KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of Imaging & Pathology
Omfs Impath Research Group

Salivary markers for detection of oral (pre)cancerous lesions

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LIST OF ABBREVIATIONS

8-OHdG: 8-Hydroxy-2-Deoxyguanosine

BMI: Body Mass Index

ELISA: Enzyme-Linked Immunoassay

EMBASE: Excerpta Medica Data

IFN-γ: Interferon-γ

IL-6: Interleukin 6

IL-8: Interleukin-8

MDA: Malondialdehyde

MEDLINE: Medical Literature Analysis and Retrieval System Online

PUBMED: Publisher Medline

SCC: Squamous Cell Carcinoma

TBA: Thiobarbituric Acid

TNF-α: Tumor Necrosis Factor Alpha

TNM: Tumor Nodes Metastasis

PX: Protoporphyrin

TUNEL: Terminal Deoxynucleotyl Transferase dUTP Nick End Labeling

IL-IB: Interleukin 1 Beta

DUSP1: Dual Specificity Protein Phosphatase 1

HA3: Hyaluronic Acid 3

OAZ1: Ornithine Decarboxylase Antizyme 1

S100P: S100 Calcium Binding Protein P

SAT: Satellite Transcript

DEFINITIONS

Sensitivity is measuring the proportion of positives that are correctly identified (1).

Specificity is measuring the proportion of negatives that are correctly identified (1).

Robustness is defined as the ability to reproduce the method in different places, i.e. laboratories or under different conditions without changes in obtained results (1).

Convenience method is a non-probability method, where subjects are selected because of their convenient accessibility and proximity to scientist (1).

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CHAPTER 1

General introduction, aims and hypothesis

Abstract

The following dissertation detailed the role of salivary biomarkers associated with oral cancer and pre-cancerous lesions. In the following section, the motivation for this project was provided underlining the Indian prospects besides global significance of oral cancer. In addition, oral cancer biology and its clinical pathology was briefly discussed. The main focus of this chapter was cancer detection and diagnosis in relation to the gold standard histological diagnosis. The need for new low-threshold and easily applicable diagnostic techniques is identified with a specific focus on salivary biomarkers.

Introduction

Oral cancer can be described as a cancer that develops in tissues of the oral cavity or the oropharynx. It is the sixth most common cancer in the world and about 90% of these are being classified as squamous cell carcinomas (1, 2). In India, oral cancer is the most common fatal cancer between 30-69 years old (23%). Tobacco-related cancer represents 42% of the male and 19% of the female cancer deaths in India, with twice as many deaths from oral cancer as from lung cancer (3). High prevalence rates of oral cancer are noted in relation to major risk factors such as chronic tobacco and alcohol consumption (4), with a pronounced synergistic effect of smoking and alcohol consumption on oral cancer risks (3). The prognosis of patients suffering from oral cancer is very poor (4), with low survival rates, notwithstanding significant advancements in diagnostics, treatment and management of oral cancer (2-4). Poor prognosis and low survival rates may be related to detection of oral pre-cancer and cancer in advanced stages. Likewise, early prevention and early detection are of outmost importance in relation to treatment, management and survival of oral cancer (5). Improvement of early detection of cancer by development of new diagnostic methods based on tumor biomarkers might aid in early diagnosis by providing molecular-level changes such as biochemical and cellular changes associated with oral carcinogenesis (6). Molecular and biochemical changes start much earlier than histological changes and clinical appearance. Hence, diagnostic tools based on biomarkers in body fluids such as saliva, could provide new ways for oral cancer detection at early stages, thus allowing earlier intervention and improved survival rate (7).

Background on oral cancer biology

***Epidemiology and etiology*:** Head and neck cancers are reported as fourth major cancer in Indian males (8). Oral cancer more specifically ranks on top of all cancers in India, accounting for more than 30% of all cancers reported (8). Also, oral cancer is also 6 times more common in India as compared to USA and European countries (8-9). The increased prevalence of oral cancer in India may be caused by exposure to sunlight, smoking and other smokeless tobacco habits, alcohol, spicy food, and neglect of oral health (8-9). It has been reported that men show greater prevalence than women considering their higher proportion of smoking and drinking habits (10). Interestingly, risk factors act individually or synergistically up to 100 times in heavy smokers and heavy drinkers (10). Furthermore, in North Indian

populations, increasing rates of cancer may also be linked to dental quackery and unsterilized methods.

***Treatment and prognosis*:** A detailed review of treatment and prognosis of oral pre-cancer and cancer is beyond the scope of this thesis and has been published in different scientific articles (5). Briefly, radiation and surgery therapies are the main treatment modalities for early stage of oral cancer with stage I-II and ≤ 4 cm in size and localized (11). Treatment of localized oral cancer with stage III-IVA/B needs a multidisciplinary approach such as combination of surgery, radiation and chemotherapy (5). The emergence of molecular targeted therapeutics i.e. Erbitux® might aid in efficacy of current treatments with decrease in treatment-related cytotoxicity (5).

***Oral cancer biomarkers*:** Recently, advanced researches based on complex cellular mechanisms associated with staging of tumorigenesis enlightening different biomarkers have been proposed (12-15). Different biomarkers have been reported in oral cancer for diagnostic, prognostic, and therapeutic potential. Biomarkers can be divided based on different staging of oral cancer such as cell proliferation, differentiation, growth and signaling pathways, apoptosis, angiogenesis, and extracellular matrix degradation and migration (6). As discussed later in the dissertation, salivary biomarkers are selected as a proof-of-concept biomarker for early detection of pre-cancer and cancer based on different proposed pathways of pathology (15-20). Evidently, carcinogenesis is a complex multi-step process and different pathways such as free radical, inflammatory etc., have been proposed. Therefore a single biomarker is not sensitive and specific enough for diagnosis or detection of oral cancers. So, there is need for potentially combining multiple biomarkers for early detection, diagnosis and screening of oral cancer (19-21). However, current diagnostic methods in conjunction with salivary biomarkers might improve early detection of oral cancer as such to not be solely depending on clinical examination and histopathological diagnosis. Different oral pre-cancer and cancer biomarkers have been identified in tissue, oral cavity and body fluids (15-23). Especially, in case of oral (pre-)cancer, saliva is an ideal fluid for biomarker identification because it is localized to the pre-cancer and cancer sites. Other methods such as exfoliated cytology uses saliva for detection of oral epithelial cells (17). Distinct biomarkers have been reported in cell signaling and tumorigenesis pathways indicating that whole cells in saliva act as useful diagnostic tools, though the usefulness of technique for early detection needs to be explored further (23). Despite available research on saliva-based biomarkers of oral pre-cancer and

