High throughput diagnostic method to early detection of oral cancer

PhD thesises

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1. Introduction

The incidency and mortality data of orofacial malignant cancer in Hungary show continously increasing tendency, these facts drive researchers and clinitians in this field to do they task urgently. These serious diseases are caused by hevy smoking, bad oral hygiene and a huge alcohol consumption, mainly the summation of these effects.

The aim of this PhD work is necessary to find a method which is simple, low cost, and available for early diagnosis of the cancers.

1.1. Ethiology

Wordwidwe 500 000 new patients are registered in statistical data of epithelium originated oral cancers. In the list of the first 6 most common group of mortality the second is the cancer mortality directly behind the cardiovascular lethality. The frequency of cancer death increased permanently, between 1965 and 1990 the worldwide data show 3 times increasing of cancer mortality.

Among the different regions of human body the oral malignomas - next to stomach, lung, breast, colon and cervix carcinomas – are in the 6th place in the frequency list of developed countries , and the 3rd place in the 3rd world. Oral cancers are 1-5% of all carcinomas in Europe, 4% in the USA, and 5 % in Hungary, it means 2500 death cases yearly.

New publicated european epidemiological data show that the morbidity and mortality of oral cancer of the both gender is the highest in Hungary among the measured 38 european countries. The death rate and the number of newly diagnosed patients are higher than any other East-European countries.

1.2. The importance of early diagnosis

The main reason of this high mortality of oral cancers, that patients are detected, diagnosed and treated in late stadium of the cancer development, but it is a fact, that the chance of the 5-years-survive is relatively high, if the cancer is localized, histologically carcinoma in situ. After the National Cancer Institut of USA statistics the ratio of 5-years-survive is 82% in cases of localized tumors, 45% in cases of regional methastasises and 21% in cases of distant methasatasises.

Because these facts mentioned above are well known, the early detected tumors are mainly curable, the ratio of survive is higly controllable, we have a question, how we can change the nowadays sensed bad situation?

The 5-10% of hungrian population never, the 50 % of people have visited the dentist in case of serious tooth ache. Though the early diagnosis of the oral and maxillo-facial tumors are not available, the half or two-third of the cancer cases are detected to late, in incurable stadium. The dental education and the oral hygienic culture of hungarian population is very low, and the people in highest cancer risk have not been examined by dentist for a long years ago. The main reason of extremly high mortality of oro-pharyngeal cancers in Hungary is the late diagnosis. The reasons of the late dagnosis are the followes:

- a lack of highly educated medical personell
- a lack of perfect diagnosis of precancerous lesion
- the rapid progression of tumors
- the non informed risk patients
- the lack of regular screening

1.3. The importance of ethiologic factors

1.3.1. Smoking

We have a worldwide known scientific data of the correlation between cancer diseases (lung, oral region) and smoking, but all researches show the conclusion that all the forms of tobacco consumption is the basic of tumor development in any localisation..

1.3.2. Alcoholic drink consumption

In all cases very important the quality and quantity of drinks: tipical costume in Hungary the high concentrated alcholic beverages and beer consumption.The data of one-time-consumpted amount is 0.47 dl/male and 0.25 dl/female.

We can see a correlation of geographic localisation between the mortality of liver fibrotic chirrhosis and oral tumor death cases. May be the ethiologic factors are the same int he both diseases.

1.3.3. Bad oral hygiene

The bad oral hygiene is an irritative agent itself, (caries teeth, radices, old-fashioned dentures, calculus or plaque accumulation). After some new scientific research we can see, that plaque bacteria are able to create acetaldehide which is an important chemical carcinogen agent.

1.3.4. Viruses

There is a significant correlation between the presence of HPV viruses or antibodies and the development of oral tumors.More virus infection and HIV positivity increases the development of oral tumors.

1.3.5. Malnutrition

1.3.6. Immunosupressed condition

Sometimes caused by medication side effect.

1.3.7. Fungal infections

Some Candida species are responsible of the development of oral tumors.

1.4. The opportunities of prevention

1.4.1. Primary prevention: to eliminate the risk factors

According to the literature the effect of smoking and alcohlic drinks not only summated, but also cumulated, their combinated impairment completely destroy the oral tissues.

1.4.2. Secondary prevention: early diagnose and treatment

The best tool of secondary prevention is the annual stomato-oncologic screening. The following methods are suggested:

1. total population
2. patients of clinics and hospitals
3. workplaces
4. risk patients
5. connected to other screenings
6. individual

The analytical laboratory methods, which are available for screening are absolute objective, standardizable and relatively low cost.

