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Grado en Odontología

SALIVA BIOMARKERS TO DETECT ORAL CANCER

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Abstract

Objectives: Oral cancer is one of the 6th most common human cancers worldwide with significant morbidity and mortality. Recent discovery regarding saliva biomarkers stressed that they play an important role in cancer detection and management. The main objective here was to determine among all saliva biomarkers which ones could be used as a predictor for early diagnosis of oral cancer. The secondary objectives are to explain by which process a biomarker needs to go by to be considered as such and their possible routine application nowadays.

Methodology: to carry out this work, a systematic review was achieved based on English articles encountered in PubMed, Research Gate and Science Direct using keywords and inclusion/exclusion criteria to sharpen the search.

Discussion of results: some biomarkers stood out from the crowd because of their specificity and sensibility to a certain oral malignant lesion as early diagnosis, but they are still few compared to the number of biomarkers able to detect oral cancer at later stages leading to a lower survival rate. Throughout my researches, I found a few biomarkers eligible for early diagnosis which is: Cytokines, Transferrin, L-leucine, L-phenylalanine, Choline, Betaine, Pipecoline acid, L-carnitine and finally Loss of heterozygosity according to the studies in comparison with countless biomarkers present in our saliva. It became a new priority to understand the delay of detection of these cancers and try to solve it by informing people, to carry out this project further screening projects must be implemented.

Conclusion: The field of early diagnosis is still vague, indeed, the assays regarding the validity of a certain biomarker to be used as an early marker of asymptomatic cancer still need some

training on more large samples, the authors still speak about these biomarkers as a probability of detecting early lesion and it is still difficult to implement them daily.

Resumen

Objetivos: El cáncer oral es uno de los sextos cánceres humanos más comunes en todo el mundo con una morbilidad y mortalidad significativas. Un descubrimiento reciente con respecto a los biomarcadores de la saliva enfatizó que desempeñan un papel importante en la detección y el tratamiento del cáncer. El objetivo principal aquí fue determinar entre todos los biomarcadores de la saliva cuáles podrían usarse como predictores para el diagnóstico precoz del cáncer oral. Los objetivos secundarios son explicar por qué proceso debe pasar un biomarcador para ser considerado como tal y su posible aplicación rutinaria en la actualidad.

Metodología: para la realización de este trabajo se realizó una revisión sistemática a partir de artículos en inglés encontrados en PubMed, Research Gate y Science Direct utilizando palabras clave y criterios de inclusión / exclusión para agudizar la búsqueda.

Discusión de los resultados: algunos biomarcadores se destacaron entre la multitud por su especificidad y sensibilidad a una determinada lesión maligna oral como diagnóstico temprano, pero aún son pocos en comparación con la cantidad de biomarcadores capaces de detectar el cáncer oral en etapas posteriores que conducen a una menor tasa de supervivencia. A lo largo de mis investigaciones, encontré solo 5 biomarcadores elegibles para el diagnóstico temprano según los estudios en comparación con innumerables biomarcadores presentes en nuestra saliva. Se convirtió en una nueva prioridad comprender el retraso en la detección de estos cánceres e intentar solucionarlo informando a las personas, para llevar a cabo este proyecto se deben implementar más proyectos de cribado.

Conclusión: el campo del diagnóstico precoz todavía es vago, de hecho, los ensayos sobre la validez de un determinado biomarcador para ser utilizado como marcador temprano de un

cáncer asintomático todavía necesitan algo de formación en muestras más grandes, los autores todavía hablan de estos biomarcadores como un probabilidad de detectar una lesión precoz, pero aún es difícil implementarlos a diario.

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1. Introduction

Oral cancer is the sixth most common human malignancy, it is nowadays a major global public health problem. Its 5 years survival rate has even remained lower in contrast to the 5 years survival rate for other body's cancers like breast and prostate cancer. Oral cancers can be classified as *precancerous lesions* such as Leukoplakia (85%), Erythroleukoplakia, Lichen Planus and as a *cancerous lesion* that can arise from precancerous lesion like Oral Squamous Cell Carcinoma (OSCC). (1)

OSCC represents 90% of all oral cancers with high morbidity and mortality rates due primarily to the delay of diagnosis because this cancer is usually first diagnosed when becoming symptomatic, unfortunately, when the patient starts presenting symptoms it means that the cancer is in late-stage with probable regional metastasis. A person dies from oral cancer every hour. (2)

The current tools for early detection, screening, prognosis and evaluation of the severity of oral cancers are insufficient. For now, the gold standard procedure is biopsy but it is not for early detection because this technique is too invasive, not comfortable, expensive, and need specially trained medical personal and equipment. As consequences of looking into non-invasive methods for early diagnosis of cancers, saliva seems to be the diagnostic's tool of choice because of its direct contact with oral cancer lesions. (3)

The saliva is 99% water including various components like DNA, RNA, proteins metabolites, and microbiota which can be used for the diagnosis purposes that we aim for. It is one of the most significant body fluids and is called the "mirror of the body", one of its greatest advantages is that unlike current methods, its collection is non-invasive, easy to be stored,

inexpensive, and comfortable for the patient. This medium can be useful regarding the research of specific biomarkers for all kind of cancers and can be a valid method to be employed to screen a larger population. (4)

1.1. Oral cancer

Alcohol and tobacco are the main etiological factors in the development of oral cancer, especially tobacco smoking that has a 75% association, studies have shown that oral cancer usually occurs after the fifth decade of life and is more common in men than women. (1)
Oral Squamous Cell Carcinoma represents 90% of all oral cancer and can be associated with premalignant lesions which are Leukoplakia, Oral Lichen Planus and Erythroplakia and so the OSCC develops in common sites as the tongue, the lips or the floor of the mouth. It appears first unnoticed because painless in the early stages but as developing, the patient may feel a burning sensation. In the oral cavity, this cancer presents itself as an ulcerative exophytic lesion, a lump red or a mixed lesion shown in figures 1,2 and 3 below. (5)

Fig. (1). OSCC of the vestibule with raised exophytic margins.

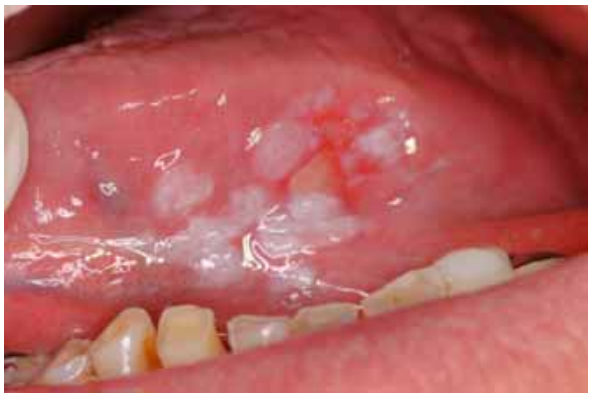

Fig. (3). Leukoplakia of the lateral surface of the tongue undergoing malignant transformation.

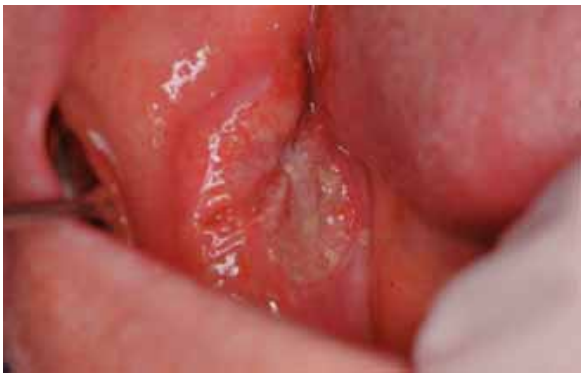

Fig. (2). OSCC of the buccal mucosa presenting as an asymptomatic ulcer.

Its evolution is staged using the TNM system, as an accurate classification, it is important for treatment selection and outcome prediction. T means “Tumor” for the extent of it, N means “Lymph Node” whether or not they are present and M means “Metastasis” for the absence M_0 or presence M_1 of metastasis. This classification is a universal language for cancer: (6)

Stage I	Stage II	Stage III	Stage IV
$T_1N_1M_0$	$T_2N_0M_0$	$T_1N_0M_0$	$T_3N_1M_0$
		$T_2N_0M_0$	T_4N_0 or N_1M_0
		$T_3N_0M_0$	Any T, N_2, or N_3M_0 Any T, any N, M_1

Table: TNM Staging System For the Oral Cavity (7)

1.2. What are biomarkers

1.2.1. Definition and use

In the medical field, a biomarker might be used for screening, diagnostics, evaluation of response or a treatment’s tolerance. A biomarker might be a molecule (hormone, enzyme, metabolite…), or a cell type, the presence of abnormal concentration of which in the blood,

urine or saliva indicates a particular event or physiological status. The discovery of a biomarker starts from a statistical correlation between the measured amount of the biomarker and the state of a disease. Thanks to the assays of this biomarker in body fluid, it is possible to trigger preventive or therapeutic interventions likely to reduce the pathology. It is very important to determine a threshold value for each biomarker, from which clinical actions will be taken. (8)

The biomarkers could be used for several purposes such as making a differential diagnosis for symptomatic patients, potential indicator to detect the recurrence of cancer as well as determinate the progression of a disease, thanks to these tumor markers we could evaluate the stage of cancer, the volume of the tumor. Moreover, regarding the treatments, these biomarkers could help to determine a direction for immunotherapy and to monitor the response to the therapies that were undertaken. (9)(2)

1.2.2. Biomarkers criteria

To be considered as such, a biomarker needs to fulfil these followings characteristics: (10)

- A major product of oxidative modification directly involved in the expansion of disease.
- Needs to be stable, insensitive to artefactual induction, difficult to lose or no modifiable during storage.
- Representative of the equality between generation and combing of oxidative damages.
- It needs to be tested by a specific, sensible, reproducible and robust analytical assay.
- Exempt of any kind of factors linked to dietary intake that could interfere.

1.2.3. Phases of development

The Early Detection Research Network (EDRN) of the American National Cancer Institute was established in 2000 and one of the first aiming to discover and validate biomarkers to assess cancer and its risks, indeed, their mission is to "implement biomarker research through systematic evidence-based discovery, development and validation for identification of cancer risk". (11)

This structure brings together laboratories, data management center, validation centers, a steering committee and a network consulting team and has not ceased to evolve since its establishment. (11)

According to EDRN recommendations, the development of a biomarker is a **five-step process** consisting of:

1. **Preclinical study:** a first phase of exploratory research patients in a "sick" group (tumor tissue) compared to a "healthy" group (nontumor tissue), consisting of evaluating the level of expression of potential biomarkers in both groups.
2. **Clinical assay and validation:** the second step will consist of collecting a sample in a non-invasive way. At the end of the clinical trial, we should be able to distinguish biomarkers for patients with and without cancer for these to be promising for screening. But, the drawback is that it doesn't imply early detection.
3. **Retrospective longitudinal trial:** should make it possible to validate the capacity of the biomarker to detect the disease before the onset of symptoms, a biomarker is considered good for early diagnosis only if the latter maintains its levels distinct long before the apparition of symptoms.

4. **Prospective screening trial:** the phase allows us to know the characteristics of the tumor detected at the moment of the screening, and also to be able to know the false positive evaluated.
5. **Cancer control trial:** is about the relevance of screening test for cancer over the population regarding the benefit, regarding the fact that sometimes cancers can regress on its own and it was not necessary to be detected earlier, the economical aspect… Is this screening test worse for the reduction of cancer's mortality?

Not all biomarkers will have to pass all the 5 phases; it will depend on the techniques used. (11,12)

1.2.4. EDRN triage system for Biomarkers

The Early detection research Network (EDRN) has set up several mechanisms to prove the usefulness of a biomarker from its discovery to its validation because nowadays many biomarkers have been known but few are useful in the detection of early-stage cancers. These steps are important because they are expensive and should therefore only test the most promising biomarkers.

Therefore, the EDRN has set up a triage system of "go or no-go decision" with some criteria like:

-Does the biomarker clearly distinguish the difference between a healthy person and one with early-stage cancer?

-Is this biomarker better than the ones already found for specific cancer, is it more valuable?

- Can the efficacy of the biomarker be imitated when verified using an independent set?

If the biomarkers cannot check these criteria, then it's a no go decision. (11,13)

Without the EDNR system, the discovery regarding new biomarkers would have been delayed and remain on the side for years more. Let's not forget that this organization has defined standards and approaches through which products can be called biomarkers and then developed and assessed, further researches have been implemented to dig into the field of early detection.

1.2.5. Techniques to identify them

Our purpose here is to determine biomarkers that appear before or with the first signs of disease. This implies having other means of identifying individuals in the early stages of the disease or being able to conduct a prospective study of a population in which a certain number of individuals will subsequently be identified as sick.

- Ultra Performance Liquid Chromatography-Mass Spectrotomy:

This trial aimed to set up a Liquid Chromatography–Electrospray Ionization–Mass Spectrometry (UPLC–ESI–MS) method to establish salivary L-leucine and L-phenylalanine as markers for early diagnosis of OSCC. This method presents some benefits because it is simple, reliable, non-invasive, rigorous and accurate. The study needs only 10 min to be led and is used for large-scale screening. (14)

- Salivary test (RNAProSAL):

Some salivary transcriptome and proteome biomarkers are unstable and so, hard to be preserved, it has been important to find the right medium to store them, therefore, RNAProSAL has taken on the challenge to provide rapid ambient temperature collection and not requiring specialized personnel and equipment. The authors have compared this study to another system: SOP (Standard Operating Procedure) comparing some proteins such as

exRNA IL-8, GAPDH…The results show us that both of these methods reach significant and similar effectiveness regarding collection and processing system to analyze saliva transcriptome and proteome even after 14 days stored at room temperature. Nevertheless, the RNAProSAL shows advantages over the SOP method such as its compatibility with self-collection at room temperature, moreover it doesn't require personnel equipment and it's fast. (15)

- Selected Reaction Monitoring (SRM):

This technique used mass spectrometry to measure precisely peptides in large samples.

The main advantages of this technique are high specificity, sensitivity, precision and multiplexing capabilities that allow it to be a method of choice among others for biomarker's validation. However, some challenges are still curbing the use of SRM because of the laborious development process for each protein so the SRM has been more focused on small numbers, moreover, it is important to identify the right proteotypic peptides set for each protein target.

This assay needs to pass first by validation and optimization that contributes to slow down its expansion. Nowadays, this method presents a certain efficacy for the measurement of saliva biomarkers for oral cancer via an SRM assay. Some biomarkers candidates have been selected using the secretomes of different classes of carcinoma including OSCC and healthy patients. (16,17)

- Two-dimensional Electrophoresis (2-DE):

There are several methods using electrophoresis such as Capillary Electrophoresis, One Dimensional Electrophoresis, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis

(SDS-PAGE), Free-low Electrophoresis to analyze saliva. In order not to explain them all, I chose to speak about a successful method in protein separation in gel: The Two Dimensional Electrophoresis (2-DE). This technique presents a high separation capability, it can be associated with a technique we have talked about: Mass Spectrometry to analyze the resultant collected previously by 2-DE. The Two Dimensional Electrophoresis method is used to evaluate the level of expression of certain gathered proteins and compare them to give information about the oral environment (healthy/not-healthy). As we already talked about, to carry out a good analysis, it is fundamental to have a good collection and storage of the elements to avoid their alteration. Unfortunately, this remains a great challenge when collecting proteins saliva sample because they enter post-translational modifications that are undesirable and provoke difficulty to further visualize them. To try to counter this effect as much as possible, some methods have been put in place such as adding elements to the preparation when stored (trifluoroacetic acid), moreover, storage at very low temperature (4°C) but the results were not very conclusive. Some staining methods have been tested to allow the visualization of certain proteins, such as Silver staining in Two-Dimensional Electrophoresis method for low-intensity protein spots. But once again, this technique used in low intensity is not appropriate to be analyzed further by Mass Spectrometry. To conclude, another technique has shown better skills among 2-DE to compare proteins among samples: Difference Gel Electrophoresis (DIGE) because this method transforms proteins before using electrophoresis by attaching the fluorescent tag. (18)

- Enzyme-Linked ImmunoSorbent Assays (ELISAs):

ELISA is the most commonly used techniques for validation of markers after collection from different fluids. Indeed, it's the tool of choice for the analysis of biomarkers in fluids like plasma and urine, its main advantages are its rapidity to analyze, its reliability…but still has its limitations regarding saliva, the manufacturer suggests that this assay is not designed specifically for this latter. The procedure consists of saliva collection, centrifugation of the sample, freezing and thawing in a hot bath. Then, a human ELISA kit is used following the manufacturer's instructions, the absorbance of the samples is measured using a spectrophotometer and intra and inter-assay variation for saliva samples were carried out for quality control. (3,19)

- Point-of-Care technology (PoCT):

Defined as simple medical care, these techniques are a non-invasive way to evaluate biomarkers in saliva outside the laboratory; they don't need previous pre-processing and screening. These tests are fast to be able to bring the results to the patient as soon as possible, using portable devices as a tester with no pain. (20)

The World Health Organization (WHO) have provided guidelines for these devices: "known as assured criteria which indicated that they had to be affordable, sensitive, specific, user-friendly, rapid and robust, with no complex equipment, and be delivered to end-users efficiently." (20)

To conclude:

As a general observation: the more markers we study, the more reliable and precise the results obtained will be in determining the diagnosis and the pathology to be treated or prevented. This is why techniques that simultaneously study multiple biomarkers (proteomics, quantitative PCR, metabolomics, etc.) are arousing growing interest in the fields of prevention and diagnosis.