cancer with diagnostic and prognostic values, (13, 14) very few or none have effectively been translated into clinical practice (14). It could be due to high complexity of many biomarker assay formats, validation of biomarkers, variation of collection, storage and analysis methods (24). Adjunctive techniques for oral cancer detection and diagnosis of oral cancer and pre-cancer such as OralCDx® Brush Biopsy (CDx, New York,USA , ViziLite® (Zila Pharmaceuticals, Phoenix, AZ), VELscope® (LED Dental, White Rock, British Columbia, Canada) and other methods have been introduced (25). It is beyond the scope of this thesis to further discuss these techniques. In brief, optical based technologies have the advantage of being real-time, minimally invasive, user-friendly, cost-effective and useful assisting biopsy (26). This technology is distinguishing diseased oral mucosa from healthy oral mucosa with sensitivity 82-100% and specificity 63-100%, but it fails to discriminate oral pre-cancer and cancer (26, 27). Toluidine Blue is metachromatic dye, which selectively stains acidic tissue components of the DNA and RNA. This technique is useful as an adjunct tool with limited specificity, considering the tendency to bind to tissues undergoing rapid cell division in case of inflammatory and regenerative tissues (28). Oral cytology techniques in which samples of oral tissues are taken for histomorphological, cytomorphometry, DNA cytometry, and immunocytochemical analysis, have limitation for detection of dysplastic lesions with variable false-positive and false-negative results (26-28).

The focus of the present thesis is put on the potential diagnostic value of salivary biomarkers in detection of oral pre-cancer and cancer. The development of salivary diagnostic tools for diagnosis is of paramount importance, especially for high-risk populations. Salivary based tools are those focused on measuring changes of specific salivary biomarkers such as proteins or nucleic acids (as fatty acids are rather scarce in saliva), vitamins, ions, genomic or proteomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratines, mRNA's, DNA aberrations, etc. (29-37). It has been reported that the levels of certain inflammatory, proangiogenic cytokines and other biomarkers in saliva and tissue specimens of patients with oral premalignant and malignant lesions are elevated. Such biomarkers show a relatively moderate sensitivity and specificity relative to diagnosis, prognosis prediction and treatment monitoring (30). Though different biomarkers have been proposed, very few studies exist on validation of salivary biomarkers for diagnosis of precancerous and cancerous lesions. Therefore, the overall purpose of this thesis was validation of different salivary biomarkers for diagnosis and monitoring of oral pre-cancerous

and cancerous lesions, with a specific focus on applying this in rural areas such as in Northern India.

Consequently, all data for this thesis have been collected from free dental camps organized in rural areas considering high prevalence of oral cancer (Schools, nearby villages such as Gaura, Paharipur, Bija, Seenpur, Rasulpur Chishti, Nurpur Dona and Sukhia Nangal, Urban slums, Orphanages and Old Age Homes) in the North India by Sant Baba Nidhan Singh Mission Hospital and JBR Health Education and Research Society. The ethical approval was taken from the Sant Baba Nidhan Singh Mission Hospital and JBR Health Education and Research Society (regd.) (Figures 1-7).

Figure 1.1. Sant Baba Nidhan Singh Mission Hospital

Figure 1.2. Laboratory

Figure 1.3. Mission Hospital


ਧਾਲੀਵਾਲ ਦੋਨਾ 'ਚ ਲਗਾਇਆ ਮੈਡੀਕਲ ਚੈਕਅੱਪ ਕੈਂਪ

ਉਦਘਾਟਨ

Figure 1.4. Media Publication of Camps

Figure 1.5. Camp in Remote Area

Figure 1.6. Patient Registration in Camp

Figure 1.7. Patients in Free Camp

General hypothesis and specific aims of the thesis

For early detection of oral pre-cancer, visual examinations often remain inadequate. That is why different examining devices have been designed to try and help serving the same aim. Meanwhile, histopathological examination is still acting as a gold standard for diagnosis of oral pre-cancer and cancer. Yet and in rural areas with a largely poor and poorly educated population, the use of the latter technique is not always straightforward in terms of economical but also cultural factors. That is why one might have to look to other diagnostic indicators, which are simple to use and easy to obtain from the patients, allowing for

screening with an aim to enable oral cancer detection at an early stage. Blood and serum based biomarkers surely offer a diagnostic potential to aid early detection of oral pre-cancer and cancer. Yet, these present challenges such as high cost, complexity of many biomarker assay formats, expert training, invasiveness and time consumption thus preventing their widespread application in oral cancer diagnostics and screening. Therefore, other approaches toward oral pre-cancer and cancer biomarker detection may be required. Indeed, similar to blood and serum based biomarkers, salivary biomarkers might offer a diagnostic potential to aid early detection of oral pre-cancer and cancer. Salivary biomarkers based technologies to early detection of oral pre-cancer and cancer exploit a combination of biomarkers deriving from a noninvasive saliva collection. As biochemical or molecular changes might occur relatively early during tumorigenesis, early detection of oral pre-cancer and cancer could be anticipated.

