. The occurrence of periodontal diseases, dental caries, stomatitis and glossitis is 2-3-fold higher than in patients suffering not from diabetes mellitus. Inflammation as a mediator of carcinogenesis is a well-known fact. Recent conducted epidemiological studies showed an increased incidence of oral premalignancies and lichen planus among diabetic patients in comparison with healthy subjects.

The purpose of this study was to compare the proteomic profile of diabetic and healthy volunteers, focusing on OSCC-referring saliva biomarkers.

2. Aims of the study

1. Are there any oral squamous cell carcinoma salivary biomarkers in the precancer state rype-2 diabetes?

2.We also were interested in the question if there are any differences in the biomarker profile of the diabetic patients suffering from the disease for a longer time.

3. Our aim was to develop a reliable and good reprocible investigation protcol which will be able to use in further examinations

3. Material and Methods

Patients selection

For this study 45 volunteers were selected, and divided in into 2 groups: patients suffering from type-2 diabetes, and the control group of healthy volunteers. Patient recruitment, sample collection and the follow-up were carried out by the examination team. The datas were recorded on a specially prepared questionnaire. Besides the relevant personal data, the recorded information of laboratory values, related to previous treatment, past medical history, as well as alcohol consumption and smoking habits were also recorded. This was followed by a total stomato-oncological screening examination. Patient exclusion criteria have been summarized in Table 1.

1.	Uncontrolled diabetes
2.	Cancer (benign or malignant), precancerous lesions
3.	Poor oral hygiene
4.	Psychiatric problems
5.	Active infection or inflammation in the oral cavity
6.	Rejecting the participation or the follow-up

Table 1.: Summary of the exclusion criterias

Between 4th January and 30th November 2012, the screenings were carried out on 45 volunteers at the 2nd Department of Internal Medicine and Nephrology, University of Pecs, Hungary. A total of 45% were women and 55 % were men. The average age was: 62.3. The control group comprised 20 adults (10 women, 10 men), their mean age was: 61.2 years.

Sample collection

All subjects were asked to attend the examination between 8 a.m. and 10 a.m., without having drink or food since the night before. First a complete stomato-oncological examination was performed; details of state on of the teeth, periodontium, mucosal lesions, precanceroses were also recorded. This was followed by mouth rinsing with tap water. The whole, unstimulated saliva samples were collected with 5 ml sterile syringes using a special tactile-induced collection method, developed by our team. The collecting area involved the buccal and the lingual fold. The saliva specimens were cooled on ice; this was followed by centrifugation at 2500 rpm for 12 min at 4°C. The supernatants of the saliva samples were stored frozen at -80°C until further analysis.

Proteomic methods

1. Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ultra Turrax homogenizer was used to homogenize the saliva samples in 20 mM Tris/HCL buffer (pH 7.4), containing 3 mM EDTA, 5 mM betamercaptoethanol and 1% sodium-dodecylsulfate (SDS). This was followed by the addition of 1% bromphenolblue, and then the samples were boiled for 2 minutes and clarified by centrifugation (8000×g for 2 minutes). SDS-PAGE was carried out on 12% gel, using the Laemmli's method. For estimating the molecular weight a low-molecular weight calibration kit (Pharmacia) was used. We stained the gels with Coomassie brilliant blue R-250 and the gels were destained with a solution of 5% (v/v) acetic acid and 16% (v/v) methanol.

2. Tryptic digestion and MALDI TOF/TOF mass spectrometry

We excised the bands of interest with a scalpel blade, placed them in Eppendorf tubes and destained them by washing three times for 10 minutes in 200 µl of 50% (v/v) acetonitrile, 50 mM NH_4HCO_3 solution. Proteins were reduced with 20 mM dithiotreitol, 100 mM NH4HCO3 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 overninght. The spots were crushed and the peptides were extracted for 15 minutes in an ultrasonic bath with 15 µL of an aquenous solution of acetonitrile and formic acid (49/50/1 v/v/v). After extraction, the solution of the peptides was lyophilizied protein tryptic digests were purified by using ZipTip C18 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 saturated matrix solution prepared fresh every day by dissolving a-cyano-4-hydroxicinnamic 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 (LBT 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 5000. Each spectrum was produced by accumulating date from 1000 consecutive laser shots.

3. Identification of the peptide fragments

Singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBInr databases 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.