1.2.6. Salivaomics: names and use

A lot of candidate biomarkers have been highlighted using Genomic, Transcriptomic, Proteomic and Microbiome approache for diagnosis of oral cancer, the ones presented in the table below are not used for early diagnosis, the latter will be discussed later.

Genomics	**Transcriptomics**	**Proteomics**	**Microbiome**	**Exosomics**
Telomerase	miRNA	Defensin-1	P.Gingivalis	Exosome
P53	mRNA	CD44	C. Albicans	
		MMP-1	S.Mitis	
		Actin & Myosin		
		ADA		
		Cyfra21.1		

- Genomics:

Telomerase: is an enzyme formed from a protein-RNA complex that aims to prevent cell apoptosis by preserving the length of the chromosome by adding a specific structure at their ends: the telomere. It is normally found to be absent in healthy tissues and is known to be induced in the malignant transformation of human oral cancer cells. Indeed, the telomerase prevents those cells to be suppressed which promotes their proliferation and so plays an essential role in the transformation of cancer to a malignant stage. The silver lining is that its activity can be blocked by chemotherapy at an early stage. (21)

p53: located in the chromosome 17p, is activated after the damage of the DNA by promoting cell apoptosis and cell cycle arrest. So its alterations are common genetic changes in case of malignancies. But nowadays it is still difficult for the studies to agreed on the action of p53 for early detection.(9,22)

- Transcriptomics:

microRNA (miRNA): are a category of little non-coding RNA, with a mean of 22 nucleotides. They are qualified as post-transcriptional regulators capable to inhibit gene expression. They are stable, easy to be identified and able to indicate if the patient is healthy or not.

So when there is deregulation, its role helps the development of cancer through deletions, mutations, amplifications…(23)

messengerRNA (mRNA): "Messenger RNA (mRNA) is a single-stranded RNA molecule that is complementary to one of the DNA strands of a gene. The mRNA is an RNA version of the gene that leaves the cell nucleus and moves to the cytoplasm where proteins are made. During

protein synthesis, an organelle called a ribosome moves along the mRNA, reads its base sequence, and uses the genetic code to translate each three-base triplet, or codon, into its corresponding amino acid." (24) Some markers have been identified to be elevated in saliva in patients with OSCC such as IL-8 (interleukin-8), SAT (spermidine/spermine N1-acetyltransferase), IL-1B or OAZ (Ornithine decarboxylase antizyme). (25)

- Proteomics:

Defensin-1: are antimicrobial peptides: widely expressed for immunity and also possess cytotoxic properties. According to studies, their level has been elevated in presence of OSCC, also it has been demonstrated that they show moderate specificity and sensitivity according to prognosis prediction.(2)

CD44: is a receptor expressing at basal epithelium's surface when an invasive OSCC arises, it has been evaluated as tumor marker in saliva for detecting oral cancer by several studies. (26)

MMP-1 (matrix metalloproteinase): along with MMP-3, they are part of the major biomarkers for the detection of OSCC. Some studies comparing healthy and OSCC patients have demonstrated the overexpression of the MMP genes. (26)

Actin and Myosin: are cytoskeletal proteins responsible for cell invasion and motility so have a central role in tumorigenesis. They show to be promising saliva biomarkers candidates with their good specificity and sensitivity to distinguish oral malignant lesions, furthermore, their expression is higher in subject suffering from OSCC compared to pre-malignant ones. (27)

Adenosine Deaminase (ADA): is an enzyme playing an important role in DNA and purine metabolism. If it gets damaged, it can transform into adenosine and deoxyadenosine that are

very toxic for the living cells. According to this study focused on Squamous Cell Carcinoma of the tongue, the levels of ADA are increasing with carcinoma staging so could be an interesting marker for the diagnosis of cancer. (28)

Cytokeratin Fragment 21.1 (Cyfra21.1): are intermediate proteins that produce filaments that provide mechanical support and perform a variety of other functions in epithelial cells. The particular nature of these heterodimers makes it possible to distinguish different epithelial cells, in which they are expressed, and has become important in the classification of cancer cells. A study successfully determined that Cyfra21.1 was a biomarker in human saliva for the diagnosis of oral cancer.(29)

- Microbiome:

This stands for all the bacteria living in our body, it is one of the most diverse and the types depend on the site where they are collected from (ex: saliva). Some of the bacteria have been enlightened by studies for being good biomarkers for oral cancer such as ***Porphyromonas gingivalis*, *Candida Albicans* or *Streptococcus mitis*** (30)

- Exosomics:

Exosomics are classified as extracellular vesicles classified into three subgroups (microvesicles, apoptotic bodies and exosomes). The exosomes have been attractive as biomarkers because of their stability, with a size varying from 30 to 100 μm which is an asset for analysis, they are then isolated by ultracentrifugation for further optimization and make them interesting to study cancer. (31)

2. Objectives

- Main objective:

-In this work, we will review which biomarkers can be a predictable lead for early oral cancer diagnosis.

- Secondary objectives:

-Through this work, we will explain how a saliva product is exploited to be finally used as a biomarker and if some have already actually been used successfully for discovering cancer at their early stages with success.

3. Material and methods

Literature searches were carried out in Pubmed, Research Gate, Science Direct using the keywords: “saliva role”, “biomarkers”, “oral cancer”, “early detection”, “biomarkers for oral cancer”, “tumor markers”.

First, when searching for article about “biomarkers” and “cancer”, I ended up with thousands of articles about it, specifying a bit adding words like “early detection” reduced drastically the number of publications.

I first selected 40 articles within those keywords and then I applied the following criteria:

Inclusion criteria:

-Papers are written in English

-Articles published between 2010-2020*

*There is one article of 2001 that had included in my bibliography because I founded it really relevant, plus, another article from 2020 that I used based its researches on it too.

Exclusion criteria:

-Biomarkers for other cancers than oral

-Articles older than 2010

4. Discussion of the results

We will explore diverse studies that have been carried out to experiment whether or not a certain saliva biomarker can be used to predict a malignant lesion at early stages.

4.1. Cytokines

Cytokines are small proteins that act as signals allowing cells to act at a distance on other cells to regulate their activity and function. They can induce growth, death or differentiation in a cell. They can be pro-inflammatory such as IL-6, IL-8, IL-1b (Interleukins), TNF-a (tumor necrotic factor) and anti-inflammatory such as IL-2, IL-12, IL-4, IL-10 (Interleukins). Some studies determined that one of the pro-inflammatory cytokines: IL-6 could have different roles like inhibiting or in the contrary promoting cell proliferation.

Regarding IL-6 presence in saliva, studies show that its level is higher in oral and malignant lesions than in normal controls. (32)(33)(34)

Lee *et al* carried out a study on the involvement of potential biomarkers in saliva and plasma for early diagnosis of Oral Squamous Cell Carcinoma (OSCC), here we will focus only on the results of the saliva samples. This study brought together 41 patients with OSCC and 24 in the control group and to evaluate 14 biomarkers for OSCC early diagnosis, and conclude that IL6 showed the highest sensitivity among other cytokines biomarkers tested, which didn't show enough sensitivity and specificity for early diagnosis. According to Lee *et al*, saliva biomarkers are more appropriate for early diagnosis of OSCC compared to plasma markers more suited for late stages. (35)

More evidence are required to conclude if cytokines can be used as a marker for early diagnosis, as the size sample in most of the studies already conducted is small. Also as

cytokines are making part of any inflammatory process, not involving, necessary, a neoplastic process, there is not a means to differentiate between these 2 processes, yet.

4.2. Transferrin

A study has been carried out by the department of medical laboratory science and biotechnology, China medical university in Taichung Taiwan, recruiting 41 patients with Oral Squamous Cell Carcinoma and 30 OSCC free control subject. The 41 patients with OSCC were classified according to the stage of cancer: T1, T2, T3 and T4 according to the UICC TNM staging. (36) The techniques used in this study to identify the potential biomarker of early-stage oral cancer is the Two-dimensional Electrophoresis (2-DE) and Mass Spectrometry (MS), then the ones selected were validated by Western blotting and ELISA. (36)

The results of this study showed that transferrin levels are elevated in the saliva of OSCC patients compared to the saliva of control subjects, furthermore, the Area Under the Receiver-Operating characteristics Curves (AUROC) was used to predict the specificity and sensitivity of transferrin-based ELISA for the detection of each stage of oral cancer. This added to the fact that there was a correlation between the increasing size of the tumor and the increase of protein level of **salivary transferrin**, which led to prove the utility of saliva transferrin as early-stage biomarker for oral cancer. (36)

4.3. L-leucine and L-phenylalanine

Wang *et al.* carried out a study using the Ultra Performance Liquid Chromatography-Mass Spectrometry method to determine the utility of **L-leucine and L-phenylalanine** for early diagnosis of oral squamous cell carcinoma. Furthermore, the AUROC (Area Under the

Receiver-Operating characteristics Curves) was used to predict the specificity and sensitivity of both biomarkers. (14)

This study targeted 30 OSCC subjects to collect saliva samples from (5 men and 25 men, 7 in stage I, 6 in stage II, 2 in stage III and 15 in stage IV) with an average age of 62 years all recruited from the West China Hospital of Stomatology West China School of Stomatology, Sichuan University. The exclusive criteria were that the patients must not have received previous chemotherapy and radiotherapy and no history of medication. For comparison, saliva samples were also collected from 60 healthy individuals (35 males and 25 females).(14)

All the saliva samples were classified into three groups such as group 1 healthy individuals, group 2 early stages (T1 and T2) and group 3 late stages (T3 and T4). Both saliva biomarkers were investigated with a non-parametric test, then the AUROC was used to evaluate the early predictive power of L-leucine and L-phenylalanine using Logistic Regression (LR) mode, classifying them conforming to their specificity and sensibility. According to it, L-Leucine appears to have better predictive power as a single biomarker for group 2 early stages (T1 and T2) whereas L-phenylalanine could be used for screening and diagnosis of group 3 late stages (T3 and T4). Following those results, the combination of both biomarkers have been tested for diagnosis of OSCC using LR model and validated by leave-one-out cross-validation, the AUROC gave the final results with more than 90% specificity and sensitivity between the three groups. In comparison with group 1 (healthy), the concentration of L-leucine and L-phenylalanine is lower in groups 2 and 3.

What led to think that the combination of both biomarkers improves the accuracy of OSCC diagnosis in early stages. (14)

4.4. Choline, Betaine, Pipecoline acid and L-carnitine

This study has been carried out by Wang *et al* to investigate the role of four biomarkers in the early detection of Oral Squamous Cell Carcinoma. These four biomarkers are Choline, Betaine, Pipecolinic acid and L-carnitine being part of the metabolomics family. **Choline** is an essential nutrient so an abnormality of its metabolism could be an indicative mark of oncogenesis and tumor progression. **Betaine** also is high quantity can be observed in cancer patients. **Pipecolinic acid** is an intermediate product during the catabolism of lysine. Finally, **L-carnitine** plays an important role in metabolism as it promotes the transport of fatty acid into the mitochondria. (37)

The study panel consisted of 30 OSCC patients in the four stages of cancer classified according to the Tumor Nodes Metastasis (TNM) (6), the patients were recruited from the West China Hospital of Stomatology, West China School of Stomatology, Sichuan University. In comparison, saliva samples were also collected from 30 healthy individuals. The biomarkers were investigated by a non-parametric test that showed significantly different concentration between OSCC and healthy patients, indeed, three of them showed higher concentration in OSCC patients compared to healthy ones: Choline, Betaine and Pipecolinic acid. Whereas L-carnitine, however, lower concentration in OSCC patients. Using AUROC these four biomarkers demonstrated high sensitivity and specificity for OSCC compared to control patients, proving their use for early diagnosis of Oral Squamous Cell Carcinoma. (37)

4.5. messengerRNA (mRNA):

mRNA as biomarkers have been already stated earlier, this time they will be considered more specifically as capable of early diagnosis. Indeed, the study by Young Oh et al, performed at

the Department of Oral and Maxillofacial Surgery of Kyungpook National University Dental Hospital from 2015 to 2017, recruited 34 non-OSCC patients as a control in the first group and 33 OSCC patients in a second group and their saliva was collected and then analysed. Then mRNA levels were compared and ROC (Receiver Operating Characteristics) was used to assess the predictive power of this biomarker. The results showed that the levels of normalized mRNA genes such as CYP27A1 (polypeptide 1) and Sialic Acid Acetyltransferase (SIAE) were lower in the saliva of OSCC patients and their combination showed the favourable result in the ROC analysis. As well, two other mRNA genes (NAB2) and monoamine oxidase B (MAOB) combined showed more predictivity in patients under 60 years old than the others. These results suggest that salivary mRNA could be potential markers for early diagnosis. (38)

4.6. Loss of heterozygosity

Loss Of Heterozygosity (LOH) is a loss of genomic material affecting specifically the single retained copy of a fundamental allele. To assess the loss of chromosomal regions containing tumor suppressor genes, Microsatellite Analysis was used. Studies have shown that LOH on four particular chromosomes such as 3p, 9p, 13q and 17p can be an early predictor to detect malignant transformation for a precancerous lesion. Mao *et al* carried out a study with 37 patients with precancerous lesion such as Leukoplakia collecting multiple samples and analyzing two microsatellite markers on chromosomes 3p14 and 9p21. The results showed that 19 of these patients presented LOH on either or both loci and 8 of them developed later Oral Squamous Cell Carcinoma. This study was supported by Rosin *et al* that analyzed LOH at 19 microsatellite loci on 7 chromosomes in 116 cases, in fact, their study has demonstrated that non progressing and progressing cases have had differences in their profiles of LOH. These

results confirmed that LOH could be a good predictor in early diagnosis of oral malignant lesions..(39)

Every time a study is carried out, it leads to the same answer: nothing is sure for certain, the results are still awaited to determine for certain the use of the biomarker that will be used for early detection. Need to develop better approaches for early diagnosis and better predictability of the tumors.

Moreover, some studies stressed that the combination of two or more biomarkers could increase their efficacity, as we talked about earlier, the example of L-Leucine associated with L-phenylalanine that were more accurate in different stages. Because sometimes one single biomarker is not strong enough to distinguish between case and control groups, what lead to believe that an association between markers increase the discriminatory power to higher performance. (14)

Once again, thanks to the AUROC that determines the predictive power of biomarkers, we could see good specificity and sensitivity when used for combined biomarkers.

4.7. Early diagnosis, the road to cure?

The main problem of oral cancer is that before months or years, their presence clinically can't be detected because of the absence of symptoms and so have metastasized at the time of discovery and therefore less chance of recovery, that is why is it important to have a set of biomarkers to detect in early stages and being able to cure oral cancer before an inevitable scenario happens. The question is, what does mean "early diagnosis"? As a reminder, we said in the introduction that Oral Squamous Cell carcinoma was the deadliest oral cancer because

of its late diagnosis, indeed the OSCC discovered at stage I (T1N0) has an 80% cure rate after 5 years survival rate level, whereas a 20% cure rate discovered at stage IV, as the OSCC is rarely diagnosed asymptomatic, studies showed that 50% of OSCC are diagnosed at advanced stages such as III and IV. (40)

We should deepen on why there is such a delay to diagnose cancer and therefore, enhance its morbidity, this delay of diagnosis is caused both by patient and doctor. The diagnosis delay can be defined as a number of days that passed since the patient noticed the first sign and/or symptoms until a final diagnosis is reached. First of all, the public awareness regarding oral cancer is limited, that leading to ignoring it could exist or fear to be diagnosed with such cancer in people's mouth, which lead to think that people need to be more informed about the existing risk to get cancer (especially heavy smokers/drinkers). Second of all, the variety of symptoms and signs possible due to cancer varies a lot and therefore becomes difficult for a doctor to make a proper (on time) diagnosis, as well as for the population itself, the ability to diagnosis oral cancer by health care people should be improved. The better choice would be instead of performing a biopsy when the cancer is already installed, to perform salivary analysis during screening programs previously to any signs and symptom to further predict a probable appearance of oral cancer and thus avoiding the discomfort of the patient. (40)

5. Conclusion

The saliva contains a wide variety of biomolecules, the aim here was to identify which of these products are currently called biomarkers and explain the steps by which they need to pass to fulfil such an important role. To deepen the subject, it has been interesting to look into which of these already specific biomarkers have been elected to play a role in the early diagnosis of oral cancer, because as it is useful to already know the richness of our saliva, it is a breakthrough to use this knowledge to detect cancer even before it has manifested itself.