As such, this PhD thesis attempts to test the hypothesis of clinical applicability of different salivary biomarkers for detection and diagnosis of oral pre-cancerous and cancerous lesions. The overall objective is to utilize previously characterized oral cancer-specific biomarkers in the saliva as a validation tool for potential future diagnosis. This thesis might thus contribute towards the growing field of in vitro diagnostics using saliva-based biomarkers. It is stressed that the latter may have a great potential for cancer screening in third world countries, or remote areas with limited access to oral health care.

Research Questions

In order to reach the goals of the present thesis, several research questions have been put forward and need to be addressed regarding selection of biomarker inputs, detection modality, accuracy of detection by comparison with currently available techniques, and potential roles in clinical practice. Research questions formulated are:

1. What is the state of the art with regard to salivary biomarkers and cancer detection? (Chapter 2)

2. Can imaging techniques, molecular techniques and saliva testing provide sufficient diagnostic validity for early clinical diagnosis of oral precancerous and cancerous lesions? (Chapter 3)

3. Can salivary biomarkers be correlated to tissue biomarkers? (Chapter 3)

4. Can a unique salivary biomarker be identified for early detection of oral precancerous and cancerous lesions? (Chapter 4, 5, 6 and 7)

5. Can a combination of salivary biomarkers be used for early detection of oral precancerous and cancerous lesions? (Chapter 7)

Hypotheses addressed in this PhD thesis

Chapter 1. To explain the usefulness of noninvasive diagnostic techniques (imaging and saliva based biomarkers) for early detection and diagnosis of pre-cancer and cancer.

Chapter 2. To probe the overview and usefulness of saliva based biomarkers in oral pre-cancer and cancer.

Hypothesis: Salivary biomarkers could be used independently for diagnosis of oral pre-cancer and cancer, but saliva collection and handling process, analysis techniques, single biomarker and combination of biomarkers need to be standardized for validation.

Chapter 3. To find out the relationship between fluorescence imaging and protoporphyrin IX levels in tissues, serum and saliva of oral precancerous and cancerous patients as well as to find out the role of combination of autofluorescence imaging and salivary protoporphyrin (PX) in detection of oral precancerous and cancerous lesions.

Hypothesis: The PX levels in serum, salivary and tissues may be higher in precancerous and cancerous lesions as compared to normal healthy tissues. The sensitivity and specificity to the discrimination of precancerous and cancerous lesions from the healthy tissues may be higher by combination approaches of salivary protoporphyrin X and VELscope® system as compared to either salivary protoporphyrin X or VELscope® approach only.

Chapter 4. To find out the salivary apoptotic cells in oral precancerous and cancerous patients and furthermore to observe the potential diagnostic value of salivary apoptotic cells in detection of oral pre-cancer and cancer.

Hypothesis: The number of apoptotic cells may be significantly less in OSCC as compared to precancerous and normal healthy tissues.

Chapter 5. To evaluate serum and salivary leptin concentrations and other factors which may be associated with weight loss in patients with squamous cell carcinoma of buccal mucosa.

Hypothesis: Salivary and serum leptin levels may be lower in patients with squamous cell carcinoma of buccal mucosa as compared to controls.

Chapter 6. To evaluate salivary and serum concentrations of interleukin-8 (IL-8), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in patients with oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis and healthy controls.

Hypothesis: Salivary and serum IL-8, IL-6, TNF-α may be higher in patients with precancerous oral lesions than in a control group.

Chapter 7. To analyze oxidative DNA and lipid damage by using Salivary 8-hydroxy-2-deoxyguanosine (8-OHdG), MDA (Malondialdehyde), Vitamin C and E in oral lichen planus lesions, oral leukoplakia, oral submucous fibrosis, oral squamous cell carcinoma (SCC) and controls and in addition to determine the value of salivary biomarkers in diagnosis of oral pre-cancer and cancer patients.

Hypothesis: Squamous cell carcinoma and precancerous patients may have increased levels of salivary 8-OhdG and MDA, while lower levels of vitamin C and E as compared to normal subjects.

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CHAPTER 2

Salivary biomarkers for oral cancer detection: a review of the literature

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Kaur J, Jacobs R, Huang Y, Salvo N, Politis C. Salivary biomarkers for oral cancer and pre-cancer screening: a review of the literature. Clin Oral Investig 2018;22:633-640.

Abstract

The objective of the study was to conduct a systematic review of the literature assessing potential salivary biomarkers of oral cancer and pre-cancer, and discuss emerging issues and challenges in relation to oral cancer and pre-cancer diagnostics.

Search for articles involved the Medline, PubMed, and EMBASE. Specific terms were used from January 1995 to March 2017 by three experts. This search collected 270 articles, of which 105 articles such as reviews, case reports, news, letter to editor in first round and 117 articles such as publications in other languages than English and non-human studies were excluded. The remaining 48 articles considered analyzing whole saliva as well as specific gland saliva. Thirty-one studies considered oral stimuli such as eating, drinking, and oral hygiene practices for varied periods of time prior to sample collection. The time of collection of saliva was morning in most studies, but the exact time of collection was not mentioned. Three studies showed to have evaluated the whole saliva without centrifugation. Two-dimensional Gel Electrophoresis and tandem Mass Spectrometry were most commonly used methods. Most of the potential salivary biomarkers of oral cancer are salivary proteins. Combination approach of salivary biomarkers could be used as screening tool to improve early detection and diagnostic precision of oral pre-cancer and cancer. The current findings are of importance for clinicians and researchers to mitigate the challenges in salivary based diagnosis of oral cancer and to evaluate reliable, specific and sensitive salivary biomarkers for oral pre-cancer and cancer diagnosis.