The proteomic measurement has benefits as followes:

1. Non invasive
2. Exact measurement because the method is simple
3. The costs of peptid analysis are relatively low, the cost/benefit ratio is good

2. Theoretical background

2.1. The steps of cell cycle

The cell cycle is a period from the end of replication till the end of the next replication.

2.2. The regulation of cell cycle

The main regulatory agents are protein complexes which contain two subcomplements: one catalytic and one regulatory part. The catalytic part is a so-called protein-kinases, the regulatory parts are the cyclines binding to each other, and creating a new molecula, the cyclin-dependent-kinases (CDK). It is shown that the level of cyclines and CDK moleculas are higher in some human cancers.

2.3. The odds between progenitor and matured cells

2.3.1. Matured cell:

Mellow cell, the biologic procedures are balanced. The protein synthesis according to the location of the cell cycle is typical, measurable and should be prognosthised.

2.3.2. P**rogenitor cell:**

The progenitor cells are able to do a specific differenciation, their task is the „predisponated" targeted differentiation. The capacity of duplication is limited. The progenitor cells are the so-called adult stem cells, their main functions are in the reparation and healing mechanisms.

2.3.3.Tumor cell:

The cycle of these cells are shortened remarkable, become irregulative and stops the typical order of transcription-translation. They synthetise substantially less proteins, and the properties of the produced peptides are different. The common property of all tumor cells , that during the irregular duplications they product more-or-less typical proteins.

2.3.4. Tumor markers

This sign moleculas show the activity of malignant (non differenciated and no controll replicated) cells. These moleculas are mainly proteins, which are produced in different position of cell cycle, and detectable from any body fluids.

2.4. The saliva, as a diagniostic material

The saliva is one of the most promising examination fluid, because the collection is non-invasive. The collection is not standardized, the most scientific research uses the common 4-5 methods written int he scientific papers.

The analitical examinations of proteins, peptides and any other pathologic biomarkers of saliva is promising.

2.4.1. The composition of saliva

The saliva contains 99,3% water, and 0,7% dry material (mainly proteins and anorganic salts). The pH is between 6,4 and 7,0. Other components are fats, hormones, growing factors, bodystranger materialstes (medicaments, viruses). The composition changes dinamically.

3. The aims of this study

1. To create and standardize a simple, fast easy-to-do saliva collection method which can shorten and cheapen the examination.

2**.** To identify a marker protein from hysologically different tumor samples using the mass spectrometry method.

3. To create a simple proteomical method.

4. The validation of this previous results in a greater tumor patient's and heathy patient's population. We made this measuring with a simple and fast protemic method called mass spectometry after tryptic digestion for high throughput screening.

4. Materials and methods

1. The patients

We started the examinations in 25 persons with clinically oral cancer and 20 non cancer persons int he same age/sex relation, and in good oral condition, as volunteers.

According to the hystopathological diagnosis following the operation three patients removed, because the clinically seems tumor didn't show malignant signs.

In the oral cancer group we had 14/8 male/female, main age was 61,6 years (46-77 years). Average age of males: 59,14 years, females: 66 years.

According to the hystological results we had: cc.planocellulare: 14 males and 6 females, epitelial metaplasia: 1 female, mucoepidermoid cc: 1 female .

The non-cancer patients were: 12/8 male/female, the age was 58,1 years(46-74 years).

4.2 Sample collection

We collected 1-1.5 ml saliva to a disposable syringe using the tactile method. The samples were taken to an Eppendorf tube, and were frozen to -80 ^{o}C urgently, than were stored until the laboratory process. The time of collection was measured.

The controll patients were asked to give a sample according to the tactile method, and with two different methods too. The time was also measured.

4.3. The methods of the laboratory process

4.3.1. Electrophoresis

.Polyacrilamid gel was used. The proteins were denaturated with sodium-dodecil-sulphate (SDS) , so they will be separable according to their mass. After visualisation (staining, autoradiography) the proteins will show a spot int he picture of gel.

4.3.2. Mass spectrometry

The mass spectrometry (MS) is an effective analitical method based on the measure of mass/charge ratio of organic and anorganic ions. The basic of the measure, that the machine creates ions of the examined material, than separates the ions according to the ratio of mass and charge. The given data are analised with computer databases.

4.3.2.1. The parts and function of MS machine

The parts: sample-preparation-lifting system, source of ions, analisator and detector, combined with the data analising and controlling system.

4.3.2.2. Sample preparation

Any kind of materials are monitorable using the MS, but it is limited because of the aggregate, than the ionosation technique. Volatile compounds (gaseous moleculas) could be driven to the space of ionisation, solide or liquide compounds must be transformed to gas phase.