A lot of studies have been carried out to explore countless numbers of biomarkers present in our saliva since their use could save lives as it could detect the presence of malignant lesion even before any symptom or manifestation appear. As a reminder, early detection of oral mucosal lesions followed by appropriate treatment can increase the recovery rate to 80-90%. A lot of progress has been made to understand the characteristics of saliva, as it is becoming the tool of choice to detect diseases thanks to its advantages to be non-invasive and carried out easily.

This new lead of detection of oral cancer is very promising, here we focus on the saliva biomarkers like Cytokines, Transferrin, L-leucine, L-phenylalanine, Choline, Betaine, Pipecoline acid, L-carnitine and Loss of heterozygosity for early diagnosis but biomarkers are also present in plasma or blood, though, oral cancer preferential biomarkers are often only present in saliva which makes it an ideal fluid to look into.

The main drawback here is that there is still weak evidence and underdevelopment of identified early biomarkers, it was quite easy to find loads of biomarkers but more difficult to find those which were intended to be used for early detection. The studies carried out so far

have been unfortunately validated on too small samples, they need to be clinically validated on large patients cohort and then implemented on routine tests.

Even though some significant progress has been made throughout the year's thanks to the Early Detection Research Network (EDRN), only a few biomarkers are promising to play a role in early prediction and need nowadays further studies to be warranted and most of all need to be routinely employed in the prediction of disease.

Responsibility:

Despite the many advantages of the discovery of these saliva biomarkers, we can't ignore the economic impact generated. As a matter of fact, all the studies that need to be carried out require laboratories, researchers, material, centers, steering committees, network consulting teams. The process is slow, requires time, bigger samples, money to be able to develop more the investigations and implementing these saliva biomarkers on a daily basis to detect oral cancer at early stages.

The use of the saliva biomarkers to detect oral cancer would be painless for the patient, faster to obtain the results unlike biopsies, more economic because could be done from home (Point-of-Care technology) instead of a laboratory, and the most important, save lives.

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7. Anexes

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CANCER OF THE ORAL CAVITY

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Keywords

oral cavity cancer; oral cancer; squamous cell carcinoma; head and neck cancer

INTRODUCTION

Cancer of the oral cavity is one of the most common malignancies,[1] especially in developing countries, but also in the developed world[2]. Squamous cell carcinoma (SCC) is the most common histology and the main etiological factors are tobacco and alcohol use[3]. Although early diagnosis is relatively easy, presentation with advanced disease is not uncommon. The standard of care is primary surgical resection with or without postoperative adjuvant therapy. Improvements in surgical techniques combined with the routine use of postoperative radiation or chemoradiation therapy have resulted in improved survival statistics over the past decade [4]. Successful treatment of patients with oral cancer is predicated on multidisciplinary treatment strategies to maximize oncologic control and minimize impact of therapy on form and function.

ANATOMY OF THE ORAL CAVITY

The oral cavity extends from the vermilion border of the lips to the circumvallate papillae of the tongue inferiorly and the junction of the hard and soft palate superiorly. The oral cavity is divided into several anatomical subsites: lip, oral tongue, floor of mouth, buccal mucosa, upper and lower gum, retromolar trigone and hard palate (Figure 1). Despite their proximity, these subsites have distinct anatomical characteristics that need to be taken into account in planning oncologic therapy.

EPIDEMIOLOGY AND ETIOLOGY

Worldwide, 405,000 new cases of oral cancer are anticipated each year, and the countries with the highest rates are Sri Lanka, India, Pakistan, Bangladesh, Hungary and France[5]

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Review Article

Salivary biomarkers in oral squamous cell carcinoma – An insight

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ABSTRACT

Oral cancer refers to the malignancies that occur in the oral cavity, lip and pharynx with 90% of oral cancers being squamous cell carcinomas (OSCC). OSCC has the highest mortality ratio compared to other carcinomas. Although oral cavity is easily accessible, most oral cancers are detected at a later stage leading to lower survival rates. Early detection of OSCC is a key factor in improving the prognosis and survival rate of the patient. Rapid advancement in the field of diagnosis has enabled early diagnosis of many potentially malignant conditions even before its clinical manifestations. One such diagnostic modality that has gained much relevance in the field of molecular biology has been the discovery of salivary biomarkers (DNA, RNA and protein markers). These salivary biomarkers have been shown to play a non-invasive role in the diagnosis and surveillance of oral cancer. The direct contact between the saliva and the oral cancer lesions makes it a most sensitive and specific, screening method in diagnosis, staging and follow-up. This review aims to discuss the effectiveness and the potential of salivary biomarkers as a screening tool in OSCC.

1. Introduction

Oral cancer ranks sixth among the cancers occurring worldwide with 90% of them being diagnosed as OSCC. However, the prognosis of OSCC is good with a survival rate of 90% in case of early detection. The gold standard for diagnosis of OSCC is biopsy followed by histopathological examination, the major drawback in this technique is delay in detection. The biomarkers which are the measurable indicators of physiological and pathological process are useful in the diagnosis and influence the prognosis of disease.

Salivary biomarkers have proven to be cost effective adjourns in diagnosis and follow-up of oral and oropharyngeal carcinoma. This review summarizes the current knowledge regarding the classification, criterias, rationale, applications, merits and demerits of salivary biomarkers in relation to oral squamous cell carcinoma.

2. Biomarkers

Biomarkers are molecular signatures that are unique to a certain disease (e.g., oral cancer), and has been defined by 'WHO' as any substance, structure or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease.[1] Biomarkers are also defined as "a characteristic that is an objectively measured and evaluated indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention" (National Institutes of Health, 1998).[2] Biomarkers could be analyzed in different analytes like blood and saliva.

3. Classification of cancer biomarkers

Biomarkers have been classified based on biomolecules and disease states (Table 1).[3]

3.1. Applications of biomarkers[4]

1. Biomarkers help in predicting the preventive measures that could be formulated.
2. Aids in detection of various stages of oral malignant transformation.
3. Evaluates the molecular changes related to oral carcinogenesis.
4. Enhances the prognosis, diagnosis, and treatment of oral carcinomas.
5. Helps in manipulating the drugs used for the treatment of cancer.

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Salivary markers for detection of oral (pre)cancerous lesions

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Review

Saliva: reflection of the body

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ABSTRACT

Saliva has become an important resource for evaluating physiological and pathological conditions in humans. The use of saliva has many advantages, including the simple and non-invasive method of collection and its easy, low-cost storage. With the addition of modern techniques and chemical instrumentation equipment, there has been an increase in its use for laboratory investigations, applicable for basic and clinical analyses in the fields of medicine and dentistry. The value of these methods for the diagnosis of oral and systemic diseases has been the subject of study by several researchers with the aim of increasing its use alongside complementary exams.

1. Introduction

Saliva is an aqueous fluid found in the oral cavity, composed of a complex mixture of secretory products (organic and inorganic products) from the salivary glands and other substances coming from the oropharynx, upper airway, gastrointestinal reflux, gingival sulcus fluid, food deposits, and blood-derived compounds.[1,2]

Saliva is one of the most complex, versatile, and important body fluids, supplying a large range of physiological needs. In the digestive tract, saliva plays an important role in esophageal physiology, the digestive process, and gastric cell protection. In the oral cavity, saliva takes part in mastication, speech, deglutition, gustatory sensitivity, tissue lubrication, mucosal protection against invasion, antibacterial, antifungal, and antiviral activity, post-eruptive maturation, ionic balance regulation at enamel remineralization, deposition of acquired enamel pellicle, and acid diffusion limitation.[3–5]

Water is the greatest component of saliva, representing 99% of its composition. The solid components, organic and inorganic molecules, are found dissolved in the aqueous component and vary widely from one individual to another, and even vary in the same individual several times during the day. The inorganic part is composed of weak and strong ions, with the most important being Na^+, K^+, Cl^-, Ca^{2+}, HCO_3, Mg^{2+}, and NH_3. The organic part contains components such as body secretion products (urea, uric acid and creatinine), putrefaction products (putrescine, cadaverine; lipids such as cholesterol and fatty acids), and more than 400 types of protein. The most relevant proteins have a glandular origin (α-amylase, histatins, cystatins, lactoferrins, lysozymes, mucins, and proline-rich proteins (PRPs)) or are plasma-derived (albumin, secretory immunoglobulin A (sIgA), transferrin).[6]

Salivary analysis has become an important resource for the evaluation of salivary conditions with physiologic and pathologic implications and is a useful tool for disease diagnosis, mainly due to its origin, composition, functions, and interactions with other organ systems. Additionally, it has a simple and non-invasive collection method, is easy to store, and is inexpensive when compared to blood collection. With the addition of modern techniques and chemical instrumentation equipment, there has recently been an observable increase in its use for laboratory investigations, applicable for basic and clinical purposes in dentistry and other medical areas. The value of saliva as a diagnostic tool for oral and systemic diseases has been an area of study for many researchers with the aim of increasing its use as a possible complementary exam.[7–10]

Recently, an increasing appreciation of the use of saliva as a mirror that reflects the normal internal characteristics and disease

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Open Access

Current Aspects on Oral Squamous Cell Carcinoma

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Abstract: Oral squamous cell carcinoma is the most common malignant epithelial neoplasm affecting the oral cavity. This article overviews the essential points of oral squamous cell carcinoma, highlighting its risk and genomic factors, the potential malignant disorders and the therapeutic approaches. It also emphasizes the importance of the early diagnosis.

Keywords: Oral squamous cell carcinoma, overview.

INTRODUCTION

Worldwide, oral cancer accounts for 2%–4% of all cancer cases. In some regions, the prevalence of oral cancer is higher, reaching the 10% of all cancers in Pakistan, and around 45% in India [1,2]. In 2004-2009 over 300,000 new cases of oral and oropharyngeal cancer were diagnosed worldwide. During the same time period, over 7,000 affected individuals died of these cancers [3].

Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms. It is estimated that more of 90% of all oral neoplasms are OSCC [4].

Despite the advances of therapeutic approaches, percentages of morbidity and mortality of OSCC have not improved significantly during the last 30 years. Percentages of morbidity and mortality in males are 6.6/100,000 and 3.1/100,000 respectively, while in females the same percentages are 2.9/100,000 and 1.4/100,000 [5]. Additionally, the incidence of OSCC is increasing among young white individuals age 18 to 44 years, particularly among white women [6]. The percentage of 5-year survival for patients with OSCC varies from 40-50%. Regardless of the easy access of oral cavity for clinical examination, OSCC is usually diagnosed in advanced stages. Most common reasons are the initial wrong diagnosis and the ignorance from the patient or from the attending physician [7].

CLINICAL FEATURES

One of the real dangers of this neoplasm, is that in its early stages, it can go unnoticed. Usually at the initial stages it is painless but may develop a burning sensation or pain when it is advanced. Common sites for OSCC to develop are on the tongue, lips and floor of the mouth. Some OSCCs arise in apparently normal mucosa, but others are preceded by clinically obvious premalignant lesions, especially erythroplakia and leukoplakia. Usually, OSCC presents as an ulcer with fissuring or raised exophytic margins (Fig. **1**). It may also present as a lump (Fig. **2**), as a red lesion (erythroplakia), as a white (Fig. **3**) or mixed white and red lesion, as a non-healing extraction socket or as a cervical lymph node enlargement, characterized by hardness or fixation. OSCC should be considered where any of these features persist for more than two weeks.

Fig. (1). OSCC of the vestibule with raised exophytic margins.

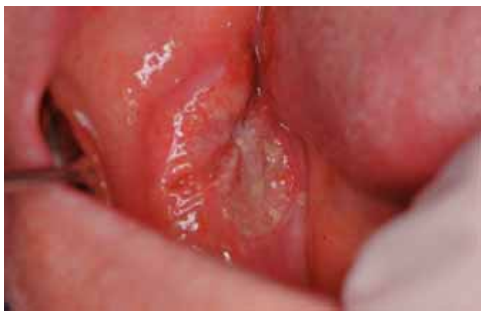

Fig. (2). OSCC of the buccal mucosa presenting as an asymptomatic ulcer.

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Head and Neck Cancer (L Licitra, Section Editor)

Overview of the 8th Edition TNM Classification for Head and Neck Cancer

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Opinion Statement

The main purpose of the TNM system is to provide an anatomic-based classification to adequately depict cancer prognosis. Accurate cancer staging is important for treatment selection and outcome prediction, research design, and cancer control activities. To maintain clinical relevance, periodical updates to TNM are necessary. The recently published 8th edition TNM classification institutes the following changes to the staging of head and neck (excluding thyroid cancer): new stage classifications [HPV-related oropharyngeal cancer (HPV+ OPC) and soft tissue sarcoma of the head and neck (HN-STS)] and modification of T and N categories [T and N categories for nasopharyngeal cancer (NPC), T categories for oral cavity squamous cell carcinomas (OSCC), N categories for non-viral related head and neck cancer and unknown primary (CUP), and T categories for head and neck cutaneous carcinoma]. These changes reflect better understanding tumor biology and clinical behavior (e.g., HPV+ OPC and HN-STS), improved outcomes associated with technical advances in diagnosis and treatment (e.g., NPC), evolving knowledge about additional prognostic factors and risk stratification from research and observation (e.g., inclusion of depth of invasion variable for OSCC, inclusion of extranodal extension variable for all non-viral head and neck cancer, and reintroduction of size criteria for non-Merkel cell cutaneous carcinoma of the head and neck). This review summarizes the changes and potential advantages and limitations/caveats associated with them. Further evidence is needed to evaluate whether these changes would result in improvement in TNM stage performance to better serve the needs for clinical care, research, and cancer control.

2 Oral Cancer

KEY WORDS

Human papillomavirus
Oral cancer
Premalignant lesions
Squamous cell carcinoma
Tobacco consumption

2.1 INTRODUCTION

The oral cavity is a part of the upper aerodigestive tract that begins at the lips and ends at the anterior surface of the faucial arch. Oral cancer is an umbrella term that consists of cancers that originate in the oral tissues. The primary site of origin of oral cancers is submucous tissues, the epithelium, and minor salivary gland. Some of the other common subsites of oral carcinoma are the alveolus, tongue, buccal mucosa, and gingivobuccal sulcus.

Oropharyngeal cancer is responsible for the deaths of thousands of people every day (Menck et al., 1991). It is very surprising that cancers of the oral cavity remain undetected in its nascent stages, despite easy accessibility of the oral cavity. Approximately 40,000 new cases of oral cancer are reported every year in the United States. Oral cancer occurs predominantly in males with a frequency of incidence over two times that of females (Neville et al., 2002; Swango, 1996; Ries et al., 1991). The incidence rate has declined annually by 1.4% in men and 1.1% in women during the past couple of decades. Age was previously regarded as one of the important causal factors; however, it has been increasingly reported among men younger than 50, which is associated with human papillomavirus (HPV) infection (WHO Fact Sheet, 2011).

2.2 EPIDEMIOLOGY

Oral cancer commonly occurs in middle-aged and older individuals, although a disturbing number of these malignancies have also been documented in younger adults in recent years (Chen et al., 1990; Llewellyn et al., 2001; Schantz and Yu, 2002). From an epidemiological and clinicopathological perspective, oral cancer can be divided into three categories: (1) carcinomas of the oral cavity proper, (2) carcinomas of the lip vermilion, and (3) carcinomas arising in the oropharynx. Males were more susceptible for oral cancer than females; however, now relatively more females are contributing toward the global oral cancer population, which can be attributed to lifestyle changes like exposure to carcinogens such as tobacco and alcohol (Silverman, 1998; Chen et al., 1990; Khan and Bisen, 2013).

Review Article

Salivary biomarkers as a diagnostic tool

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Abstract
Saliva, a biological fluid is steadily emerging as a potent diagnostic tool in modern day health and disease. Advances in molecular biology have led to the invent of certain markers for diagnosis of conditions like oral cancer and dental caries to name a few. In addition, saliva based biomarkers are cost– effective, accurate and provide a noninvasive diagnostic approach. Detecting pathologies at earlier stages can significantly increase survival rates and affect treatment prognosis. The present article discusses various salivary biomarkers that can aid in the diagnosis of many such systemic conditions.

Keywords: Saliva, Biomarkers, Systemic Conditions.

Introduction
Saliva is a dilute aqueous solution that contains both inorganic & organic constituents and plays an essential role during mastication, swallowing and speech.

The term biomarker refers to any measurable and quantifiable biological entity than can serve as an indicator for health related assessments.[1] Salivary diagnostics is indeed a dynamic and emerging field that takes into consideration the concept of molecular diagnostics that aids in the diagnosis of several oral and systemic diseases using salivary biomarkers. Saliva is a readily available and easily fetched specimen, which can be collected by non – invasive procedures and contains many hormones and antibodies that have proven to be an inevitable aid in screening and diagnosis. Salivary diagnostics has undoubtedly evolved overtime and serves as a subset of the larger field of molecular diagnostics, now recognized in a wide variety of clinical areas.

Listed below are few systemic conditions and the role of salivary biomarkers in their detection:

Oral Cancer
Oral cancer is one of the globally concerned health problems. Increased incidences of tobacco consumption among young population nowadays are an alarming scenario. Delayed diagnosis is the major causative factor for high morbidity rates in oral squamous cell carcinoma.[2] Studies conducted in the field of molecular biology help in assessment of cancer risk as well as prediction of prognosis.