Introduction

Oral cancer is a serious global public health problem and it is the sixth most common human malignancy with a five-year mortality rate of approximately 50% [1]. In India, the standardized incidence rate of oral cancer is 12.6 per 100,000 inhabitants [2]. Tobacco consumption, alcohol, and human papilloma virus infections are major risk factors for oral squamous cell carcinoma [3]. Most oral carcinomas develop from oral premalignant lesions such as leukoplakia, erythroplakia, and lichen planus [4,5]. Oral cancer is detected at a late stage due to the lack of awareness of the symptoms and risk factors among the public as well as lack of prevention and early detection by oral physician and healthcare providers [6,7]. Latest innovations in oral cancer research have directed to the development of possible useful diagnostic tools at the clinical and molecular level for the early detection and diagnosis of oral cancer. Tissue biopsy with histological assessment is a gold standard for oral cancer diagnosis, but this technique requires specific training and is invasive, painful, time consuming and expensive [8]. Clinical diagnostic technologies for early detection of oral cancer are oral transepithelial brush biopsy kits such as. Oral CDx® brush biopsy, tolonium chloride or toluidine blue dye, salivary diagnostics and lastly optical imaging systems [9-11]. All these methods have their own advantages and disadvantages; unfortunately, these non-invasive tools have failed in their practical implication in the community setup as well as remote areas, as patients are still being diagnosed in advanced stages of oral cancer. Taking into account all mentioned criteria, there is need of early detection of pre-cancer and cancer by developing salivary biomarkers [12-15]. Various saliva based biomarkers for early detection of oral pre-cancer and cancer have been proposed [13-37]. From the late 1992 until present, more than 100 studies have been published on more than 120 biomarkers of saliva (salivary constituents, proteomic, transcriptomic, genomic and metabolomic analyses) and these have been suggested as potential diagnostic tools of oral cancer and pre-cancer diagnosis. Many reviews have been published on evolution and advancement of salivary proteomic, metabolomic, genomic and transcriptomic research for diagnosis and detection of oral cancer and pre-cancer. This review offers an up-to-date list of potential saliva based biomarkers of oral pre-cancer and cancer. The main objective of this review is to assess the issues concerning saliva based diagnosis in oral cancer and pre-cancer which can further contribute to find the reliable salivary biomarkers for early detection of oral pre-cancer and cancer.

Materials and Methods

The following databases were searched: Medline, PubMed, and EMBASE (January 1995 to March 2017). Search terms of “saliva”; “salivary”; “biomarkers”; “leukoplakia”; “erythroplakia”; “lichen planus”; “non-organic”; “peptide or protein”; “DNA”; “mRNA”; “microRNA”; “metabolomics”; precancerous”; “cancerous”, or “proteomics” in various combinations including and/or were used.

The search was done by two researchers, limited by only including literature involving human saliva based studies; English published articles and only enclosing literature published between the years 1995 and March 2017. Review articles, editorials, opinions, comments, author response, case reports, letter to the editors, news, and technological note articles were excluded. Studies based on salivary biomarkers from a disease group and control group were chosen for full text assessment. A third reviewer was asked to review the articles if there was a disagreement between the two reviewers. Final articles were selected with agreement of the three reviewers. Information on subject population, salivary sample collection and processing, biomarkers in oral cancer and pre-cancer used, as well as biomarkers identified were incorporated in the abstract.

Results:

This search collected 270 articles. After an initial selection by title, 105 articles such as reviews, case reports, news, letter to editor, opinions and technical reports were excluded. During further title and abstract reading, an additional amount of 117 articles were excluded for the following reasons: 1. publications in other languages than English, 2. non-human studies, 3. studies lacking controls or patient groups, 4. Studies lacking a clearly described methodology. As a result, forty-eight articles were chosen for the final review. Database search process, excluded and included articles, as well as reasons for the exclusion are shown in Figure 2.1.