4.3.2.3. Sources of ions

We need the ionisation of detectable particles. To choose the best ionisation technique is determinated according to the measurable molecula on surrounding matrix. No universal technique of ionisation, though the speed and severity of change the ion sources is deteminative in the quality of the machine.

4.3.2.4. Analisator

The analisator separates the ions according to their mass/charge ratio. As the smaller the mass and as the bigger the charge of the ion, as higher speed could be fastened in the flying tube.

We can do qualitative analisis, the machine is available to do the high throughput measuring. Daily 6500 measuring is possible in automatised way.

4.3.3. The separation of proteines

SDS gel electrophoresis

4.3.4. Tryptic digestion and MALDI TOF/TOF MS

After the electrophoretic procedure the extra bands were seen in the samples of the tumor patients, we sectioned. Every columns were sectioned in the 10mm, 22,5mm and 36,5mm height. The band were destained, an incubated in trypsine solution and after some laboratory procedure were dropped to the target plate. To MS analisis we used Autoflex II TOF/TOF type machine. To make the MALDI TOF PMF we used a LIFT mode for PSD (post source decay) and CID (collision-induced decay) fragmentation in automatised way with controlling of FlexControl 2.4 software. We detected the spectra between m/z 800 and 5000.

The PMF of proteins were detected with MSDB and NCBInr databases, and MASCOT and Bruker BioTools 3.0 software. With MALDI-TOF/TOF MS we identified the proteins, which were detectable in case of tumor manifestation, or which would have diagnostic benefits. During the identiication at first we made a primary mass spectra, following this procedure we dissociated towards the diagnostic important or high intensity peptides. we could determinate the perfect primary structure and variations of peptides.

4.3.5. The valuation of spectra

The mass and the apportionment of the peptides are detectable on the curves. With the comparison of these spectra and the database of the used software we could identify the proteins of the saliva samples.

4.3.6. Targeted peptide analisis

We diluted 50 µl of saliva samples in 100 µl, 50 mM concentrated NH_4HCO_3 solution, than we made the trypsine digestion, and MALDI TOF/TOF MS analisis according to written above.

5. Results

5.1. The simplifying of collection

We presented, that our sample collection needs shorter time and less equipment:

	Stimulation	**Wax method**	**Tactile method**
Average time	6 min	7 min	1 min
Equipment which is necessary	toothbrush, toothpaste, mug, sterile container for collection, Eppendorf tube	toothbrush, toothpaste, dental wax, mug, sterile container for collection, Eppendorf tube	Mug, disposable syringe, Eppendorf tube

5.2. The examination of representative samples

According to the hystological groups we made ELFO and staining of proteins of 4 tumor and 5 healthy patient's samples. There was a difference of pattern between healthy and tumor patients. We cut this bands using simple blades, and made the mass spectrometry. We found the proteines (Annexin 1, Peroxiredoxin-2) which were seen in all tumor samples, and were absent from all non-tumor samples.

5.2.1. The appointed tumor marker proteins

After identification we collected the results of tumor and controll samples in a chart. This chart is the evidence, that there were two proteins, which were seen in all tumor samples, and were absent from all non-tumor samples. The both proteins have operative role in tumorgenesis and tumor progression.

name	***Control patientsl***	***Tumorpatients***
Annexin 1 xxx	0/5	4/4
Peroxiredoxin-2 xxx	0/5	4/4

5.2.2. The identified proteins

5.2. 3.1. Annexin group

5.2.3.2. Cornulin group

5.2.3.3. Peroxiredoxin group

5.2.3.4. A thioredoxin system

5.3. Triptic digestion of native saliva

We diluted the 50 uL of saliva with NH_4HCO_3 solution, than digested.

5.4. The examination of the total sample collection with targeted peptide analisis

The third aim of this study was a targeted analisis of the choosen peptides in all samples. We asked that is it possible to detect the peptide sequence from native samples, only following a simple, easy-to-automatise triptic digestion using MALDI-TOF. We focused to the tipical peptide fragments of the two previously detected proteins. We found the peptides in all tumor samples, and the occurence of the same peptides in controll samples were under the sensitivity of detection.

6. Discussion

7. The summary of the results

1. We elaborated a simple, low cost collection method, and demonstrated that this method is available to collect the saliva samples of numerous patients.
2. We identified two tipical oral cancer proteins from tumor patients samples using the mass spectometry.
3. We elaborated and used at first a direct triptic digestion method in native saliva examination.
4. To compare the results of the native and preparated samples, we presented the same proteins.
5. We validated the representative results in numerous oral cancer and healthy population. On behalf of the high throughput screening this measuring was made with a simplified and shorten proteomic method, and the spectra were analisad in blind method. According to our examinations this automatisable method added valuable results and these outcomes project the possibilities of simple and low cost screening method..