Tumor Marker: A tumor marker is defined as a substance found in any body fluid that may be an indication of cancer or certain noncancerous conditions.[3] They maybe unique genes or their products which are present only in cancer cells.[4] Tumor markers have been reported to be significantly increased in the saliva of patients diagnosed with oral cancer.[5]

Alterations in Host Cellular DNA: DNA markers originate from dead cells and are detected in the early stages of tumorigenesis. However, tissue specificity of DNA markers is very low.[6] Studies have suggested that premalignant lesions with aneuploidy have a higher transformation rate into malignancy as compared to lesions with normal DNA content.[7] Studies have also demonstrated that loss of heterozygosity in regions that contain a known human suppressor gene is an early predictor of malignant transformation of precancerous lesion.[8] Mitochondrial DNA mutations have proven to be useful for detection of exfoliated oral squamous cell carcinoma [OSCC] cells in saliva.[9]

RNA as a Biomarker: RNA has been found to be an informative marker for the identification of oral cancer. Scientists have earlier compared the clinical accuracy of saliva with that of blood RNA biomarker for oral cancer detection and discovered four RNA biomarkers having a sensitivity and specificity of 91% and 71% respectively.[10]

Protein Markers: Salivary protein markers have shown moderate sensitivity and specificity as prognostic markers.[9] Few studies have indicated that saliva contains specific proteins that may serve as potential biomarkers for OSCC. This can be attributed to the fact that 46 proteins were found at contrasting levels between OSCC and control groups.[11] Metalloproteinases such as MMP-11 and MMP-2 were found to be significantly altered in OSCC.[12] Shpitzer, *et al.* in their study found a 39% increase in MMP-9 with a sensitivity and specificity of 100% & 79% respectively in OSCC patients.[13]

Markopoulos *et al*[9] have summarized various molecular markers useful for early diagnosis of OSCC [Table 1]

Review Article

A Review of Salivary Biomarker: A Tool for Early Oral Cancer Diagnosis

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Abstract

The oral squamous cell carcinoma (OSCC) is one of the most common epithelial malignancies with significant morbidity and mortality. Recent observations indicate that the clinical and histological appearance of oral mucosa may not truly depict the damage occurring at the genetic level. This phenotypic and genotypic disparity may account in part for the failure to establish effective screening and surveillance protocols, based on the traditional clinical and microscopic examination. The tumor markers are playing an increasingly important role in cancer detection and management. These laboratory-based tests are potentially useful in screening for early malignancy, aiding in cancer diagnosis, determining prognosis, surveillance following curative surgery for cancer, up-front predicting drug response or resistance, and monitoring therapy in advanced disease. A systematic review of the literature was performed based on the English titles listed in the PubMed, EBSCO, Cochrane, Science Direct, ISI web Science, and SciELO databases using the keywords. Abstracts and full-text articles were assessed. This article may help to identify the potential biomarkers for screening and the molecular pathology analysis in the high-risk patients with the OSCC.

Keywords: *DNA marker, oral squamous cell carcinoma, protein marker, RNA marker, saliva*

Introduction

The head and neck cancers are one of the most common causes of cancer death worldwide with incidence rate varying in different regions in the Southeast Asia and Africa, the head and neck cancers accounts for approximately 8–10% of all cancers.[1] The primary anatomic sites of the oral squamous cell carcinoma (OSCC) are buccal mucosa, lip, alveolar ridge, retromolar trigone, hard palate, floor of the mouth, the ventral two-thirds of the tongue, and oropharynx.[2]

The key challenge to reduce the mortality and morbidity of this disease is to develop strategies to identify and detect the OSCC when it is at a very early stage, which will enable effective intervention and therapy. Detection of the OSCC is currently based on the expert clinical examination and histological analysis of suspicious areas, but it may be undetectable in hidden sites. Therefore, sensitive and specific biomarkers for OSCC may be helpful in screening high-risk patients. The biomarkers for early cancer detection must meet the following criteria: (a) the altered can be objectively measured; (b) must be measurable in small specimens; (c) must be altered in the high-risk tissues, but not in the normal tissues; and (d) must be altered in the early stages of cancer development. Unlike the other deep cancers, the OSCC occurring in the oral cavity is much easier to be monitored, specimens are easier to be collected for diagnosis, and the treatment is easier to be applied.[3]

Tumor cells inhabit or produce biochemical substances which are referred to as tumor markers. These can be normal endogenous products that are produced at a greater rate in the cancer cells or the products of newly switched on genes that remain quiescent in the normal cells.[4] The tumor markers may be present as intracellular substances in tissues or as released substances in the circulating body fluids such as serum, urine, cerebrospinal fluid, and saliva. Examples of using body fluids for tumor detection include sputum for the lung cancer diagnosis,[5] urine for the urologic tumors,[6] saliva for the OSCC,[7] breast fluid,[8] as well as serum or plasma for almost all types of cancers. With the recent diagnostic technological advances, however, the role of saliva as a tool for diagnosis has advanced exponentially.

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Review

Saliva: A potential media for disease diagnostics and monitoring

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SUMMARY

Within the past 10 years, the use of saliva as a diagnostic tool has gained considerable attention and become a well-accepted method. As a diagnostic fluid, saliva offers superiority over serum due to both a noninvasive collection method by specially trained persons and a cost-effective approach for screening of large populations. Collection of saliva offers a reduced risk of infection compared to the collection of serum. Moreover, obtaining saliva samples from infant, disabled or anxious patients, is much easier than obtaining other samples. There is a lot of useful components-changing information in saliva when a person is in sick. Therefore, we define these changing components as "biomarkers". The utilization of biomarkers as early predictors for clinical disease not only contributes to the effective prevention and treatment of diseases, but also enhances the assessment of potential health risks. In this article, we have reviewed the properties of saliva, the salivary analysis method for biomarker discovery, and the diagnostic potentials of salivary biomarkers in monitoring and detecting periodontal disease, Oral and Breast cancers, and Sjögren's syndrome. We also discussed some barriers of applications of saliva as a diagnostic media as well as recent improvements. We also prospected the future processing directions of using biomarkers in disease diagnosis and draw a conclusion that saliva is indeed an effective media in various disease monitoring and diagnosis.

Introduction

Early detection of disease plays a significant role in successful clinical treatment. In most cases of various diseases, early detection and diagnosis lead to a greater survival rate with a reduced chance of the disease re-emerging. Successful monitoring of a disease, especially in its early stage, may also reduce any severe impacts on a patient's health or help to prevent and/or delay succeeding complications. The ability to evaluate physiological conditions, trace disease progression, and monitor post-treatment therapeutic resulting through a noninvasive method is one of the primary objectives in the field of healthcare research. Saliva, a multi-constituent oral fluid that can be collected through noninvasive means, has considerable potential for the surveillance of general health and disease. Human saliva contains many kinds of proteins and peptides, each of them carries several significant biological functions. With the advancement of novel technological means (such as bioinformatics, metabolomics, genomics and proteomics), saliva, as a clinical tool, has become a more and more attractive option because of its ability to mirror both oral and systemic health conditions.[1] But in order for saliva-based diagnostics to be useful, two prerequisites must be fulfilled: (1) discovering biomarkers for various diseases among the complicated composition of saliva, and (2) evaluating the sensitivity and specificity of biomarkers through a series of continuous developments.[2]

Saliva profile

Water is the most abundant component in saliva, representing 99% of saliva's total composition. The solid components soluble in the aqueous phase differ from person to person, and can even vary in the same individual at distinct times during a day. The inorganic species are mainly composed of weak and strong ions including Na^+, K^+, Cl^-, Ca^{2+}, HPO_3^{2-}, HCO_3^-, Mg^{2+}, and NH_3. The organic species (see Table 1) consist of body secretion products (urea, uric acid and creatinine); putrefaction products (putrescine and cadaverine); lipids (cholesterol and fatty acids), and more than 400 types of protein. Among those proteins, the most relevant ones are glandular in origin (alphaamylase, histatins, cystatins, lactoferrins, lysozymes, mucins, and proline-rich proteins (PRPs)) or are plasma-derivatives (albumin, secretory immunoglobulin A (sIgA), and transferrin).[3]

Human saliva proteome (HSP) analysis is inherently challenging because human saliva contains an inherently large variety of proteins with an equally wide range of concentrations. For example, α-amylase, the most abundant protein in human saliva, is at mg/ml level, whereas cytokines are typically within the range of pg/ml.[4]

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The EARLY DETECTION RESEARCH NETWORK: A National Infrastructure to Support the Discovery, Development and Validation of Cancer Biomarkers

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Statement of Conflicts of Interest

The authors declare no potential conflicts of interest.

COMMENTARY

Phases of Biomarker Development for Early Detection of Cancer

Margaret Sullivan Pepe, Ruth Etzioni, Ziding Feng, John D. Potter, Mary Lou Thompson, Mark Thornquist, Marcy Winget, Yutaka Yasui

1) Introduction

Recent developments in such areas of research as gene-expression microarrays, proteomics, and immunology offer new approaches to cancer screening *(1).* The surge in research to develop cancer-screening biomarkers prompted the establishment of the Early Detection Research Network (EDRN) by the National Cancer Institute *(2).* The purpose of the EDRN is to coordinate research among biomarker-development laboratories, biomarker-validation laboratories, clinical repositories, and population-screening programs. By coordination of research efforts, the hope is to facilitate collaboration and to promote efficiency and rigor in research.

With the goals of the EDRN in mind, the purpose of this commentary is to define a formal structure to guide the process of biomarker development. We categorize the development into five phases that a biomarker needs to pass through to produce a useful population-screening tool. The phases of research are generally ordered according to the strength of evidence that each provides in favor of the biomarker, from weakest to strongest. In addition, the results of earlier phases are generally necessary to design later phases.

Therapeutic drug development has had such a structure in place for some time *(3).* The clinical phases of testing a new cancer drug are as follows: phase 1, determinations of toxicity, pharmacokinetics, and optimal dose levels; phase 2, determinations of biologic efficacy; and phase 3, definitive controlled trials of effects on clinical endpoints. For each phase, guidelines exist for subject selection, outcome measures, relevant comparisons for evaluating study results, and so forth. Although deviations are common, the basic structure facilitates coherent, thorough, and efficient development of new therapies. A phased approach has also been proposed for prevention trials *(4,5).*

In a similar vein, we hope that our proposed guidelines or some related construct will facilitate the development of biomarker-based screening tools for early detection of cancer. Although deviations from these guidelines may be necessary in specific applications, our proposal will, at the minimum, provide a checklist of issues that should be addressed at each phase of development before proceeding to the next.

2) Objectives of Population Screening

The goal of a cancer-screening program is to detect tumors at a stage early enough that treatment is likely to be successful. Moreover, the screening tool must be sufficiently noninvasive and inexpensive to allow widespread applicability. A substance secreted by tumor tissue, not secreted by nontumor tissue, and easily and cheaply detectable in serum or urine is, therefore, an ideal biomarker because the cancer is detected specifically and noninvasively. Biomarkers, however, may be more complicated and/or indirect, involving, for example, measures of immune response to a developing tumor, hormonal changes induced by a tumor, or mass spectrometry profiles of serum protein. In this commentary, we use the term "biomarker" for cancer detection in a broad sense.

Cancer is a diverse disease, and it is unlikely that a single biomarker will detect all cancer of a particular organ with high specificity and sensitivity. Indeed, biomarkers, such as prostate-specific antigen (PSA), that purport to have high sensitivity tend to have low specificity because they do not detect cancer *per se* but rather a more general process. We note that maintaining high specificity (low false-positive rates) is a very high priority for population screening. Even a small false-positive rate translates into a large number of people subjected to unnecessary costly diagnostic procedures and psychologic stress. Thus, biomarkers need to be highly specific for cancer, and the use of several such biomarkers of cancer will likely be necessary for an overall screening program that is both sensitive and specific.

3) Five Phases of Screening Biomarker Development

We propose that biomarker development be conceptualized as occurring in five consecutive phases as depicted in Fig. 1. In this section, we outline the key objectives of each phase and discuss aspects of study design for achieving the primary aim.

3.1) Phase 1—Preclinical Exploratory Studies

The first step in the search for biomarkers often begins with preclinical studies, comparing tumor tissue with nontumor tissue. These are exploratory studies to identify characteristics unique to tumor tissue that might lead to ideas for clinical tests for detecting cancer. Immunohistochemistry and western blots have been extensively used for this purpose. More recent technology includes gene-expression profiles based on microarrays that yield information regarding expression for thousands of genes *(6),* protein expression profiles based on mass spectroscopy *(7),* and levels of circulating antibodies against thousands

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Mini-Reviews

The Early Detection Research Network: 10-Year Outlook

Sudhir Srivastava[1*]

BACKGROUND: The National Cancer Institute's Early Detection Research Network (EDRN) has made significant progress in developing an organized effort for discovering and validating biomarkers, building resources to support this effort, demonstrating the capabilities of several genomic and proteomic platforms, identifying candidate biomarkers, and undertaking multicenter validation studies. In its first 10 years, the EDRN went from a groundbreaking concept to an operational success.

CONTENTS: The EDRN has established clear milestones for reaching a decision of "go" or "no go" during the biomarker development process. Milestones are established on the basis of statistical criteria, performance characteristics of biomarkers, and anticipated clinical use. More than 300 biomarkers have been stopped from further development. To date, the EDRN has prioritized more than 300 biomarkers and has completed more than 10 validation studies. The US Food and Drug Administration has now cleared 5 biomarkers for various clinical endpoints.

SUMMARY: The EDRN today combines numerous collaborative and multidisciplinary investigator-initiated projects with a strong national administrative and data infrastructure. The EDRN has created a rigorous peer-review system that ensures that preliminary data—analytical, clinical, and quantitative—are of excellent quality. The process begins with an internal review with clinical, biostatistical, and analytical expertise. The project then receives external peer review and, finally, National Cancer Institute program staff review, resulting in an exceptionally robust and high-quality validation trial.

The Early Detection Research Network (EDRN)[2] is a pioneering effort by the National Cancer Institute (NCI) that is designed to discover and validate biomarkers for assessment of cancer and cancer risk. First launched in 2000, the EDRN provides a vertically integrated network of academic- and industry-based scientists collaborating to meet the challenge of developing new cancer-screening and early detection products. The mission of the EDRN is to implement biomarker research through systematic evidence-based discovery, development, and validation of biomarkers for identification of cancer risk, early detection, diagnosis, and prognosis determination to reduce cancer morbidity and mortality (Tables 1, 2, and 3).

The identification of biomarkers involves a rigorous process that begins with discovery, which leads to development, validation, and application. EDRN has fulfilled these expectations by establishing a process for biomarker development by using a multidisciplinary and multiinstitutional approach. This infrastructure, combined with the development of highly interactive databases and informatics systems, serves as a model for the conduct of translational research that is fully aligned with the goals and objectives of the NCI and the NIH communities *(1)*.

The EDRN has implemented pioneering solutions to enable data sharing between laboratories using common data elements, thus ensuring and expediting consistent data description across institutions. Research collaborations occur within an environment of teamwork across different disciplines and laboratories focused on achieving the following common goals *(2)*:

- Developing and testing promising biomarkers and technologies to obtain preliminary information to guide further testing;
- Evaluating promising, analytically proven biomarkers and technologies, such as measures of accuracy, sensitivity, and specificity and, when possible, poten-

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[2] Nonstandard abbreviations: EDRN, Early Detection Research Network; NCI, National Cancer Institute; HUPO, Human Proteome Organisation; MS, mass spectrometry; TSP1, thrombospondin 1; EPCA-2, early prostate cancer antigen-2; proPSA, prostate-specific antigen precursor; CA125, cancer antigen 125; DCP, des-γ carboxyprothrombin; AFP, α-fetoprotein; HCC, hepatocellular carcinoma; HE4, human epididymis protein 4.

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Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography–mass spectrometry

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ABSTRACT

This study aimed to set-up an ultra performance liquid chromatography–electrospray ionization–mass spectrometry (UPLC–ESI–MS) method for the determination of salivary L-phenylalanine and L-leucine for early diagnosis of oral squamous cell carcinoma (OSCC). In addition, the diagnostic accuracy for both biomarkers was established by using receiver operating characteristic (ROC) analysis. Mean recoveries of L-phenylalanine and L-leucine ranged from 88.9 to 108.6% were obtained. Intra- and inter-day precision for both amino acids was less than 7%, with acceptable accuracy. Linear regression coefficients of both biomarkers were greater than 0.99. The diagnostic accuracy for both biomarkers was established by analyzing 60 samples from apparently healthy individuals and 30 samples from OSCC patients. Both potential biomarkers demonstrated significant differences in concentrations in distinguishing OSCC from control ($P < 0.05$). As a single biomarker, L-leucine might have better predictive power in OSCC with T1–2 (early stage of OSCC including stage I and II), and L-phenylalanine might be used for screening and diagnosis of OSCC with T3–4 (advanced stage of OSCC including stage III and IV). The combination of L-phenylalanine and L-leucine will improve the sensitivity (92.3%) and specificity (91.7%) for early diagnosis of OSCC. The possibility of salivary metabolite biomarkers for OSCC diagnosis is successfully demonstrated in this study. This developed method shows advantages with non-invasive, simple, reliable, and also provides lower detection limits and excellent precision and accuracy. These non-invasive salivary biomarkers may lead to a simple clinical tool for the early diagnosis of OSCC.