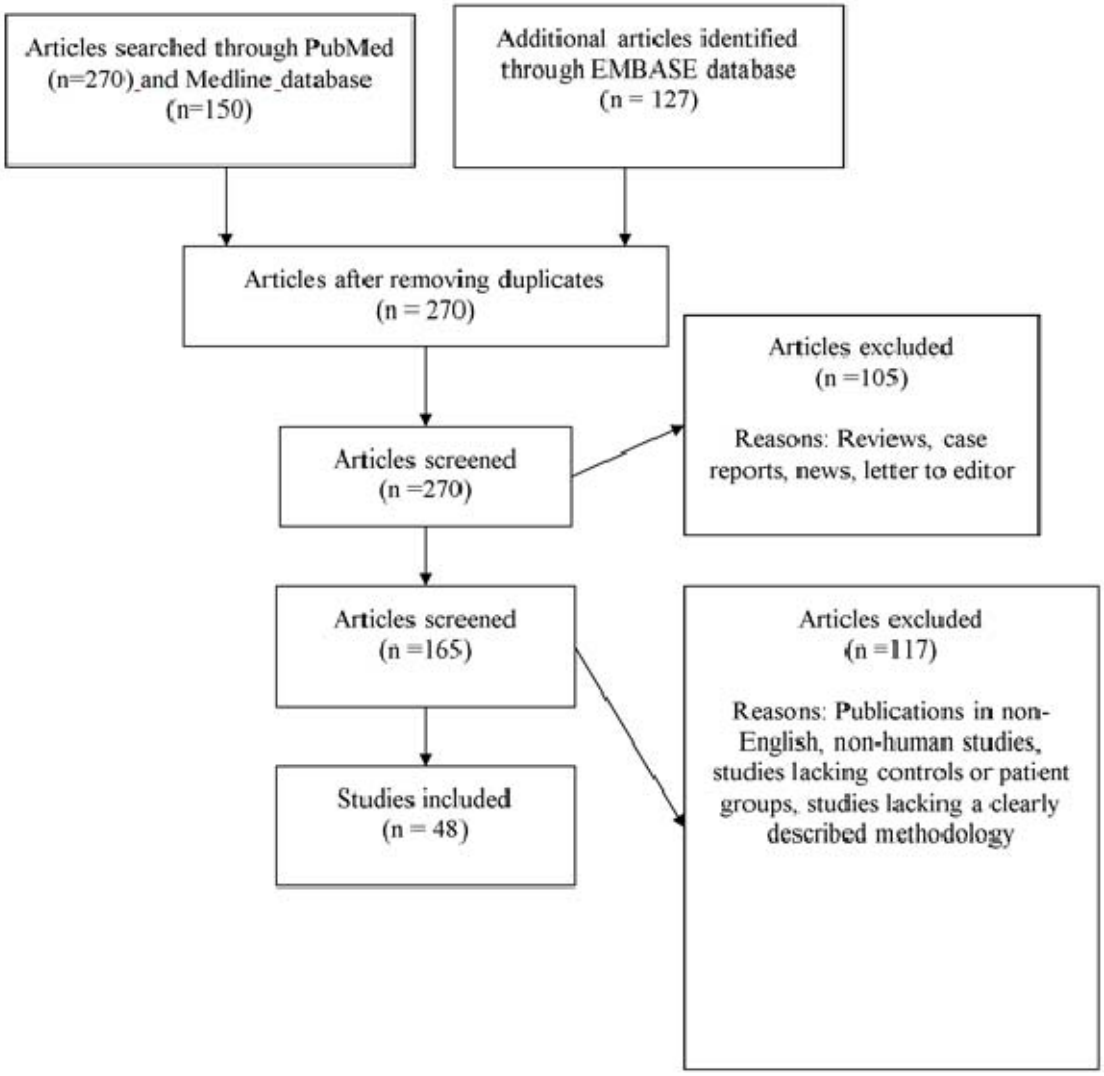

Figure 2.1. Research methodology and studies included in the review

Different issues in saliva diagnosis need to be addressed, such as lack of standardization of saliva sample collection, processing, and storage; wide inconsistency in the levels of potential salivary biomarkers of oral pre-cancer and cancer in non-cancerous individuals, healthy and oral precancerous & cancerous patients; and need of standardization of oral salivary biomarkers with different chronic oral inflammatory diseases and other types of cancer patients. All of these factors were assessed to enhance the use of saliva-based biomarkers for diagnosis of oral pre-cancer and cancer technology. Variations in saliva collection methods, such as whole saliva and specific salivary gland saliva, stimulated and unstimulated could affect levels of salivary biomarkers. Thirty-five articles considered analyzing whole saliva as

well as specific gland saliva. Between analyzing unstimulated and stimulated saliva, thirty-two studies used this second one. Thirty-one studies considered oral stimuli such as eating, drinking, and oral hygiene practices for varied periods of time prior to sample collection. The time of collection of saliva was morning in most studies, but the exact time of collection was not mentioned. A centrifugation method is normally done to remove the solid constituents including food debris after saliva sampling. Different speed centrifugations such as 2000 g for 10 minutes, 2600 g for 15 minutes, 2000 rpm for 5 minutes, 14,000 rpm for 20 minutes and 800 g for 10 minutes were used in the reviewed studies [16-25]. Three studies showed to have evaluated the whole saliva without centrifugation [18,19]. Samples were frozen until further analysis was done after centrifugation. Most salivary biomarker studies have analyzed only cell free portion of the saliva samples. In few studies RNase or proteinase inhibitors were used to preserve salivary proteins and RNAs, respectively [21-23], however, most studies were performed without any inhibitors. Most of these studies used supernatant samples after centrifugation were stored at -4 to -80°C [21]. In only three studies, the samples were analyzed immediately without storing. Patient selection criteria were not specified in most of the studies. Two-dimensional Gel Electrophoresis and tandem Mass Spectrometry were most commonly used methods. Flame photometry, atomic absorption and spectrophotometry were used for analysis of salivary biomarkers. Different biomarkers have been proposed as shown in Table 2.1 below.

Table 2.1. Salivary biomarkers for oral cancer detection

Authors	Category	Biomarkers	Methods	Results
Szanto et al. 2012	Proteins	Annexin 1 and peroxiredoxin 2	Gel Electrophoresis	Annexin 1 and peroxiredoxin 2: present in oral cancer
Camisasca et al. 2017		Keratin 10 (CK10)	2DE	Immunopositivity for CK10 in oral leukoplakia
Li et al. 2004 Zimmer-mann et al. 2007		IL-8	ELISA	IL-8 levels higher in oral cancer as compared to healthy controls (300 - 785pg/ml)
Arellano-Garcia et al. 2010		Tetranectin protein	Gel Electrophoresis	Under-expression of tetranectin protein in metastatic oral squamous cell carcinoma
Brailo et al. 2012		TNF-α, IL-6	ELISA	Salivary IL-1β and IL-6 significantly higher in oral cancer (10-25 pg/ml)
Li et al. 2004		IL8, IL1B, DUSP1, HA3, OAZ1, S100P, SAT	Human Genome U133A microarray, ELISA	Elevated levels of IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT in oral cancer
Arellano-García et al. 2008		IL-8, IL-1beta	ELISA, luminex multianalytic profiling	IL-8 and IL-1beta expressed at higher levels in oral squamous cell carcinoma (187.5 - 675 pg/ml)
Brailo et al. 2012		IL-6	ELISA	IL-6 levels higher in oral leukoplakia and oral cancer