4. Statistical analysis

The statistical analysis was carried out by the ClinProTools 3.0 software, which is easy-to-use data post-processing software for visualization, data reduction, data

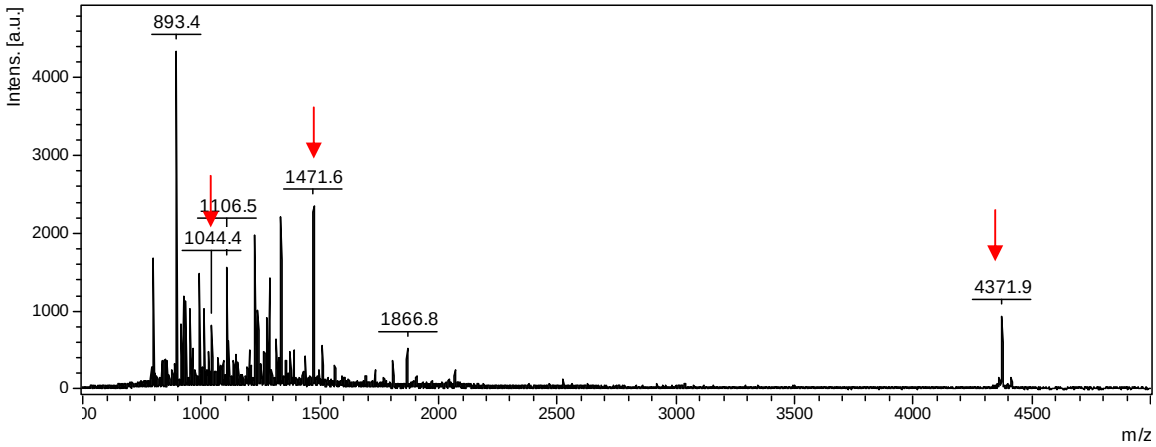
mining and building predictive models from protein profiling data using Bruker's MALDI-TOF mass spectrometers. Supervised classification model generation and validation using different sophisticated mathematical and bioinformatic algorithms like t-probe or the Wilcoxon-test. ClinProTools combines intuitive visualization features and multiple mathematical algorithms to generate pattern recognition models for classification and prediction of e.g. disease from mass spectrometry based profiling data. ClinProTools uses a range of algorithms for generating classification models.

4. The Results

Focusing on the potential carcinogenetic effect of diabetes we analyzed human saliva samples for detecting oral squamous cell carcinoma biomarkers. According to the comparing SDS- PAGE gel the protein content of the diabetic and healthy samples was different. The potentially oral squamous cell-related protein spots were excised and digested with trypsin. The resulting peptides were analyzed by MALDI TOF/TOF MS for protein indication. We could indentify over 900 peptides. There were also obvious differences in the amount of oral squamous cell carcinoma biomarkers. The differences in the spectras are marked in Figure 1.

Figure 1.: Differences in the spectras. Picture A show the spectras in the saliva samples of a diabetic patient suffering from the disease over 18 years. All 3 predictible biomarker are present. While on Picture B the saliva sample of a healthy subject shows none of the biomarkers.

Picture A: Diabetic sample, biomarker spectras marked



Picture B: Saliva sample of a healthy 58 years old women contain none of the typical biomarkers