1. Introduction

In recent years, there is a growing interest among researchers to use salivary biomarkers in investigation of disease diagnosis, such as lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4–6], sjögren's syndrome [7], etc. Oral cancer, one of the six most common human cancers, refers to all malignancies arising from the lips, the oral cavity, and pharynx [8,9]. The World Health Organization has reported oral cancer as having one of the highest mortality ratios amongst other malignancies with a death rate of 45% at five years from diagnosis [10]. Approximately 300,000 individuals worldwide are diagnosed with oral cancer annually. More than 90% of oral cancer is squamous cell carcinoma (OSCC). At present, once OSCC detected, it will be at advanced stage, which would generally result in a poor prognosis and a low survival rate. Therefore, early detection of OSCC as well as the screening of high risk populations with precancerous lesions remains to be an urgent need.

Currently, the most definitive method for oral cancer diagnosis and screening is a scalpel biopsy. It is time-consuming and needs extensive experience. In addition, it is also impractical to use imaging techniques for oral cancer screening, since they are expensive and insensitivity for small lesions [11]. Therefore, a number of molecular-based diagnostic markers have been used to detect the presence of OSCC with varying degrees of sensitivity and specificity. Compared with blood samples, using saliva for clinical diagnostics have attracted more and more research scientists and clinical doctors.

Human saliva, a multi-constituent oral fluid, is secreted primarily by three major glands namely parotid gland, submandibular gland and sublingual gland [12,13]. Generally, salivary glands produce about 1–1.5 L of saliva daily. It contains approximately 99% water with minerals, nucleic acids, electrolytes, mucus and proteins [14]. It is one of the most complex, versatile, and important body fluids, supplying a large range of physiological needs. Therefore, saliva is also called the "mirror of the body" or

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RNAPro•SAL: A device for rapid and standardized collection of saliva RNA and proteins

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Abstract

The stabilization and processing of salivary transcriptome and proteome biomarkers is a critical challenge due to the ubiquitous nature of nucleases and proteases as well as the inherent instability of these biomarkers. Furthermore, extension of salivary transcriptome and proteome analysis to point-of-care and remote sites requires the availability of self-administered ambient temperature collection and storage tools. To address these challenges, a self-contained whole saliva collection and extraction system, RNAPro•SAL, has been developed that provides rapid ambient temperature collection along with concurrent processing and stabilization of extracellular RNA (exRNA) and proteins. The system was compared to the University of California, Los Angeles (UCLA) standard clinical collection process (standard operating procedure, SOP). Both systems measured total RNA and protein, and exRNA IL-8, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin and ribosomal protein S9 (RPS9) by qPCR. Proteome analysis was measured by EIA analysis of interleukin-8 (IL-8), and β-actin, as well as total protein. Over 97% of viable cells were removed by both methods. The system compared favorably to the labor-intensive clinical SOP, which requires low-temperature collection and isolation, yielding samples with similar protein and exRNA recovery and stability.

Keywords

saliva diagnostic tool; salivary protein; salivary extracellular RNA; ambient temperature; point-of-care settings

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Author contributions

S.H.C. conceived of and designed the study, acquired the data, analyzed and interpreted the data, drafted the article, and provided critical revision. G.A.T. and A.N.H. analyzed and interpreted the data and provided critical revision. W.L. conceived the study. T.G. analyzed and interpreted the data. R.L.B. and M.J.L. analyzed and interpreted the data and provided critical revision. L.F., M.Y., and C.S. acquired the data. F.W., D.E., P.D.S., and D.T.W.W. conceived of the study and interpreted the data and provided critical revision and general supervision.

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A targeted proteomic strategy for the measurement of oral cancer candidate biomarkers in human saliva

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Abstract

Head and neck cancers, including oral squamous cell carcinoma (OSCC), are the sixth most common malignancy in the world and are characterized by poor prognosis and a low survival rate. Saliva is oral fluid with intimate contact with OSCC. Besides non-invasive, simple, and rapid to collect, saliva is a potential source of biomarkers. In this study, we build an SRM assay that targets fourteen OSCC candidate biomarker proteins, which were evaluated in a set of clinically-derived saliva samples. Using Skyline software package, we demonstrated a statistically significant higher abundance of the C1R, LCN2, SLPI, FAM49B, TAGLN2, CFB, C3, C4B, LRG1, SERPINA1 candidate biomarkers in the saliva of OSCC patients. Furthermore, our study also demonstrated that CFB, C3, C4B, SERPINA1 and LRG1 are associated with the risk of developing OSCC. Overall, this study successfully used targeted proteomics to measure in saliva a panel of biomarker candidates for OSCC.

Keywords

Selected reaction monitoring; saliva; oral cancer; skyline

1. Introduction

Head and neck cancers are the sixth most common malignant tumors worldwide [1]. Oral cancer is the most frequent subtype among these with oral squamous cell carcinoma (OSCC) annually affecting over 300,000 people worldwide [2]. Despite advancements in oral cancer prevention and multimodality treatments, the 5-year survival rate of OSCC patients is less

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Conflict of interest statement

The authors declare no competing financial interest.

High-throughput generation of selected reaction-monitoring assays for proteins and proteomes

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Selected reaction monitoring (SRM) uses sensitive and specific mass spectrometric assays to measure target analytes across multiple samples, but it has not been broadly applied in proteomics owing to the tedious assay development process for each protein. We describe a method based on crude synthetic peptide libraries for the high-throughput development of SRM assays. We illustrate the power of the approach by generating and applying validated SRM assays for all *Saccharomyces cerevisiae* kinases and phosphatases.

Selected reaction monitoring (SRM; plural, multiple reaction monitoring)[1,2] has recently emerged as a targeted proteomic technology for the consistent detection and accurate quantification of specific, predetermined sets of proteins in a complex background and in multiple samples. It exploits the capability of triple quadrupole (QQQ) mass spectrometers to selectively isolate precursor ions corresponding to the mass of the targeted peptides and to selectively monitor peptide-specific fragment ion(s). Suitable sets of precursor and fragment ion masses for a given peptide, called SRM transitions, constitute definitive mass spectrometry (MS) assays that identify a peptide and, by inference, the corresponding protein in proteome digests[3]. SRM has high sensitivity (low-attomolar) and a broad dynamic range (up to five orders of magnitude), and it is quantitative[4,5]. Once SRM assays have been established for a set of peptides, they can be used in a highly multiplexed manner (>1,000 SRM transitions per hour)[4] and with great reproducibility, even if the measurements are carried out in different laboratories[6] The consistency, sensitivity and completeness of datasets generated by SRM measurements compare favorably with the data generated with shotgun proteomic methods in which precursor ions are stochastically selected for fragmentation[1,7].

In spite of these favorable properties, SRM has not been broadly used in proteomics, and SRM-based studies have mostly focused on small numbers of proteins[4,5,7]. The effort required to develop a high-quality SRM assay for a protein has prevented the broader application of this technology. Assay development involves first the validation of the assay to confirm that it selectively monitors the analyte of interest and, second, optimization of the assay to maximize its sensitivity[8]. Optimization is achieved by determining the most suitable SRM transitions for each target peptide, along with other associated liquid chromatography (LC)-MS parameters, and is a lengthy and iterative process. Assay validation typically relies on the acquisition of full-scan MS/MS spectra for the targeted peptide on the same MS platform that will be used to deploy the assay, that is, a QQQ instrument. Acquisition of reliable MS/MS spectra of peptides in biological samples is strongly compromised by complex backgrounds that obscure the fragmentation pattern and limit the dynamic range, thus making the validation of transitions for low-abundance peptides extremely challenging. Additionally, MS/MS spectra acquisition on QQQ instruments is slow compared to fast scanning mass spectrometers such as linear ion traps. This creates the paradoxical situation that highly sensitive SRM assays have to be developed and validated by a method that has a substantially lower sensitivity and dynamic range than the SRM assay itself, which has prevented the routine development of SRM assays for low-abundance proteins.

Here we present a method for generating validated SRM assays for sets of proteins, subproteomes or whole proteomes that overcomes this limitation. It is based on the use of low-cost libraries of crude, unpurified synthetic peptides as a reference for validating and optimizing SRM assays and on a MS method to generate the assays at a throughput exceeding 100 per hour.

The method consists of the following steps (**Supplementary Fig. 1**). (i) A set of proteotypic peptides[9] is selected for each target protein based on empirical data in prior proteomic datasets, such as those contained in repositories like PeptideAtlas[10] or by bioinformatic prediction[11]. (ii) The selected peptides are synthesized by Spot synthesis[12,13] on a microscale and recovered from the synthesis support in a crude, unpurified form. (iii) Pools consisting of ~100 such synthesis products are analyzed by a SRM-triggered MS/MS method, whereby the detection of any of a few anticipated transitions for each peptide triggers the acquisition of a full MS/MS spectrum for the target peptide. (iv) MS/MS spectra, optionally consensus MS/MS spectra in which multiple spectra per peptide are acquired, are used to both validate the assays and extract the most favorable SRM coordinates for each peptide, such as highest-intensity fragment ions, peptide elution time and fragment

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Chapter 4

Protein Electrophoresis in Saliva Study

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Rui Vitorino and Francisco Amado

Additional information is available at the end of the chapter

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1. Introduction

Saliva started for been less studied than other body fluids, but in the last years it has being receiving an increased attention. Until now, more than 2000 different proteins and peptides have been identified in whole saliva and salivary glandular secretions [1]. From these, more than 90% derive from the secretion of the three pairs of "major" salivary glands (parotid, submandibular and sublingual glands). The remaining 10% derives from "minor" salivary glands and from extra-glandular sources, namely gingival crevicular fluid, mucosal transudations, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, and food debris [2].

Saliva secretion is mainly under autonomic nervous system regulation. Sympathetic and parasympathetic stimulation have different effects on the flow rate and composition of saliva secreted. Whereas parasympathetic stimulation results in the production of a high volume of saliva with low protein concentration, stimulation of the sympathetic branch of the autonomic nervous system is responsible for the secretion of a small amount of saliva with increased protein concentration. Besides this distinctive characteristic, and inversely to what is observed for the majority of body systems, the effects of parasympathetic and sympathetic innervations are not antagonic but rather exert relatively independent effects in which the activity of one branch may synergistically augment the effect of the other [3,4]. Despite the thought of an exclusive nervous regulation, recent in vivo animal experiments indicate a short-term endocrine regulation of salivary glandular activities as well [5-9].

The primordial function of saliva is to aid in the moistening and preprocessing of food, aiding in deglutition. Besides this, other important functions exist for saliva, which can generally be grouped in digestive (and ingestive) and protection [10]. For digestive (and ingestive) purposes, saliva contains enzymes, including proteases, lipases and glycohydrolases, which initiate partial break-down of food components. Among these

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Technical note

Validation and quality control of ELISAs for the use with human saliva samples

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ABSTRACT

Enzyme-linked immunosorbent assays (ELISAs) have proven to be a powerful tool for fast and reliable sample analysis, in both clinical diagnostics and in research. Most assays are now available for use with a range of different analytical fluids, including serum, plasma or urine. In recent years, saliva has drawn attention as a potentially valuable diagnostic fluid; however few ELISAs have been validated for use with saliva, or their validation is often incomplete. Saliva has a number of different physical characteristics than, for example, cell culture medium or serum and assuming an ELISA which works well with serum samples will also do so with saliva potentially could lead to erroneous data and conclusions.

In this report, we provide a detailed protocol to validate any ELISA for use with saliva samples and show the results of validation procedures for 13 ELISAs for using saliva. Our findings suggest that the majority of ELISAs work reliably with saliva, even if the assay was not specifically designed for this biological fluid. However, we also report a few cases where recovery or intra-and inter-assay variations were unexpectedly high, emphasising the importance of performing a validation procedure for each assay before using it with saliva to ensure accurate and reliable data.

1. Introduction

In recent years, saliva has gained considerable attention as a possible alternate diagnostic fluid to blood. Saliva is easily accessible and convenient to collect. Collection can take place in just about any environment and is painless, which, especially when considering collection from children, could be very advantageous compared to obtaining blood samples. For example, salivary diagnostics are now being used in tests for various hormones, HIV and alcohol and there is a growing scientific literature describing the use of salivary biomarkers to determine diabetic and cardiac related risk factors (Dodds et al., 2000; Rao et al., 2009).

For most biomarker analyses, ELISAs are still widely used as the method of choice. ELISAs are accurate techniques, which generally allow for reliable, rapid and high throughput sample analysis. Conveniently, commercial ELISA kits come supplied with instructions for the assay procedure and often extensive validation and quality control data are provided. However, in our experience, it is these validation and quality control data which have limited value for a number of reasons.

First, most commercially available ELISAs are designed for analysis of cell culture or serum samples, and even if assays are recommended for use with saliva, quality control data such as spike/recovery, linearity, intra-and inter-assay variation or assay sensitivity have seldom been performed with saliva samples. However, differences in the composition of saliva compared to serum potentially all can interfere with the assay and influence assay performance. Assay interferences (termed "matrix effects") can be defined as "the sum of effects of all components (both qualitative and quantitative) in a system, with the exception of the analyte to be measured" (Wood, 1991). Therefore, differences in pH, proteolytic enzymes or viscosity between serum and saliva could all be potential causes for matrix effects which have not been considered in an assay developed for using serum samples. Second, saliva collection and storage methods can significantly

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diagnostics

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Review

Advancing Point-of-Care (PoC) Testing Using Human Saliva as Liquid Biopsy

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Abstract: Salivary diagnostics is an emerging field for the encroachment of point of care technology (PoCT). The necessity of the development of point-of-care (PoC) technology, the potential of saliva, identification and validation of biomarkers through salivary diagnostic toolboxes, and a broad overview of emerging technologies is discussed in this review. Furthermore, novel advanced techniques incorporated in devices for the early detection and diagnosis of several oral and systemic diseases in a non-invasive, easily-monitored, less time consuming, and in a personalised way is explicated. The latest technology detection systems and clinical utilities of saliva as a liquid biopsy, electric field-induced release and measurement (EFIRM), biosensors, smartphone technology, microfluidics, paper-based technology, and how their futuristic perspectives can improve salivary diagnostics and reduce hospital stays by replacing it with chairside screening is also highlighted.

Keywords: saliva; diagnostic toolboxes; biomarkers; the point of care; diseases

1. Introduction

Laboratory testing remains the dominant mainstay for analytical processes of a large number of samples involving the disciplines of biochemistry, haematology, microbiology, anatomical pathology, and much more [1]. Due to the limitations and pressure on healthcare budgets faced by a very large number of countries, primary care is best suited for the world to reduce expenses instead of secondary and tertiary hospitals. Poverty, chronic disease, infections lead to significant problems in developing the world, and adequate diagnostic testing turns out to be difficult to meet the needs. Hence, consequently, initiatives in making solid models using point-of-care technology (PoCT) came into existence [2].

1.1. Paradigm Shift from Central Laboratory (CL) to Point-of-Care

A self-monitoring blood glucose meter, coagulation (INR), and pregnancy testing kits using urine samples are well-known examples of PoCT and has become over-the-counter products to be sold in the market. Saliva is predicted to be a substitute for blood, collected non-invasively for the diagnosis of oral and systemic diseases. Thus, PoCT replaces the specialist testing centres by using the samples other than blood and urine [3]. For the development of PoCT devices, minimum risk of infection with no mental and physical pain is of utmost importance to consider, in addition to automation, integration, multiplexed detection ability, quick analysis, small sample size, and minimal training as the primary goals of modern medicine [4]. With the advent of the struggle in the growing potential of developing PoCT, the World Health Organization (WHO) provided guidelines which had

Telomerase in saliva: An assistant marker for oral squamous cell carcinoma

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Author information ▸ Article notes ▸ Copyright and License information Disclaimer

ABSTRACT

Go to:

Introduction:

Telomerase is a ribonucleoprotein complex responsible for de novo telomere synthesis and addition of telomeric repeats to existing telomeres. Telomerase activity is generally found to be absent in normal tissues. Telomerase is known to be induced upon malignant transformation of human cells.

Method:

In the present study, we analyzed both telomere length and telomerase activity in saliva samples from oral carcinoma patients. The study was done to investigate the presence of telomerase activity in oral squamous cell carcinoma by TRAP assay.

Result:

Telomerase activity was detectable in 79 of 100 human OSCC and 51 of 100 premalignant cases and 8 of 100 normal patients.

Conclusion:

These results indicate that telomerase is activated frequently during the late stage of oral premalignancy and may play a crucial role in OSCC.

Keywords: Oral squamous cell carcinoma, saliva, telomerase, telomere

Short Communication
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Is salivary evaluation of P53 and MMP-3 a good tool for early detection of oral squamous cell carcinoma?