Balan et al. 2012		Cancer Antigen 125	ELISA	Cancer antigen 125 levels higher in oral cancer
He et al. 2009		CA-50, CEA	ELISA, immune-radiometric analysis	CEA and CA-50 levels significantly higher in malignant tumors (22-87 ng/ml)
Stoott-Miller et al. 2012		Metal-loproteinase (MMP-1, MMP-10, MMP-12, MMP-3)	Array and quantitative reverse transcription-PCR	Salivary concentrations of MMP1 and MMP3 in squamous cell carcinoma were 6.2 x and 14.8 x higher, respectively, as compared to controls MMP 3 (0.21-1.78ng/ml) MMP-1 (0.1-2.3 ng/ml)
Ghallab and Shaker 2017		Chemerin and MMP-9	Enzyme-linked immunosorbent assays	Salivary MMP-9 and chemerin showed AUC of 0.99, and 0.88 in distinguishing oral squamous cell carcinoma from oral premalignant lesions MMP-9 (10-60 ng/ml)
Shintani et al. 2010		Cystatin SA-1	Lasers desorption/ ionization time of flight mass spectrometry	Truncated cystatin SA-I expressed in oral cancer
Jou et al. 2012		Transferrin	Gel Electrophoresis, MALDI TOF MS, Western blot, ELISA	Transferrin levels higher in oral cancer
Giebulto-wicz et al. 2008		ALDH (aldehyde dehydrogenas)	Fluorimetric method	ALDH activity higher in oral squamous cell carcinoma
Rai et al. 2011	DNA	Adenosine deaminase (ADA)	ELISA	Higher levels of ADA in tongue carcinoma
Nagata et al. 2012		ECAD, TMEFF2, MGMT	Methylation specific PCR, microchip, Gel Electrophoresis	ECAD, TMEFF2 and MGMT had high sensitivity (>75%) and specificity for detection of oral cancer combination of ECAD, TMEFF2, RARβ, and MGMT leads to 100% sensitivity and 87.5% specificity for oral squamous cell carcinoma
Park et al. 2009	Micro-RNAs	Micro-RNA 125a, Micro-200a	Quantitative RT-PCR	miRNAs, miR-200a and miR-125a present at significantly lower levels in saliva of oral squamous cell carcinoma

Combination of salivary biomarkers				
Zimmermann et al. 2007, Li et al. 2004	mRNAs	DUSP1, GADD45B, H3F3A, IL1B, IL8, OAZ1, RGS2, S100P and SAT	Microarrays	DUSP1, GADD45B, H3F3A, IL1B, IL8, OAZ1, RGS2, S100P and SAT were significantly elevated in saliva of oral squamous cell carcinoma Combination of IL8, SAT and H3F3A increased overall sensitivity to 90.6% for oral squamous cell carcinoma prediction
De Jong et al. 2010	Salivary actin and myosin	actin and myosin	Mass spectrometry	Sensitivity and specificity values to distinguish pre-malignant and malignant oral lesions were 100% and 75% for actin, respectively; 67% and 83% for myosin, respectively
Michailidou et al. 2016	mRNAs	IL-1B, IL-8, OAZ and SAT mRNAs	real-time RT-PCR	IL-1B, IL-8, OAZ and SAT mRNAs biomarkers combined show a good predictive probability up to 80% with oral squamous cell carcinoma
Brinkmann et al. 2011 Elasboff et al. 2012	mRNAs	RNA, SAT, OAZ1, H3F3A, DUSP, S100P	Quantitative PCR microarray analysis	IL-8, IL-IB, SAT, OAZ1, HA3, and DUSP1 levels significantly elevated in oral cancer
Zhong et al. 2005	Other	Telomerase	PCR-ELISA	Expression of telomerase activity in oral cancer
Liu et al. 2012		MiR-31	RT-PCR	Median fold change of miR-31 significantly higher in oral cancer
Tang et at. 2013	Long noncoding RNAs	lncRNA	RT-PCR	Whole saliva consists of a detectable amount of lncRNA
Chitra etal. 2012	Oxidative stress-related molecules	Lipid peroxidase, hydroxyl radicals, super-oxide dismutase	Tissue micro-array, ELISA	Increased levels of hydroxyl and hydrogen peroxides radicals in Oral Squamous Cell Carcinoma
Bernabe et al. 2012	Glucocor-ticoid	Cortisol	ELISA	Higher levels of salivary cortisol in oropharyngeal Squamous Cell Carcinoma:
Sartini et al. 2012	Meta-bolomics	Nicotinamide	Western blot	Downregulation of nicotinamide methyltransferase
Cheng et al. 2014		Apoptosis cells	TUNEL assay	Number of apoptotic cells: significantly less in oral Squamous Cell Carcinoma as compared to controls

Most of the potential salivary biomarkers of oral cancer are salivary proteins (Table 2.1), but the analysis of salivary proteins requires high sensitivity methods and technologies due a very low concentration in saliva (Table 2.1). Few studies were conducted on combinations of salivary biomarkers in detection of oral cancer as shown in Table 2.1. In general, studies also like methods for standardizing salivary diagnostics in research and clinical environment (Table 2.2).

Table 2.2. Different issues related to salivary diagnostic standardization

Sr. No.	Issue
1	Standardization of methods of saliva sample collection, processing and analysis
2	Different levels of possible salivary biomarkers in both non-cancerous individuals and cancerous patients
3	Validation of salivary biomarkers

Discussion

This review was aimed to evaluate saliva biomarkers and their relationship with oral cancer and pre-cancer lesions in saliva based studies. Molecular changes for diagnosis of oral cancer and pre-cancer can be divided into different levels, such as changes in cellular DNA can lead to mRNA levels and altered protein levels may further lead to changes at enzymatic levels. Numerous biomarkers have been studied for detection of oral pre-cancer and cancer, yet no further studies have been done. Most of the studies were conducted using whole saliva. Whole saliva consists of fluids from all major and minor salivary glands, as well as fluids from mucosal and periodontal tissues, which are influenced by oral and systemic environments and by host immune responses in oral and systemic diseases. Furthermore, easy collection of saliva, cost effective, less time consumption without requirement of special collection device as well special training makes this methodology easily accepted. Collection of saliva from specific salivary glands such as parotid gland, submandibular and sublingual glands may provide specific information, considering an eventual salivary gland disease. This procedure requires special collection devices and trained personnel, yet it can be a time-consuming procedure and difficult to be achieved.