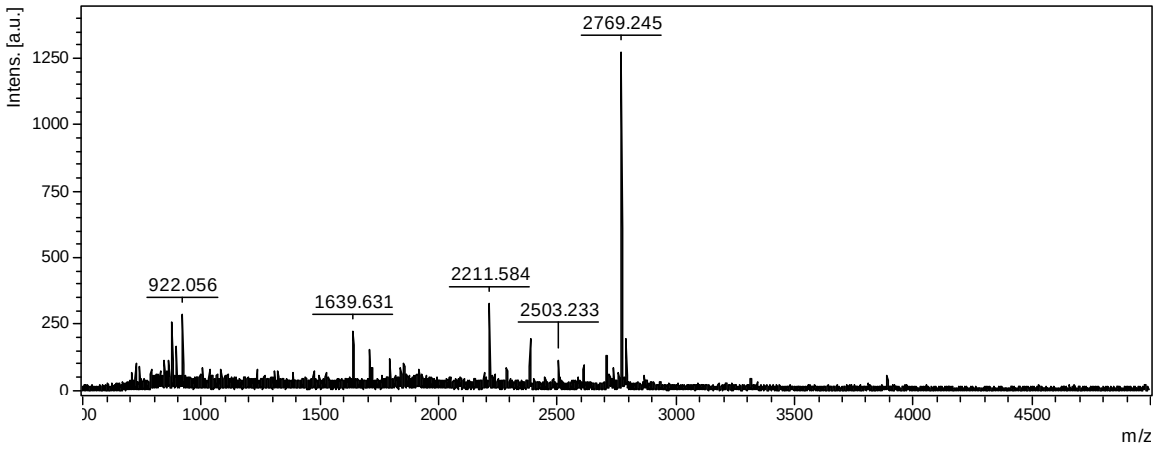


Table 2. The list of the detected potential OSCC biomarkers

Nr	Name	ID Code	Theoretical weight (Da)	Sequence covarage%
1.	Annexin A8-like 2 [Homo sapiens]	gi|55666310	36,84	47,63
2.	Annexin A8-like Homo sapiens	Q5T2P8_HUMAN	36,86	32,72
3.	Tyrosine kinase	gi|473882	7,36	46,88
4.	AX969656 NID Homo sapiens	CAF14764	14,82	26,61
5.	Protein kinase [Homo sapiens]	gi|9886711	86,35	31,59
6.	Peroxiredoxin-2	gi|2507169	21,7	64
7.	Annexin A2	gi|113950	38,44	30

Table 3. Comparison of the OSCC biomarker profile of both groups. 1- Annexin A8, 2-Peroxiredoxin 2, 3- Tirozin protein kinase

Diabetes		**Control**	
n=25	**Biomarker**	**n=20**	**Biomarker**
D001	1,2,3	H001	neg
D002	1,2,3	H002	neg
D003	1,3	H003	neg
D004	1,2,3	H004	neg
D005	1,2	H005	neg
D006	1,2,3	H006	neg
D007	1,2,3	H007	neg
D008	1,2,3	H008	neg
D009	1,2,3	H009	neg
D010	1,2,3	H010	neg
D011	1,3	H011	neg
D012	1,2,3	H012	neg
D013	1,2,3	H013	neg
D014	1,2,3	H014	neg
D015	1,3	H015	neg
D016	1,2,3	H016	neg
D017	1,2	H017	neg
D018	1,2	H018	neg
D019	1,2,3	H019	neg
D020	1,2,3	H020	neg
D021	1,2,3		
D022	1,2,3		
D023	1,2		

D024	1,2,3		
D025	1,3		

Introducing the identified biomarkers

Annexin A8

Annexins are important in various cellular and physiological processes such as providing a membrane scaffold, which is relevant to changes in the cell's shape. Also, annexins have been shown to be involved in trafficking and organization of vesicles, exocytosis, endocytosis and also calcium ion channel formation. Annexins have also been found outside the cell in the extracellular space and have been linked to fibrinolysis, coagulation, inflammation and apoptosis. Annexins have many forms; Annexin A11 binds specifically to calcyclin in a calcium-dependent manner and is required for midbody formation and completion of the terminal phase of cytokinesis. According to this Annexin A11 and A8 is involved in cell growth and a reduction in annexin A11 expression using RNAi stops cell division. Annexins have been implicated in several disease processes, including inflammation and neoplasia. Alterations in the expression of individual annexins have been associated with tumorigenesis in several types of tumour. Loss of Annexin A1 has been found to be an early event in oesophageal squamous cell carcinoma. According to our study it may seem that an overexpression of Annexin A11 and A8 may not only related with colorectal cancer as described previously, but may be also an indicator for development of OSCC.

Tyrosine protein kinase

Tyrosine-specific protein kinases are enzymes which catalyze the addition of a phosphate group from tyrosine specific amino residues. These enzymes are key regulatory components in signal transduction, regulating cell division, cellular differentiation, and morphogenesis. A mutation that causes certain tyrosine kinases to be constitutively active has been previously associated with several cancers. Their exact role in the carcinogenesis and in the development of OSCC needs to be analyzed in more detail in our future investigations.

Peroxiredoxin 2

The thioredoxin peroxidase family, the also called peroxiredoxins, efficiently reduces the intracellular level of H_2O_2 produced in those cells stimulated by various cell surface ligands. The peroxiredoxin family was reported to be closely related to various causes of liver fibrosis, and also of oral squamous cell carcinoma. The oxidation kinetics of all peroxiredoxin was extremely rapid and sensitive, occurring at H_2O_2 doses unable to affect common markers of cellular oxidative stress. In our research, Peroxiredoxin-2 has shown a significant up-regulation at patients, suffering longer time (up to 10 years) from type-2 diabetes, which indicated that it is an early protein target of OSCC. This result is summarized in table 4.