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Objective Oral squamous cell carcinoma (OSCC) is one of the most common malignancies around the world. Despite the advancement in treatment methods, the prognosis is still not good. Based on clinicians' idea, the early diagnosis of the lesion can lead to better prognosis. Some salivary biomarkers such as matrix metalloproteinase-3 and P53 may detect OSCC in early stages. In this study, we wanted to compare salivary MMP-3 and P53 levels in OSCC patients and control group.
Methods Fifteen patients with OSCC (9 male and 6 female) were selected from Oral Pathology Department, Babol, Iran. Salivary MMP-3 and P53 were measured by ELISA and compared with control group. Data was analyzed by ANOVA, *t*-test, and Mann–Whitney.
Result There was no significant differences between salivary MMP-3 and P53 concentration in patients with OSCC and healthy individuals.
Conclusion Based on our findings and other similar studies, salivary MMP-3 and P53 levels might not be accurate enough to detect early stages of OSCC. But there are controversial statements about these questions. So, supplementary studies are needed to be done in future.
Keywords OSCC, saliva, P53, MMP3, ELISA9

Introduction

Head and neck cancer including oral cavity, pharynx, hypopharynx, and larynx is one of the most common malignancies around the world.[1] Moreover, 90% of head and neck cancers have been diagnosed as squamous cell carcinoma (SCC).[2] Cancer is a major cause of mortality worldwide and a lot of new cases occur every year.[3]

The process of oral carcinogenesis is multifactorial and multistep, and the exact sequence is almost unknown.[4,5] In spite of presence of risk factors such as tobacco and alcohol in some patients, most of them ultimately are not diagnosed as malignancies. This implies that genetic has an important role in susceptibility to cancers.[6]

Unfortunately, despite the advanced methods in surgery, radiotherapy and chemotherapy, the prognosis of oral SCC (OSCC) is not good yet. But in case of early detection, the prognosis could be better.

Biopsy followed by histopathological evaluation is gold standard for diagnosis of OSCC, but it is more helpful in late stages of disease. Biomarkers are measurable indicators which can be useful for early diagnosis of some lesions.[7] The most common laboratory diagnostic procedures involve the chemical and cellular analysis of blood. Other biologic fluids like saliva are also used in diagnostic tests.[8] Salivary biomarkers in oropharyngeal carcinoma are cost-effective. Saliva contains a wide range of components and is easy access, so patients feel more comfortable. The method is also non-invasive and handling is safe. There are also other advantages for using saliva instead of blood.[7] Therefore, nowadays there is a tendency to use of salivary biomarkers as diagnostic and prognostic factors.

The matrix metalloproteinase (MMP) family involves diverse substrates.[9] They are large family of zinc-dependent endopeptidase, and they have ability to digest extracellular matrix.[10] Generally, MMPs are made up of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain. They are secreted from the cell or anchored to the plasma membrane. MMPs have six separated groups: Collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others.[11]

Matrix metalloproteinase-3 (stromelysin-1) is a secretory enzyme which several growth factors regulate its expression and the process of wound healing can stimulate its secretion.[12] It is expressed by keratinocytes, fibroblasts, and chondrocytes.[13] It has found that overexpression of MMP3 is in association with developing malignancies including head and neck carcinomas.[14,15]

The P53 protein is activated after DNA damage or oncogenic signals. It has an important role in cell cycle control, DNA repair, and apoptosis.[16] Altered expression and functional loss of P53 are common genetic changes in human malignancies.[17,18]

Until now, conflicting results have been obtained from the study on MMP-3 and P53 markers in OSCC patients, which could be due to differences and limitations in the immunohistochemical and RT-PCR methods in semi-quantitative cytokine assessments. In addition, these two markers have not been evaluated simultaneously in patients with OSCC. Therefore, in this study, we decided to use a completely quantitative method to examine the levels of salivary P53 and MMP-3 in OSCC patients to find their real changes in OSCC affected patients. Among the available methods, studies can perform on the tissue blocks, blood, and other biologic fluids. Saliva is believed to be a reliable tool for diagnosis of OSCC because it is in direct contact with cancerous tissue.[7]

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REVIEW ARTICLE

The utilization of saliva as an early diagnostic tool for oral cancer: microRNA as a biomarker

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Abstract
Recently, dentistry presents a preventive philosophy, seeking early diagnoses and minimally traumatic treatments for patients. Cancer is known for its aggressive nature, where its signals and symptoms may only appear in advanced stages of the disease, therefore, reducing the possibility of using atraumatic treatment options and patient survival. Saliva has in its composition substances which can be used as biomarkers for disease diagnoses, one of those being microRNA. microRNAs are a group of small RNA molecules with 18–24 nucleotides which have functions such as the degradation of oncogenes transcripted mRNA. The aim of this paper is to explore all theoretical possibilities that microRNA offers as an early diagnostic tool for oral cancer. Studies show that microRNA can be directly linked with cancer gene regulation. Because microRNA is more specific to tissues and diseases than mRNA, it holds the premise of being a feasible, non-invasive, and stable biomarker for early diagnosis of oral cancer. The fact that miRNA can be found in saliva makes it an extremely affordable and feasible option as a biomarker to be used. Since it is linked to regulating functions of cancer genes, it also brings hope that in the near future, it could be used as a reliable biomarker.

Keywords miRNA · Biomarker · Cancer · Saliva · Oral cancer · Early diagnostic

Introduction

Dentistry has undergone a major evolution. During its earliest days, dentistry was a mostly "reparative" science, with very little focus on prevention. This focus has since changed, not only with working professionals, but also with patients who now worry more about prevention and care more for their oral health [1].

In regards to cancer, prevention is relatively difficult due to its association with variables such as personal habits, genetic predisposition, exposure to factors that cause cancer, and other variables that sometimes are not under the control of patients and professionals. In addition to these, the stage in which the diagnosis of cancer is detected and confirmed is also a variable. The earlier the diagnosis and start of treatment, the greater the probability of eliminating the cancer lesion and survival of the affected patient [2, 3].

Basal cell carcinoma (BCC) is the most affecting oral cancer, and constitutes about 90% of cancer lesions (BCC), resulting in about 8000 deaths per year. These numbers could be lower if there was a way to perform an early diagnosis [4].

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Salivary mRNA markers having the potential to detect oral squamous cell carcinoma segregated from oral leukoplakia with dysplasia

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ABSTRACT

Background: In the current study the presence of extracellular IL-1B, IL-8, OAZ and SAT mRNAs in the saliva was evaluated as a tool in the early detection of oral squamous cell carcinoma.
Methods: 34 patients with primary oral squamous cell carcinoma stage $T_1N_0M_0/T_2N_0M_0$, 20 patients with oral leukoplakia and dysplasia (15 patients with mild dysplasia and 5 with severe dysplasia/*in situ* carcinoma) and 31 matched healthy-control subjects were included in the study. The presence of IL-1B, IL-8, OAZ and SAT mRNA was evaluated in extracellular RNA isolated from saliva samples using sequence-specific primers and real-time RT-PCR. ROC curve analysis was used to estimate the ability of the biomarkers to detect oral squamous cell carcinoma patients.
Results: The data reveal that the combination of these four biomarkers provides a good predictive probability of up to 80% (AUC = 0.799, p = 0.002) for patients with oral squamous cell carcinoma but not patients suffering from oral leukoplakia with dysplasia. Moreover, the combination of only the two biomarkers (SAT and IL-8) also raises a high predictive ability of 75.5% (AUC = 0.755, p = 0.007) approximately equal to the four biomarkers suggesting the use of the two biomarkers only in the prediction model for oral squamous cell carcinoma patients limiting the economic and health cost in half.
Conclusion: SAT and IL-8 mRNAs are present in the saliva in high quality and quantity, with a good discriminatory ability for oral squamous cell carcinoma patients only but not for patients with oral leukoplakia and dysplasia an oral potentially malignant disorder.

1. Introduction

In 2015, over 450,000 new individuals are going to be diagnosed worldwide with oral cancer [1] and suffer from it. Despite the fact that oral cancer is really easily accessible because it is in the oral cavity, its five year survival rate (about 50–62%) still remains low [2,3], mainly because of its late diagnosis due to being fully asymptomatic in its early stages. The World Health Organisation WHO particularly stresses that early detection of oral cancer is the key to its management and successful therapeutic approach (www.who.int/cancer/detection/en) [4].

There are several types of oral cancer, but approximately 90% are oral squamous cell carcinomas [5,6]. Until now, there have been several attempts to develop biomarkers in order to detect oral squamous cell carcinomas early in their progression, the latest of which use saliva as a source [7–10]. Saliva is a valuable body fluid that has been used long ago for drug testing [11,12], therapeutic monitoring [13], and disease diagnosis [14–17]. It seems to be preferable than peripheral blood for disease detection and diagnosis [18] due to the non-invasive nature of its collection.

Various biomarkers have been proposed for oral squamous cell carcinoma detection and use for screening purposes [19,20] but all the biomarkers for OSCC detection that have been reported in articles until now have not been studied extensively or in a larger scale. This study focuses on the analysis of four mRNA markers in

Abbreviations: OSCC, Oral squamous cell carcinoma; OAZ, Ornithine decarboxylase antizyme 1; SAT, Spermidine/spermine N1-acetyltransferase 1; IL-8, (interleukin 8); IL-1B, interleukin 1B; ROC curve, receiver operating curve; AUC, Area under the curve.

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REVIEW ARTICLE

Biomarkers in saliva for the detection of oral squamous cell carcinoma and their potential use for early diagnosis: A systematic review

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ABSTRACT

Objective: To determine the capacity of salivary biomarkers in the early diagnosis of oral squamous cell carcinoma. *Study design*: A systematic review of the literature was performed based on the English titles listed in the PubMed, EBSCO, Cochrane, Science Direct, ISI web Science and SciELO databases using the following search descriptors: Oral cancer, diagnosis, biomarkers, saliva and oral squamous cell carcinoma. Abstracts and full-text articles were assessed independently by two reviewers. International checklists for assessment of methodological quality were used. Levels of evidence and grades of recommendation through the Scottish Intercollegiate Guidelines Network (SIGN) template were recognized. The units of analysis were identified through a reference matrix. *Results*: Through the research strategy and after application of different filters and considering choosing criteria, six studies were obtained for analysis. Salivary biomarkers for oral cancer most frequently found were mRNA and proteins for IL-8, CD44, MMP-1 and MMP-3. New peptide-biomarkers such as Cyfra 21-1 and ZNF510 were found. ZNF 510 was the only biomarker which increased in the population with tumour stage T1 + T2 and T3 + T4. Only one study showed a sensitivity and specificity of 96% when the biomarker ZNF 510 is employed to discriminate early and late tumour stages. *Conclusions*: There is no sufficient scientific evidence to support the capacity of the identified salivary biomarkers for the early diagnosis of oral cancer (sub-clinical stages of the pathogenic period before cancer phenotypes are manifested). Salivary biomarkers, however, may be employed to discriminate between healthy and cancer patients.

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Biomarkers, diagnosis, oral cancer, saliva

Introduction

Oral cancer is the sixth most common form of human cancer. At the same time, oral squamous cell carcinoma (OSCC) is the commonest form of head and neck cancer (HNC) and its mortality rate at 5 years is ~60% [1]. Regrettably, OSCC is usually diagnosed in advanced stages of malignant development. Despite improvements in therapeutic strategies, its poor survival rate has not ameliorated over the last 30 years. The challenge of this decade, therefore, is to reduce both mortality and morbidity of this disease through the development of strategies that detect OSCC in its early stages. In addition to the conventional clinical oral examination, currently there are no scientifically validated techniques for the early detection of OSCC [1,2].

The application of advanced biochemical methods and molecular biology techniques to detect biomarkers may contribute, but they must progress to the identification of potential biomarkers and their eventual application in clinical trials to improve early diagnosis, intervention and treatment [3,4]. One critical point is the absence of routine oral cancer screening, which is neither invasive nor expensive [5]. According to some studies, identification and diagnosis can take as long as 6 months [3,6]. Due to its relatively low incidence (4.6–6.9; ASR per 100 000 individuals age standardized rate), it is difficult to educate the public on oral cancer, which would reduce the time of diagnosis [7].

Early detection of pre-malignant lesions is critical for prognosis and survival rates. In the case of OSCC, if the malignancy is detected in the T1 stage, the survival rate at 5 years is 80%, while if it is detected in T3 and T4 stage it is 20–40% [8]. The literature suggests various methods for such detection. Self-fluorescent visualization has been used as an adjunct to white light visualization in monitoring oral cancer. However, said methods are sensitive but not specific enough to detect pre-malignant lesions in patients at high risk of oral cancer [9]. The sensitivity and specificity of oral cytology is weak, but has improved with the advent of molecular tools [10].

Recently, saliva has taken an important role as a diagnostic fluid because sampling is inexpensive, easy and not invasive. Moreover, potential biomarkers for diseases such as periodontitis, breast cancer, oral cancer and Sjögren Syndrome have been identified in saliva [11–13]. Salivary biomarkers for OSCC include biomolecules such as DNA, RNA and proteins that can

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Quantitative Proteomics Reveals Myosin and Actin as Promising Saliva Biomarkers for Distinguishing Pre-Malignant and Malignant Oral Lesions

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Abstract

Background: Oral cancer survival rates increase significantly when it is detected and treated early. Unfortunately, clinicians now lack tests which easily and reliably distinguish pre-malignant oral lesions from those already transitioned to malignancy. A test for proteins, ones found in non-invasively-collected whole saliva and whose abundances distinguish these lesion types, would meet this critical need.

Methodology/Principal Findings: To discover such proteins, in a first-of-its-kind study we used advanced mass spectrometry-based quantitative proteomics analysis of the pooled soluble fraction of whole saliva from four subjects with pre-malignant lesions and four with malignant lesions. We prioritized candidate biomarkers via bioinformatics and validated selected proteins by western blotting. Bioinformatic analysis of differentially abundant proteins and initial western blotting revealed increased abundance of myosin and actin in patients with malignant lesions. We validated those results by additional western blotting of individual whole saliva samples from twelve other subjects with pre-malignant oral lesions and twelve with malignant oral lesions. Sensitivity/specificity values for distinguishing between different lesion types were 100%/75% ($p = 0.002$) for actin, and 67%/83% ($p < 0.00001$) for myosin in soluble saliva. Exfoliated epithelial cells from subjects' saliva also showed increased myosin and actin abundance in those with malignant lesions, linking our observations in soluble saliva to abundance differences between pre-malignant and malignant cells.

Conclusions/Significance: Salivary actin and myosin abundances distinguish oral lesion types with sensitivity and specificity rivaling other non-invasive oral cancer tests. Our findings provide a promising starting point for the development of non-invasive and inexpensive salivary tests to reliably detect oral cancer early.

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Introduction

Oral cancer develops in stages, transitioning from a normal oral epithelium, to a pre-malignant, dysplastic oral lesion, to a malignant lesion, most commonly in the form of oral squamous cell carcinoma (OSCC). For those who develop OSCC, the overall 5- year survival rate is approximately 50%, unchanged over the last 30 years[1]. For those where malignancy is detected early, soon after transitioning from pre-malignancy, treatment is more effective, and consequently the survival rate increases to about 80%[2]. Clearly, the ability to distinguish between pre-malignant and malignant oral lesions is crucial[1,3].

Unfortunately, pre-malignant and malignant lesion types cannot be distinguished simply by visual inspection; instead invasive tests are used. The current gold standard for characterizing lesions, histological analysis of tissue biopsies[3], has several disadvantages:

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ORIGINAL ARTICLE

Adenosine deaminase in saliva as a diagnostic marker of squamous cell carcinoma of tongue

Balwant Rai · Jasdeep Kaur · Reinhilde Jacobs · Suresh Chander Anand

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Abstract Tongue cancer is amongst the most common and fatal types of cancers in the world. The abnormalities in purine metabolism are characteristic features of many human tumors. Little is known about the correlation between the activities of key enzymes of purine nucleotide pathway and clinical indicators of tongue cancer invasiveness and aggressiveness. Fifty patients (M: F 25:25; mean age: 55.6 years (range 45-60; SD 1.8)) with diagnosed squamous cell carcinoma of the tongue (test group) and 30 normal subjects (M: F 15:15) without any systemic disease (control group) were recruited after obtaining informed consent. All patients were staged by the TNM classification. Salivary adenosine deaminase (ADA) activity was assessed in cancerous patients (test group) and normal healthy subjects (control group). Statistically significant differences between test and control groups were observed in salivary ADA ($P<0.001$). Furthermore, serum ADA levels significantly increased as the disease stage progressed from stage I to stage III of squamous cell carcinoma of the tongue in both genders ($P<0.001$). Salivary ADA might be used as a diagnostic tool for early detection of squamous cell carcinoma of tongue.