Unstimulated saliva has no effect on flow rate and salivary composition of salivary glands. So, it might be considered as an ideal sample. Stimulated saliva may provide more accurate detection of cancer biomarkers [26]. Very few studies were conducted on the effect of stimulated and unstimulated saliva on salivary biomarkers of cancer. Therefore, it needs to be explored to further standardize the salivary biomarkers. Normally saliva samples were collected in the morning 60 to 90 minutes after eating or drinking [21-26]. Variation in time of saliva collection, handling, processing and method of analysis can affect the biomarker's analysis since it is most important to avoid changes after collection of samples [27]. Thus, saliva must be collected under standardized protocols due to salivary flow rate and compositional influences affected by circadian rhythms and other factors. Saliva is very sensitive sample, which is affected by systemic, physiological and biochemical changes in oral cavity. It also changes with collection time, type of saliva collection method, temperature and storage methods [28-32]. Whole saliva consists of epithelial cells, different microbial, and remnants of food and liquid ingestion. Consequently, it is very important to centrifuge the whole samples to remove food and other debris material [32]. A centrifugation method is done to eliminate the solid constituents including food debris after saliva sampling. Various degrees of centrifugal force and various lengths of time were used [21-30] but no study was conducted on effect of various degrees of centrifugal force and various lengths of time on different levels of salivary biomarkers. RNase inhibitors or proteinase inhibitors were used to preserve salivary RNAs and proteins respectively in few studies [21-23], but most studies were conducted without using any inhibitors. No study was conducted on the effect of inhibitors and without inhibitors on salivary biomarkers levels. For saliva sample storage, different scientists stored the saliva samples at −80°C (21-27), though few others stored samples at 4°C or -20°C [15-20]. Schipper et al. reported that storage at -80°C gives better results as compared to -20°C [31]. There should be minimum time elapse between sample collection and analysis to avoid an altered result. Reducing storage time before centrifugation and storing samples in low temperature avoiding degradation of sensitive biomarkers including proteomics is imperative [21,31]. Two-dimensional gel electrophoresis and tandem MS are most commonly used. It has been reported that several biomarkers found in most studies were not confirmed by other methods [32]. Different studies came up with different questions as to whether the level of the salivary biomarkers of oral pre-cancer and cancer should be investigated in different laboratories following similar or different protocols. In reality, potential biomarkers of pre-cancer observed by more than one study showed a wide

variability in the levels of the diseased and control groups as well as in different laboratories. This can be explained due to differences in methods used for collecting, handling, processing and analyzing salivary samples. Thus, further studies are required for standardization of saliva collection, processing, storage methods and methods of analysis for standardization of salivary biomarkers.

Selection of appropriate controls and diseases is critical to avoid false positive and negative results of biomarkers, as most studies did not mention inclusion and exclusion criteria [32]. Analysis of human saliva biomarkers is a very delicate procedure since it contains large number of components with different concentration. Our review shows that there are different methods used in biomarker screening and identification (Table 1) [21].

Variability in the levels of different salivary biomarkers of oral pre-cancer and cancer in both non-cancerous individuals and oral cancer patients needs to be standardized. The reference levels vary so much in different studies that it is unfeasible to decide what ranges of salivary 8-OhdG and MDA levels likely show oral development. It could be due to different analytical methods used, different individuals, different groups, intra- and inter-subject variability, differences in ethnic backgrounds, dietary habits etc. Reference level of salivary biomarkers of oral cancer varies different research groups, so it creates obscurities in the estimation and diagnosis of oral cancer. For example, salivary 8-OhdG and MDA levels are studied not only for possible risks of oral cancer and precancerous lesions detection but also for monitoring disease activity of periodontitis and other chronic inflammatory diseases [15,18,19,33-37].

Standardization of salivary diagnostics requires high specificity and sensitivity to differentiate oral cancer and other common oral inflammatory diseases. Oral cavity trauma, dental plaque, gingivitis, periodontitis, fungal infections and other muco-cutaneous inflammatory diseases affect the level of potential salivary biomarkers of oral cancer and precancerous lesions and needs to be explored in detail. It has been suggested possible salivary biomarker levels in oral cancer and pre-cancer patients and controls without taking into account other inflammatory conditions. Yet, salivary biomarkers of oral cancer and pre-cancer might be altered in the presence of oral inflammation. An inflammatory condition could give false results and reduce the diagnostic value of particular biomarker in clinical use for oral pre-cancer and cancerous lesions detection. Therefore, research that confirms any possible salivary biomarker of oral cancer with individuals having common non-neoplastic oral inflammatory diseases is required in order to validate the reliability of that specific salivary biomarker in oral cancer. On the other hand, numerous possible salivary biomarkers

of oral cancer such as 8-OHdG and MDA are also altered in different cancers such as lung cancer and breast cancer [33,34]. Hence, several salivary biomarkers might be significantly altered by the presence of more than one type of cancer.

Biomarkers can be divided into four categories: normal health, general, specific and non-specific salivary biomarkers of oral cancer and pre-cancer [JBR biomarkers Group].

Biomarkers such as methylation markers, IL-8, actin, myosin, miRNAs are very speculative and remain without sufficient scientific evidence when it comes to oral cancer and pre-cancer detection using body fluids [22, 25, 35-37]. Salivary peptides with sensitivity approximating 90% and specificity 80% for oral cancer diagnosis have been described [38]. Different salivary myeloid related protein 14, Mac-2 binding protein, Profilin 1, CD59, and catalase proteins have been used to differentiate oral cancer patients from healthy controls [39]. Salivary defensin-1 levels were higher in oral cancer patients compared to the healthy controls [40]. More recently, oral cancer detection was also described using capillary electrophoresis and time-of-flight mass spectrometry based metabolomics [41]. In such analyses, oral cancer tissue demonstrated decreased levels of glucose and glutamine while increased levels of lactate suggesting that lactate production and glucose consumption were enhanced, denoted as the Warburg effect [42]. Metabolomics and proteomic approaches are considered the new era in salivary biomarker diagnosis. These advances may thus bridge a gap for diagnosis in early oral cancer stages, most often described as hardly possible by a conventional approach.

