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RESEARCH

Salivary Proteomic Analysis of Diabetic Patients for Possible Oral Squamous Cell Carcinoma Biomarkers

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Abstract Since oral squamous cell carcinoma (OSCC) is one of the most important causes of death worldwide, the prevention and early detection plays a crucial role. Recent epidemiological studies have incriminated diabetes as a risk factor for the development of OSCC, as well as oral premalignant lesions. As for the last 20 years diabetes and oral squamous cell carcinoma rates have been increasing rapidly, therefore a reliable detection method of major saliva proteins as possible biomarkers for OSCC is of key priority. In this study we collected whole saliva samples from patients with diabetes and from healthy subjects. To reduce the risk of failure and to keep the investigation good reproducible, we proposed an examination and saliva collecting technique. The proteins were analyzed using SDS-PAGE and MALDI TOF/TOF mass spectrometry. Our findings show that the expression of Annexin A8, Peroxiredoxin-2 and Tyrosine kinase is elevated by patients having diabetes. All these proteins have been previously described in cancer saliva samples also in OSCC. Our current findings showed that testing saliva may be an effective and reliable method for detecting oral cancer in early stages.

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Introduction

Clinical proteomics is a relatively young discipline, but it has developed considerably in the last 10 years. Because it's easy and non-invasive collection in large quantities-saliva seems to be an ideal diagnostic target [1]. 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 [2–4].

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 [5]. 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 [6]. 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 [7].

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 [8–11]. Inflammation as a mediator of carcinogenesis is a well-known fact [12]. Recently conducted epidemiological studies showed an increased incidence of oral premalignancies and lichen planus among diabetic patients in comparison with healthy subjects [13, 14].

The purpose of this study was to compare the proteomic profile of diabetic and healthy volunteers, focusing on OSCC-referring saliva biomarkers. This paper presents our preliminary 12-month results.

Material and Methods

For this study 45 volunteers were selected and divided in into two 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 Selection

Patient inclusion was based on the following criteria: the age of the volunteers should have been between 25 and 70 years. A good oral hygiene and general good health was also an important criterion for the participation. Patient exclusion criteria have been summarized in Table 1.

Between 4th January and 30th November 2012, the screenings were carried out on 25 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, ranging from 25 to 70 years. Table 2 shows the detailed parameters of the diabetic participants. The control group comprised 20 adults (10 female, 10 male), their mean age was: 61.2 years.

Table 1 Parameters for patient exclusion

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 2 Parameters of the diabetic patients

Data	*n* %
HbA1c level	
<7 %	10 (41 %)
> 7 %	15 (59 %)
DM2 diagnozed since	
<10 years	8 (32 %)
> 10 years	17 (68 %)
Medication	
Insulin	24 (96 %)

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, and precanceroses were also recorded. This was followed by mouth rinsing with tap water [15]. 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 2,500 rpm for 12 min at 4 °C. The supernatants of the saliva samples were stored frozen at −80 °C until further analysis.

MALDI TOF/TOF Mass Spectrometry-Based Protein Identification

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 min and clarified by centrifugation (8,000×g for 2 min). SDS-PAGE was carried out on 12 % gel, using the Laemmli's method [16]. 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.

We excised the bands of interest with a scalpel blade, placed them in Eppendorf tubes and destained them by washing three times for 10 min in 200 μl of 50 % (v/v) acetonitrile, 50 mM NH_4HCO_3 solution. Proteins were 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 overninght. The spots were crushed and the

peptides were extracted for 15 min 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 800–5,000 m/z. Each spectrum was produced by accumulating date from 1,000 consecutive laser shots. 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.

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 showed significant differences. 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. There were also obvious differences in the amount of oral squamous cell carcinoma biomarkers. Increased levels of Annexin A8, Tyrosine kinase, Peroxiredoxin-2 could be seen. While the healthy samples showed no sign of the previously detected biomarkers. We present our results in Tables 3 and 4.

Discussion

Inflammatory processes have a well known carcinogenetic role. It is proven that patients suffering from inflammatory diseases in the gastrointestinal tract (ulcerative colitis, Crohn disease) have a higher risk for gastrointestinal cancer. Considering this fact, the occurrence of the type-2 diabetes correlated inflammatory processes; diabetic patients are possible targets of carcinogenesis [17].

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, and Tyrosine kinase almost in all diabetic samples. An increased level of several common glucopeptides was also detected.

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

Table 3 Up-regulated salivary proteins in diabetic patients

No.	Name	Accession number	Matched peptides	Theoretical MW (kDa)	Sequence coverage %
1	Annexin A8-like 2	gi|55666310	11	36.84	47.63
2	Annexin A8-like 1	Q5T2P8_HUMAN	12	36.86	32.72
3	Tyrosine kinase	gi|473882	4	7.36	46.88
4	AX969656 NID	CAF14764	4	14.82	26.61
5	Protein kinase	gi|9886711	4	86.35	31.59
6	Peroxiredoxin-2	gi|2507169	4	21.7	64.00
7	Annexin A2	gi|113950	8	38.44	30.00

formation and completion of the terminal phase of cytokinesis. According to this Annexin A11 and A8 is involved in cell growth [18] and a reduction in annexin A11 expression using RNAi stops cell division [19].

Annexins have been implicated in several disease processes, including inflammation and neoplasia [20]. Alterations in the expression of individual annexins have been associated with tumorigenesis in several types of tumor. Loss of Annexin A1 has been found to be an early event in oesophageal squamous cell carcinoma [21]. 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.

Table 4 Comparing the appearance of specific biomarkers in diabetic patients and healthy participants (1-Annexin A8, 2-Peroxiredoxin 2, 3-Tyrosine kinase)

No. diabetic	Biomarker	Nr. healthy	Biomarker
D001	1, 2, 3	H001	negative
D002	1, 2, 3	H002	negative
D003	1, 3	H003	negative
D004	1, 2, 3	H004	negative
D005	1, 2	H005	negative
D006	1, 2, 3	H006	negative
D007	1, 2, 3	H007	negative
D008	1, 2, 3	H008	negative
D009	1, 2	H009	negative
D010	1, 2, 3	H010	negative
D011	1, 3	H011	negative
D012	1, 2, 3	H012	negative
D013	1, 2, 3	H013	negative
D014	1, 2, 3	H014	negative
D015	1, 3	H015	negative
D016	1, 2, 3	H016	negative
D017	1, 2	H017	negative
D018	1, 2	H018	negative
D019	1, 2, 3	H019	negative
D020	1, 2, 3	H020	negative
D021	1, 2, 3		
D022	1, 2, 3		
D023	1, 2		
D024	1, 2, 3		
D025	1, 3		

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 [22]. The peroxiredoxin family was reported to be closely related to various causes of liver fibrosis, and also of oral squamous cell carcinoma [23]. 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.

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 [24, 25]. Their exact role in the carcinogenesis and in the development of OSCC needs to be analyzed in more detail in our future investigations.

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. Specific analyses and confirmations are required to validate their exact role in the pathogenesis of oral squamous cell carcinoma.

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