Keywords Saliva · Adenosine deaminase · Squamous cell carcinoma · Tongue cancer · Staging of cancer · Diagnostic marker

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Introduction

Adenosine deaminase (ADA) is an important enzyme participating in purine and DNA metabolism [1]. In the purine salvage pathway, it catalyzes the irreversible conversion of either adenosine or deoxyadenosine to inosine and deoxyinosine. Defects in this enzyme often result in an intracellular accumulation of substrates of adenosine deaminase, namely, adenosine and deoxyadenosine. These substrates are very toxic to living cells [2]. It has been suggested that deoxyadenosine toxicity causes dATP(2′-deoxyadenosine 5′-triphosphate) accumulation. The latter is a strong inhibitor of ribonucleotide reductase, causing some aberrations in DNA synthesis [3]. There is some ongoing debate as to whether it has been reported that adenosine deaminase activity is augmented in the cancerous tissues and cells [4], while other studies oppose this concept 5. ADA is also involved in the development of B and T lymphocytes, as it is evident from the fact that ADA deficient animals suffer from B and T lymphopaenia [1–5]. The levels of enzymes in T-lymphocytes vary according to

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Non-invasive bioassay of Cytokeratin Fragment 21.1 (Cyfra 21.1) protein in human saliva samples using immunoreaction method: An efficient platform for early-stage diagnosis of oral cancer based on biomedicine

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ABSTRACT

Oral cancer (OC) is considered as sixth most common cancer in the world. The challenge facing oral cancer is the lack of non-invasive, rapid, sensitive, accurate, and inexpensive screening and diagnosis methods. Given the increasing importance of prevention, prognosis, and early-stage diagnosis of cancer in improving of survival rate, the use of efficient diagnostic devices is essential. In this study, novel bioassay based on antigen and antibody immunocomplex was proposed for early stage diagnosis of OC. For the first time, an efficient immunosensor (Cys-GA-anti-Cyfra21.1-BSA-Cyfra21.1 antigen/AuE) was successfully designed and developed to the detection and determination of the Cyfra21.1 biomarker in unprocessed human saliva samples. The Au electrode was modified by Cysteamine (CysA) and Glutaraldehyde (GA) respectively via self-assembly as a substrate to immobilize the biological agents. The engineered immunosensor exhibit an excellent ability to detect and determine of Cyfra21.1 biomarker in low concentrations in unprocessed human saliva samples. Under the optimized operating conditions, the results demonstrate that the desired platform has a good sensitivity in the detecting of Cyfra21.1 with the low limit of quantitation (LLOQ) of 2.5 ng/mL, which this evaluation was performed at a wide linear range of 2.5−50 ng/mL. The use of the CysA-GA nano-hybrid as extraordinary stable substrate and extensive platform to place recognition elements was investigated using various electrochemical methods including cyclic voltammetry (CV) and square wave voltammetry (SWV). In this study, the engineered biosensor was used to non-invasive detection of Cyfra21.1 in unprocessed human saliva sample. Based on results, CysA-GA-anti-Cyfra21.1 antibody-BSA- Cyfra21.1 antigen/AuE with significantly high current intensity can provide appropriate, reliable, affordable, quick, and user-friendly diagnostic device to monitoring oral abnormality by detection and determination of Cyfra21.1 biomarker in human real sample. Above all, the easy to prepared designed immunosensor can be an extremely promising candidate to specific and favorable for a vast range of clinical diagnosis of OC in near future.

1. Introduction

Oral cancer (OC) is considered as sixth most common cancer in the world [1,2] and anatomically involves all malignancies in the lips, oral cavity, pharynx, and nasopharynx [3]. Oral squamous cell carcinoma (OSCC) is the most common and severe type of the disease [4] and generally occurs in the lateral edge of the tongue, the mouth floor, buccal mucosa and gingiva [4–6]. Furthermore this type of cancer accounts for 90 % of OC diagnostic cases [3]. Based on GLOBOCAN worldwide statistics on OC in 2012, estimated that OC accounted for 529,500 incident cases and 292,300 deaths, which is dedicated the 3.8 % of all cancer incidences, and 3.6 % of cancer deaths to itself. Also, the rate of incidence in men (4%) is two times more than women (2%), which can be related to more high-risk habits among men [7,8]. Geographical distribution is very various for the incidence of oral cancer worldwide, and the regions with the highest rates of incidences are in South and Southeast Asia. Sri Lanka, Bangladesh, Pakistan, and India are known as the countries with the highest rates of oral cancer for men, which 25 % of all cancer cases in these countries diagnosis as OC [9]. Due to the aging population and their exposure to many risk factors, it is estimated that the number of OC patients will reach 856,000 by 2035 [8].

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REVIEW

Salivaomics in oral cancer

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Purpose of review
The goal of cancer screening is to detect tumor at an early stage, and early cancer detection is the hallmark of successful treatment. In addition to traditional tissue biopsy-based diagnostics, more reliable, inexpensive, and noninvasive methods are required for early diagnosis of cancer. In this review, we highlight some of the recent advancements in the field of salivary diagnostics in oral cancer.

Recent findings
'Salivaomics' is a broad collection of technologies used to explore different types of molecules contained in saliva. Although many protein and mRNA salivary biomarkers have been identified that can detect oral squamous cell carcinoma (OSCC), none have so far been validated for current clinical use. As the heterogeneity in carcinogenesis and multifactorial cause for OSCC, the most reliable results are gathered with the use of multiple biomarker candidates to improve accuracy and sensitivity of the test used. This further requires sensitive technology to detect salivary biomarkers in low quantities.

Summary
Large scale studies that incorporate proteomic, transcriptomic, and additional 'omics,' need to be initiated to bring technology to clinical point-of-care applications.

Keywords
early detection, oral cancer, point of care, salivary diagnostics

INTRODUCTION

Oral cancer is the sixth most common cancer but carries improved prognosis when detected at an early stage. Nevertheless, early diagnosis of tumors at this site may be difficult because oral cancer may present as an asymptomatic lesion or it may locate in hard to find regions. Current diagnostics rely on tissue biopsy and histopathology, which may cause significant delays in treatment and undermine the importance of tumor heterogeneity. Understanding the molecular biology and pathogenesis behind oral cavity cancer is vital to be able to develop new therapies and thus, improve prognosis and quality of life for our patients. Point-of-care applications are required for diagnostics and management, and even for screening purposes. According to the National Institutes of Health [1], a biomarker is a characteristic that is objectively measured and evaluated as an indicator of a normal biological or pathogenic process, or pharmaceutical response to therapeutic intervention. Before any biomarker becomes accepted for its use in clinical assays, it must be verified and validated.

Saliva is an inexhaustible biofluid. It includes various components, including DNA, RNA, proteins, metabolites, and microbiota, which may be utilized for diagnostic purposes with unprecedentedly rich genetic information. It also provides real-time data of the patient's health status with a variety of possible translational applications. Saliva collection is easily accessible, repeatable, and noninvasive without any extensive equipment and handling. Therefore, saliva presents as an attractive source of disease biomarkers. In this review, we highlight some of the recent advancements in the diagnostics of oral cancer through salivary biomarkers.

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Role of Saliva and Salivary Diagnostics in the Advancement of Oral Health

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C. Dawes[1] and D.T.W. Wong[2]

Abstract

The objective of this article was to provide an account of some of the developments related to saliva over the first 100 years of the *Journal of Dental Research* and to outline some of the many biomarkers identified in saliva in the last few years. The first section covers findings in salivary physiology, biochemistry, calcium phosphate chemistry related to saliva, microbiology, and the role of saliva in maintaining oral health. The second section highlights salivary diagnostics, salivaomics, and saliva exosomics in the context of the emerging theme of personalized and precision medicine.

Keywords: parotid, submandibular/sublingual, exosomics, proteomics, salivaomics, transcriptomics

Salivary Physiology

Prior to the 20th century, little was known about human saliva physiology. However, it was known that saliva contained amylase, and the parasympathetic and sympathetic nerve supplies to most of the salivary glands had been determined, primarily by studies on animals. A few years before the first issue of the *Journal of Dental Research* in 1919, Carlson and Crittenden (1910) developed a collection device for parotid saliva (Fig. 1), which allowed the study of secretion from an individual salivary gland. However, it was not until 1955 that Schneyer developed a device for collection of submandibular and sublingual saliva, and an improved version was described by Truelove et al. (1967). The composition of secretions from minor salivary glands of the lips was first described by Dawes and Wood (1973). Veerman et al. (1996) collected and compared the compositions of stimulated parotid, submandibular, sublingual, and palatine secretions. A major stimulus for salivary physiology research, although primarily in animals, was the monograph by Burgen and Emmelin (1961).

Two pioneers who studied variations in flow rate and calcium and phosphate concentrations in human whole saliva were Becks and Wainwright, who published a series of articles in the *Journal of Dental Research* in the 1930s and 1940s. Their 1943 paper on the normal unstimulated flow rate of whole saliva is still widely quoted, and others have since confirmed their finding that about 10% of the population has an unstimulated salivary flow rate ≤0.1 mL/min, whereas the mean value in the population is 0.3 to 0.4 mL/min. However, flow rate is virtually zero during sleep (Schneyer et al. 1956).

An important study by Thaysen et al. (1954) on 3 young women showed that the concentrations of the main electrolytes (sodium, potassium, bicarbonate, and chloride) in parotid saliva elicited by beta-methyl-acetyl-choline were very dependent on flow rate. Since this is a key factor influencing saliva composition, development of a negative-feedback technique (Dawes 1967) for maintaining a constant stimulated flow rate, up to the physiologic limit of the gland, allowed study of the effects of other physiologic variables—such as flow rate itself, duration of stimulation, nature of the stimulus, circadian rhythms, previous stimulation, exercise, and stop-flow conditions—on human salivary composition.

Because of variation in nomenclature used in different branches of salivary research, a group of researchers in the field recommended a standard nomenclature (Atkinson et al. 1993), which seems to have been generally accepted.

The mechanisms by which salivary glands secrete electrolytes from plasma into saliva are rather complex, but a recent mathematical model (Vera-Sigüenza et al. 2018) appears to fit the theoretical processes and the experimental data quite well. Several neurotransmitters—including acetyl choline, norepinephrine, vasoactive intestinal peptide, substance P, and nitric oxide—act as transmitters in salivary secretion (Pedersen et al. 2018), but there is still much to be learned about the factors influencing the superior and inferior salivary nuclei in the pons and medulla. The mechanisms involved in secretion of protein by pancreatic cells (which also apply to salivary acini) were described by Jamieson and Palade (1967a, 1967b).

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Review Article

Chemokines and Cytokines as Salivary Biomarkers for the Early Diagnosis of Oral Cancer

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Chemokines have been shown to be important in both inflammation and carcinogenesis and are able to be measured in saliva with relatively robust methods including enzyme-linked immunosorbent assays (ELISA). Thus it has been hypothesized that patients with oral cancer and oral potentially malignant lesions will have elevated levels of specific chemokines in oral fluids and that this may be used as a marker of both the early detection of malignant disease and progression to malignancy. The concept that salivary biomarkers can be easily measured and indicate disease states has profound consequences for clinical practice and may open up new strategies for the diagnosis, prognosis, and potential therapy of oral squamous cell carcinoma (OSCC). This review focuses on our understanding of cytokines and chemokines and the potential role that they may have in clinical practice.

1. Introduction

Oral cancer is the eleventh most prevalent cancer worldwide [1]. Oral cancers in Australia account for approximately 2-3% of all cancers and approximately 1% of all cancer deaths, with an increasing incidence over the past decades [2]. The most common oral cancer is oral squamous cell carcinoma (OSCC), which makes up 90% of all oral cancers [3], and if diagnosed early has a five-year survival rate of around 85% [4]. However, the early phase of oral cancer is often asymptomatic. Mortality for oral cancer is high because most patients seek care only when they experience late-stage symptoms (pain, persistent ulceration, unexplained bleeding, or an oral or neck mass), at which stage the disease is advanced and the survival rate decreases as low as 15–50%. Early detection of oral cancer is therefore paramount for improving survival rates and prognosis for patients with the disease.

Current diagnostic techniques focus on detection of malignant and potentially premalignant lesions in the oral cavity. Early lesions may present as unhealing lesions, mucosal colour changes, pain, tenderness or numbness, protuberances, or rough, thickened, crusted, or eroded areas [5]. Typically, premalignant and malignant lesions begin as a subtle red or white patch (erythroplakia or leukoplakia) that eventually ulcerates and progresses to an exophytic mass [6]. Regular comprehensive examinations of the oral cavity form the backbone of oral cancer screening and are especially critical in patients with identified risk habits and factors such as tobacco smoking, excessive alcohol consumption, and human papilloma virus infection [7].

The advantage of the standard visual and tactile examination is that it is simple to perform and requires no added equipment. However, subtle lesions may pass undetected, and it is difficult to make a visual distinction between benign, premalignant, and malignant lesions. Adjunctive techniques have been developed in recent years to facilitate making this distinction and enhance the effectiveness of oral examinations. Techniques such as vital staining (Toluidine Blue) and visualisation adjuncts (VELscope and ViziLite) highlight abnormal mucosa by targeting tissues undergoing rapid cell division and areas of high metabolic turnover [8]. Another adjunctive technique employs transepithelial sampling of the oral mucosa for cytologic analysis (OralCDx Brush Test system). While promising, these emergent technologies have yet to reproduce the sensitivity and specificity of examination via tissue biopsy and histopathological examination, which remains the gold standard for oral cancer diagnosis [8].

Changes in saliva interleukin-6 levels in patients with oral squamous cell carcinoma

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Objective. The aim of this study was to elucidate changes in interleukin-6 (IL-6) levels in whole saliva during the treatment of patients with oral squamous cell carcinoma (OSCC).
Study design. Twenty-nine consecutive inpatients with OSCC were enrolled. Stimulated saliva was collected just after hospitalization (period 1), just before main treatment (surgery in 26 cases; period 2), and at the time of discharge (period 3). The mean intervals were 11 ± 8 days between periods 1 and 2 and 30 ± 18 days between periods 2 and 3. Nineteen age-matched healthy control subjects were also recruited. Interleukin-6 concentrations were measured by a highly sensitive chemiluminescent enzyme immunoassay.
Results. Interleukin-6 was detected in 23 out of 29 samples in the OSCC group in period 1. The concentration of IL-6 was significantly higher in the OSCC group (mean 20.1 ± 36.3 pg/mL) than in the control subjects (0.6 ± 0.8 pg/mL; $P = .003$). The mean concentration of IL-6 at period 2 was 43.6 ± 95.6 pg/mL, significantly higher than at period 1 ($P = .002$), and at period 3 was 17.1 ± 27.6 pg/mL ($P = .52$ [compared with period 2]).
Conclusions. Interleukin-6 was up-regulated in saliva in the OSCC patients. The IL-6 level tended to increase before treatment, and it returned to baseline levels after treatment. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:330-336)**

The number of patients with oral cancer is increasing gradually, especially in younger people.[1] Although the diagnostic modalities and therapeutic management of oral cancer is improving, the treatment outcome and prognosis of oral cancer have improved little.[2] The overall 5-year survival rates for oral cancer have remained low at ~30%-50%.[3] Some researchers have pointed out the need for a sensitive biomarker to improve early detection of oral cancer.[1-4] Circulatory tumor markers for oral squamous cell carcinoma (OSCC) were investigated in various studies and showed relatively moderate sensitivity and specificity values in relation to diagnosis, prognosis predicting, or treatment monitoring.[1-6] Saliva has many advantages as a sample over both serum and tissues.[7] Saliva is relatively easy to collect in sufficient quantities for analysis even in the small clinic or the laboratory.

It is recognized that numerous cytokines have various roles in the diseases of the oral mucosa.[8,9] Interleukin-6 (IL-6) is a multifunctional cytokine that participates in the inflammatory and immune responses.[10-12] Proinflammatory cytokines, such as IL-6, have been shown to directly promote the growth of certain types of cancer and are associated with an increased rate of metastasis.[2,13] However, IL-6 has dual effects, inhibiting the growth of some cells while stimulating the growth of others.[14] IL-6 is currently being assessed for its ability to promote or inhibit various types of tumors.[2,4,9] Studies indicate that the concentrations of IL-6 in serum and saliva are significantly elevated in patients with oral neoplastic and preneoplastic lesions compared with control subjects.[8,9,15-18] Rhodus et al.[9] reported that patients with OSCC, as well as oral preneoplastic lesions, had significantly higher salivary levels of IL-6 than control subjects. They suggested that the progression of OSCC may be enhanced by the continued expression of the proinflammatory cytokines. St. John et al.[2] examined IL-6 at the mRNA and protein levels in both the serum and the unstimulated saliva of OSCC patients and age- and gender-matched controls. They demonstrated that IL-6 at both mRNA and protein levels was detected in higher concentrations in the

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Review

The Challenges of OSCC Diagnosis: Salivary Cytokines as Potential Biomarkers

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Abstract: Fast, economic, and noninvasive, molecular analysis of saliva has the potential to become a diagnostic tool of reference for several local and systemic diseases, oral cancer included. The diagnosis of Oral Squamous Cell Carcinoma (OSCC) can be performed using high specificity and sensibility biomarkers that can be encountered in the biological fluids. Recent advances in salivary proteomics have underlined the potential use of salivary biomarkers as early diagnosis screening tools for oral neoplasia. In this respect, over 100 salivary molecules have been described and proposed as oral cancer biomarkers, out of which cytokines are among the most promising. Besides being directly involved in inflammation and immune response, the role of salivary cytokines in tumor growth and progression linked them to the incidence of oral malignant lesions. This review summarizes the existing studies based on the use of salivary cytokines as potential oral cancer biomarkers, their involvement in the malignant process based on their type, and ther influence upon prognostic and metastatic rates.