Table 4: Occurance of peroxiredoxin 2 in diabetic patients

Code	Gender	Diagnosed with DM Since (years)
D001	female	13
D002	female	9
D004	male	15
D005	male	13
D006	female	12
D007	female	6
D008	male	25
D009	female	11
D010	male	19
D012	female	13
D013	female	18
D014	female	25
D016	female	8
D017	female	10
D018	male	6
D019	female	14
D020	male	7
D021	female	15
D022	male	16
D023	male	12
D024	male	5

5. Discussion

Previous epidemiological studies revealed that the incidence of benign tumors, leukoplakia and malignancies were 2-3 times higher than by the healthy control group. Accordingly, in this present study our aim was to identify several OSCC-regulated salivary proteins as potential biomarkers.

Biomarkers are important tools for cancer detection and monitoring. By understanding their role in cancer development, would improve the possibility of early detection of OSCC. The analysis of human saliva has become one of the most promising approaches. Saliva as a diagnostic fluid is widely used in the diagnostics of HIV, various forms of cancer, arthritis and much more.

The main advantage of saliva is that the sample collection is non-invasive, safe and inexpensive. A major drawback to using saliva as a diagnostic fluid has been notion that informative analytes are present with lower amounts than in the serum. But over the past few years the development of proteomic technologies has made it possible to use saliva for detecting cancer markers.

The great advantage of proteomics lies in the fact, that it is able to screen rapidly global and specific changes in gene expression that occur in the cancer cells. The mass-spectrometry driven analysis is potentially important tool for the rapid identification of specific biomarkers and proteomic patterns in the proteomes of body fluids. In this study we discovered specific OSCC-related biomarkers such as Peroxiredoxin-2, Annexin A8, Tyrosine kinase almost in all diabetic samples. An increased level of several common glucopeptides was also detected.

In conclusion, this study has shown that an elevated level of Annexin A8, Peroxiredoxin-2 may seem to be correlated with the premalignant state type-2 diabetes. Their role in the possible development of OSCC should not be underestimated.

Our further plan is to investigate on these proteins, on larger population. But specific analyses and confirmations are required to validate their exact role in the pathogenesis of oral squamous cell carcinoma.

6. Novel findings

1. In our study we could identify 3 salivary biomarkers in diabetic patients which are related and previously described in oral squamous cell carcinoma. Their exact role in the development of the disease needs further analysis, but according to our datas these biomarkers should be taken as predictible biomarkers of OSCC.

2. Our study revealed that peroxiredoxin 2 occurs more often in patients suffering from type-2 diabetes for over 10 or more years.

3. Our methods and investigation protocol is standardized and very good reprocible which could serve as a basis for the further investigations.

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I would like to give my special thanks to my family whose patient love enabled me to complete this work.

8. List of publications

8.1. This work is based on the following articles

1.**Jancsik VA**, Gelencser G, Maasz G, Schmidt J, Molnár GA, Wittmann I, Olasz L, Mark L:

Salivary proteomic analysis of diabetic patients for possible oral squamous cell carcinoma biomarkers

Pathology and Oncology Research, Vol. 19, Issue 4, Oct. 2013. DOI: 10.1007/s12253-013-9736-8, IF: 1,555

2.**Jancsik VA,** Márk L, Molnár GA, Wittmann I, Olasz L:

Nyálból izolált szájüregi laphámkarcinóma biomarkerek vizsgálata 2-es típusú diabéteszes betegekben

Fogorvosi Szemle, 106. évf. 3. sz. 2013.

3. **Jancsik VA,** Márk L, Molnár GA, Wittmann I, Olasz L:

A szájüregi rák megelőzésének lehetőségei, új módszerek a diagnosztikában

Magyar Epidemiológia, 2013.

8.2. List of abstracts

1.**Jancsik VÁ**, Márk L, Wittmann I, Olasz L:

Application Proteomic methods in early cancer-related biomarkers in diabetic patients

J Dent Res 93(Spec Iss Abstract Book): a.id:182583, 2013. IF:3.826

2.**Jancsik VA,** Molnár GA, Wittmann I, Olasz L, Mark L:

Is Type 2 Diabetes a Risk Factor for Oral Squamous Cell Carcinoma? Analyzing Alterations of Salivary Biomarkers

73rd Scientific Sessions Abstract Book, the July 2013 supplement to the Journal Diabetes®a.id:2718-PO, IF:8.3

Impact factor.: 1,555

Total impact factor: 16,681