Keywords: saliva; cytokines; biomolecules; biomarkers; oral cancer; early diagnosis; screening; noninvasive collection

1. Introduction

Diagnostics based on the analysis of saliva represent one of the most important promises of modern personalized medicine, with a potential impact on specific areas like screening, early diagnosis, therapy and post-therapy monitoring, and prognostic.

Salivary diagnosis has multiple advantages over traditional serum and tissue samples [1]. Besides being fully noninvasive and requiring little effort from the patient, analysis of salivary samples is a cost-effective approach, mainly due to easy harvesting, storage, and transfer. Saliva-based diagnostics has been the focus of present researches due to both proven good correlations of existing salivary molecules with blood levels and the existing connections with several systemic diseases, including oral cancer [1,2]. The permanent contact between saliva and the oral environment, especially malignant lesions, creates an opportunity for the development of screening, diagnostic, and monitoring tools with high sensitivity and specificity [3].

Oral cancer is a major global public health problem, ranking sixth among human malignancies, with a 5-year mortality rate, close to 50% [4]. Oral malignancies include all lesions encountered in the

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Research Paper
Head and Neck Oncology

Evaluation of saliva and plasma cytokine biomarkers in patients with oral squamous cell carcinoma

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Abstract. The aim of this study was to investigate potential biomarkers in human saliva and plasma to aid in the early diagnosis of oral squamous cell carcinoma (OSCC). Saliva and plasma samples obtained from OSCC patients (n = 41) and non-oral cancer patients (n = 24) were analyzed by Luminex Bead-based Multiplex Assay. Data were analyzed using the non-parametric Mann–Whitney U-test, Kruskal–Wallis test, and receiver operating characteristics curve (ROC) to evaluate the predictive power of 14 biomarkers individually for OSCC diagnosis. The plasma level of IP-10 in early OSCC differed significantly from that in controls. Among the salivary biomarkers, IL-1β, IL-6, IL-8, MIP-1β, eotaxin and IFN-γ and TNF-α showed significant differences between OSCC patients and controls. With respect to carcinogenesis, significant differences in plasma levels of eotaxin, G-CSF, and IL-6 were found between OSCC stages III/IV and OSCC stages I/II. The area under the curve (AUC) for OSCC vs. control was greater than 0.7 for plasma IP-10 and saliva IL-1β, IL-6, IL-8, and TNF-α. The study findings indicate that salivary biomarkers may serve a useful role as a complementary adjunct for the early detection of oral OSCC. With regard to the evaluation of tumour progression, plasma eotaxin, G-CSF, and IL-6 may help in the detection of advanced OSCC. However, the correlation between saliva and plasma biomarkers in OSCC was weak.

Key words: Luminex Bead-based Multiplex Assay; salivary biomarker; plasma biomarker; oral squamous cell carcinoma; ROC.

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According to the Health Promotion Administration of Taiwan, oral cancer is the fifth most prevalent cancer in the country (ranking fourth in males). When oral squamous cell carcinoma (OSCC) patients are diagnosed in the advanced stage, the prognosis is poor. Early diagnosis and early treatment are important, resulting in superior outcomes in OSCC patients. Risks factors for OSCC include alcohol consumption, betel nut chewing, and cigarette smoking. OSCC may also arise from a molecular mutation that leads to carcinoma[1].

[a] Man-Yee Chan and Kuo-Wei Chang contributed equally to the study.

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Proteomic identification of salivary transferrin as a biomarker for early detection of oral cancer

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ABSTRACT

Oral cancer has a low five-year survival rate. Early detection of oral cancer could reduce the mortality and morbidity associated with this disease. Saliva, which can be sampled non-invasively and is less complex than blood, is a good potential source of oral cancer biomarkers. Proteomic analysis of saliva from oral cancer patients and control subjects was performed to identify salivary biomarkers of early stage oral cancer in humans. The protein profile of pooled salivary samples from patients with oral squamous cell carcinoma (OSCC) or OSCC-free control subjects was analyzed using two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. Potential biomarkers were verified by Western blotting and ELISA assays. Transferrin levels were elevated in the saliva of OSCC patients as determined using 2DE followed by MALDI-TOF MS and confirmed by MALDI-TOF/TOF MS, Western blotting and ELISA. The increase in salivary transferrin levels in OSCC patients strongly correlated with the size and stage of the tumor. The area under the receiver-operating characteristics curves showed that salivary transferrin-based ELISA was highly specific, sensitive and accurate for the early detection of oral cancer. We have identified salivary transferrin as a biomarker for the detection of early stage oral cancer. This finding provides a promising basis for the development of a non-invasive diagnostic test for early stage oral cancer.

1. Introduction

Oral cancer accounts for 2–3% of all malignancies [1]. There are more than 300,000 patients newly diagnosed with oral cancer annually worldwide [2]. Squamous cell carcinoma accounts for 90% of these cases [3]. Common risk factors for oral cancer include tobacco, alcohol and chewing betel quid. The risks are synergistic and might result in large areas of mucosal change or stimulate carcinogenesis in the oral cavity [4,5]. Therefore, oral cancer patients carry a high risk of developing a secondary cancer in the upper aerodigestive tract [6–9]. In accordance with the multi-step theory of carcinogenesis, oral cancer develops from premalignant lesions and causes serial histological and clinical changes [10]. Clinically, premalignant lesions might present as leukoplakia or erythroplakia, but histologically these lesions have various manifestations such as hyperkeratosis, dysplasia, or even carcinoma [10].

The five-year survival rate of patients suffering from oral cancer is as low as 50%, and has not improved significantly in recent decades, despite advances in surgery, radiotherapy and chemotherapy [11–13]. The expected survival rate for patients with advanced staged oral cancer is far lower than that of laryngeal or nasopharyngeal carcinoma, even for those patients achieving complete clinical remission after local therapy [14]. Early detection of oral mucosal lesions followed by appropriate treatment can increase the recovery rate to 80–90% [12]. Consequently, early diagnosis of malignant or premalignant lesions could reduce the mortality and morbidity associated with oral cancer.

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Investigation and identification of potential biomarkers in human saliva for the early diagnosis of oral squamous cell carcinoma

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ABSTRACT

Background: Oral cancer is 1 of the 6 most common human cancers, with an annual incidence of >300,000 cases worldwide. This study aimed to investigate potential biomarkers in human saliva to facilitate the early diagnosis of oral squamous cell carcinoma (OSCC).
Methods: Unstimulated whole saliva obtained from OSCC patients (n = 30) and apparently healthy individuals (n = 30) were assayed with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) in hydrophilic interaction chromatography mode. The data were analyzed using a nonparametric Mann–Whitney *U* test, logistic regression, and the receiver operating characteristic (ROC) to evaluate the predictive power of each of 4 biomarkers, or combinations of biomarkers, for OSCC screening.
Results: Four potential salivary biomarkers demonstrated significant differences ($P < 0.05$) in concentrations between patients at stages I–II and the healthy individuals. The area under the curve (AUC) values in control vs OSCC I–II mode based on choline, betaine, pipecolinic acid, and L-carnitine were 0.926, 0.759, 0.994, and 0.708, respectively. Four salivary biomarkers in combination yielded satisfactory accuracy (0.997), sensitivity (100%), and specificity (96.7%) in distinguishing OSCC I–II from control.
Conclusions: Salivary metabolite biomarkers for the early diagnosis of OSCC were verified in this study. The proposed approach is expected to be applied as a potential technique of preclinical screening of OSCC.

1. Introduction

Human saliva is a multi-component oral fluid, which has high potential for the early diagnosis of diseases. In recent years, there have been many studies of disease diagnosis using salivary biomarkers in lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4], Sjögren's syndrome [5], etc. Over 90% of oral cancers are oral squamous cell carcinoma (OSCC), which is one of the six most common human cancers, with an annual incidence of over 300,000 cases worldwide [6,7]. OSCC occurs in the lips, oral cavity, and pharynx, and has a relatively high rate of related morbidity. The World Health Organization has reported that OSCC has one of the highest mortality rates among other malignancies, with a 5-year mortality rate of approximately 50% [8]. Therefore, the early detection or prevention of this disease and the screening of high risk populations with precancerous lesions will be the most effective strategy.

The increasing worldwide incidence of OSCC urgently demands the discovery of new biomarkers. Saliva is a noninvasive and stress-free alternative to plasma and serum, and is widely accepted as a potential medium for clinical diagnostics. It also has the advantages of being simple to collect, easy to store, and less expensive compared with blood sample collection [9–11]. Saliva is secreted primarily by three major glands, i.e., the parotid gland, submandibular gland, and sublingual gland [12,13]. In general, the flow rate of unstimulated saliva is 0.3 ml/min. Saliva contains approximately 99% water as well as minerals, nucleic acids, electrolytes, mucus, and proteins [14]. It is one of the most complex, versatile, and important body fluids, which reflects a large range of physiological needs and information. Therefore, saliva is also known as the "mirror of the body".

At present, the standard method for OSCC diagnosis and screening is time-consuming and requires extensive experience. Therefore, modern high-throughput metabolomics approaches have been used extensively to observe the altered expressions of various metabolites in a range of cancers, including OSCC, with varying degrees of sensitivity and specificity. Metabolomics is a new platform for studying systems biology, which facilitates high throughput screening processes in the pharmaceutical industry and in clinical diagnosis [15,16]. The major analytical techniques used for metabolomics investigations are based on ^{1}H nuclear magnetic resonance (NMR) spectroscopy [17–19], LC–MS [20–22], and GC–MS [23]. Principal component analysis and orthogonal partial least squares discriminant analysis are used most frequently to screen for biomarkers of disease [24]. Sugimoto et al. used capillary electrophoresis mass spectrometry to discriminate individuals with oral cancer from healthy control and 28 salivary metabolite biomarker

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Potential Salivary mRNA Biomarkers for Early Detection of Oral Cancer

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Abstract: We evaluated potential biomarkers in human whole saliva for the early diagnosis of oral squamous cell carcinoma (OSCC). We selected 30 candidate genes with relevance to cancer from recent reports in PubMed. Saliva samples were obtained from 34 non-tumor control and 33 OSCC patients. Real-time PCR was performed, and mRNA levels were compared. Normalized mRNA levels of six genes (NGFI-A binding protein 2 (NAB2), cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1), nuclear pore complex interacting protein family, member B4 (NPIPB4), monoamine oxidase B (MAOB), sialic acid acetyltransferase (SIAE), and collagen, type III, alpha 1 (COL3A1)) were significantly lower in saliva of OSCC patients. Receiver operating characteristics (ROC) analysis was used to individually evaluate the predictive power of the potential biomarkers for OSCC diagnosis. The area under the curve (AUC) values were evaluated for the OSCC vs. non-tumor groups via univariate ROC analyses, as well as multivariate ROC analyses of combinations of multiple potential biomarkers. The combination of CYP27A1 + SIAE showed a favorable AUC value of 0.84. When we divided saliva samples into two groups according to age using a 60-year cut-off, with OSCC patients and controls evaluated together, the AUC of MAOB–NAB2 was more predictive of OSCC in the under-60 group (AUC, 0.91; sensitivity, 0.92; and specificity, 0.86) than any other gene combination. These results are expected to aid the early diagnosis of OSCC, especially in patients under 60 years of age. While more studies with larger numbers of patients are necessary, our result suggest that salivary mRNA would be a potent biomarker for early OSCC diagnosis.

Keywords: oral squamous cell carcinoma; saliva; early diagnosis; mRNA; area under the curve

1. Introduction

Despite the many advances in cancer treatment, the five-year survival rate for patients with oral squamous cell carcinoma (OSCC) has improved only marginally. Therefore, there is critical need to identify markers for the early diagnosis of OSCC. Biomarkers are molecular signatures and indicators of normal biological and pathological process, and thus may provide useful information for the detection, diagnosis, and prognosis of disease. Many studies have attempted to identify cancer biomarkers in non-invasive samples [1–4]. Saliva has direct contact with OSCC lesions, which gives it particular potential as a specific and sensitive screening tool. While more than 100 potential salivary biomarkers (DNA, RNA, mRNA, miRNA, and protein) have been previously identified for OSCC (reviewed in [5–8]), further research is required to validate biomarkers for clinical applications.

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Loss of heterozygosity as a marker to predict progression of oral epithelial dysplasia to oral squamous cell carcinoma

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Head and neck squamous cell carcinoma is the sixth most common malignancy, with an annual incidence of 300,000 new cases[1,2,3] diagnosed worldwide, with particularly high incidence rates in South and Southeast Asia, Europe, Latin America and the Caribbean and Pacific nations. Globally varying studies have been addressing the fundamental aspects of this malignancy with respect to its prevention, early diagnosis and management.[1,2,3,4] Although the effort taken is phenomenal, the morbidity and mortality associated with oral squamous cell carcinoma (OSCC) is discouraging.[5] The survival rate is directly proportional to the stage of the disease at the time of diagnosis. It is 80% for Stage I cancers but drops to 20% for Stage IV cancers.[6] Unfavorable outcome due to the disease is further burdened by the morbidities, accompanying deformities due to surgery and those seen after radiation as complications, namely, mucositis and osteoradionecrosis, which have a deleterious impact on the quality of life of the affected individual.[3]

The term oral potentially malignant disorders (OPMDs) was recommended at the World Health Organization (WHO) workshop held in 2005.[7] An oral premalignant lesion is defined as any lesion or condition of the oral mucosa that has the potential for malignant transformation (MT). This encompasses a number of oral lesions, such as leukoplakia, erythroplakia, erythroleukoplakia, erosive lichen planus, oral submucous fibrosis and oral dysplasia. OPMDs are a spectrum of lesions and conditions of the oral mucosa, which are characterized by an increased risk of MT to OSCC of which leukoplakia and atrophy, hyperplasia or dysplasia. It has been established that OPMDs[11] could be the precursor lesions of OSCC.[12] These lesions are predominantly associated with habits, namely, chronic use of tobacco and excess consumption of alcohol.[13] OPMDs are also described as a group of disorders of varying etiologies, characterized by mutagen associated, spontaneous or hereditary alterations or mutations in the genetic material of oral epithelial cells with or without clinical and histopathological alterations that may transform to OSCC.[14] Of the OPMDs, oral leukoplakia is the most prevalent one with a prevalence ranging from 0.4% to 2.6% of the population worldwide, with a MT rate between 3.0% and 17.5%.[8,15,16]

Preferred and accepted marker to assess the risk of an OPMD eventually undergoing MT is the presence and grade of dysplasia in the lesion. Dysplasia is defined as the presence of specific epithelial architectural and cytologic changes and is graded as mild, moderate, or severe based on the depth and severity of the changes. It is frequently assumed that oral carcinogenesis involves OPMDs that undergo a gradual progression beginning with hyperplasia and evolving through stages of mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma *in situ* and finally carcinoma after cellular invasion through the basement membrane. Till today, the pronounced and accountable predictor of MT in a mucosal lesion is epithelial dysplasia which is described by WHO as a spectrum of architectural and cytological epithelial changes caused by accumulation of genetic changes, associated with risk of progression to OSCC. The WHO has now introduced the binary system of dysplasia grading into high grade and low grade, to address and overcome the challenges and limitations of the three grading systems.[17] The truth, sometimes, is that the progress of dysplasia to cancer does not necessarily occur in a systematic way and dysplasia in all OPMDs does not progress to OSCC.

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Early diagnosis in primary oral cancer: is it possible?

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Abstract

In this treatise oral carcinogenesis is briefly discussed, particularly with regard to the number of cell divisions that is required before cancer reaches a measurable size. At that stage, metastatic spread may have already taken place. Therefore, the term "early diagnosis" is somewhat misleading.

The delay in diagnosis of oral cancer is caused both by patients' delay and doctors' delay. The total delay, including scheduling delay, work-up delay and treatment planning delay, varies in different studies, but averages some six months. The total delay is more or less evenly distributed between patients' and doctors' delay and is partly due to the unawareness of oral cancer among the public and professionals, and partly to barriers in the health care system that may prevent patients from seeking dental and medical care. Due to the relatively low incidence of oral cancer it will be difficult to increase the awareness of this cancer type among the public, thereby reducing patients' delay. However, it should be possible to considerably reduce doctors' delay by increasing the awareness of oral cancer among professionals and by improving their diagnostic ability.

Population-based annual or semi-annual screening for oral cancer is not cost-effective, high-risk groups such as heavy smokers and drinkers perhaps excluded. Dentists and physicians, and also oral hygienists and nurse practitioners, may play a valuable role in such screening programs.

Key words: *Oral cancer, early detection of cancer, diagnostic cancer delay.*