Combination of different salivary biomarkers showed improvement in diagnosis of oral cancer. Salivary combination of mRNAs collectively had a discriminatory power of 91% sensitivity and specificity for oral cancer detection [17]. Methylation-specific PCR had a highly specific tool (96%) for HNSCC detection by using saliva samples [23]. Salivary transcriptomes such as IL-8, IL-IB, DUSP1, HA3, OAZ1, S100P, SAT were highly specific (91%) and sensitive (91%) for oral cancer detection [24]. De Jong et al. in 2010 reported that five salivary biomarkers panel such as M2BP, Profilin, CD59, MRP14, catalase had a discriminatory power of 83% sensitivity and 90% specificity for oral cancer detection. In this particular study, elevated salivary actin and myosin levels indicated an increased specificity (100 and 67 % respectively) and sensitivity (75 and 83 % respectively) for oral cancer detection [25]. Furthermore, a combination of salivary IL-1B, IL-8, OAZ and SAT mRNAs biomarkers may provide a good predictive probability up to 80% for patients with oral

squamous cell carcinoma (versus precancerous patients) [36]. Consequently, a combination approach of salivary biomarkers could be used as screening tool to improve early detection and diagnostic precision of oral pre-cancer and cancer.

Conclusions

Different assured salivary biomarkers for early detection of oral cancer and pre-cancer were reviewed, most of which had limitations in the application of clinical diagnosis. Nevertheless, some issues (eg. lack of standardization of saliva sampling, processing methodology, variation in the level of possible salivary biomarkers of oral cancer in non-cancerous, inflammatory and other types of cancer) are addressed, which are required to be solved in order to validate this methodology which could be reliable, highly specific and sensitive for use in clinical setup. The best possible diagnosis in saliva should be based on the combination of biomarker panels after standardization of the procedure, which could be used as effective screening tool to improve early detection and diagnostic precision. Furthermore, the combination of biomarkers with conventional technologies might provide additive and powerful diagnostic values for early detection of pre-cancer and cancer lesions in the oral cavity.

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CHAPTER 3

Combination of autofluorescence imaging and salivary protoporphyrin in oral precancerous and cancerous lesions: non-invasive tools

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Abstract

The aim of this chapter was to find out potential relationships between serum, salivary and tissue protoporphyrin IX (PX) levels in subjects with or without oral precancerous and cancerous lesions, and to find out diagnostic value of fluorescence imaging (VELscope® system, LED Dental Inc., White Rock, B.C.) and salivary protoporphyrin IX (PX) in oral precancerous and cancerous lesions. Furthermore, this chapter attempted to find out diagnostic value of combination approach of fluorescence imaging and salivary protoporphyrin for detection of oral precancerous and cancerous lesions. The sample comprised 3 test groups, with biopsy confirmed precancerous (leukoplakia and lichen planus), cancerous lesions (squamous cell carcinoma) and one control group of 25 healthy individuals. To find out sensitivity and specificity, another 100 patients presenting for routine dental care were selected and clinical examinations were followed by fluorescence imaging and normal photography, which were finally confirmed by biopsy. The clinical and histopathogical examinations were done in conjunction with photography of the oral cavity using digital camera and fluorescence imaging. Serum, tissue and salivary protoporphyrin (PX) levels were measured. Using fluorescence imaging, oral cancerous and precancerous lesions showed deep purple to deep brown and dark green colour respectively, while normal tissues showed pale green colour in contrast. The PX levels in serum, salivary and tissues were significantly higher in precancerous and cancerous lesions as compared to normal healthy tissues. Salivary and serum PX levels were highly correlated in all groups. The sensitivity and specificity to the discrimination of precancerous and cancerous lesions from the healthy tissues was higher by combination approach of salivary protoporphyrin X and VELscope® system as compared to individual approach. Combination approach of salivary protoporphyrin X and VELscope® system was more sensitive and specific to discriminate precancerous and cancerous lesions from the healthy tissues as compared to individual approach. Further studies are required on large samples of oral precancerous and cancerous lesions to test sensitivity and specificity and thus validate the clinical applicability of fluorescence imaging in (pre)cancerous diagnostics.

Introduction

Head and neck cancers comprise a large proportion of cancers in India accounting for 23% of all cancers in males and 6% in females (1). The vast majority of these cancers are related to consumption of tobacco, alcohol, poor hygiene, diet and viral infections (2, 3). Early detection and diagnosis of premalignant and malignant oral mucosal lesions have the potential to significantly reduce patient morbidity and mortality. Unfortunately, the clinical appearance of these lesions can often be so understated that these may remain unnoticed or ignored by patients or dentists. Furthermore, the lesions are difficult to clinically differentiate from common benign tissue changes, such as those associated with infectious or inflammatory sources or post-surgical alterations (4-6). A non-invasive and reliable adjunctive tool that directs clinicians towards sites suspicious for pre-malignancy could lead to significant advancements in early detection and diagnosis of oral precancerous and cancerous lesions.

In recent years, visual tools in diagnosis of oral cancer have made significant advancements by adding luminous detection systems (chemoluminescence and tissue fluorescence techniques) to increase the capacity to identify potential malignant lesions (6-12). The VELscope® system (Visually Enhanced Lesion Scope; LED Dental Inc., White Rock, B.C.) is a simple handheld device detecting the loss of fluorescence in visible and non-visible high-risk oral lesions by applying direct fluorescence. The loss of fluorescence reflects a complex mixture of alterations to the intrinsic tissue distribution of fluorophores (7-13). Hence, early biochemical changes are detected by their more evident appearance, permitting early detection of pathological lesions (14). In the past, few studies have evaluated this system (11-12). Reported sensitivity values ranged from 97% to 98% and specificity from 94% to 100% (8-19). Preliminary results were promising, yet information regarding the ability of VELscope to identify premalignant regions within Class II (innocuous) lesions or to reveal lesions otherwise visually undetectable is limited. The auto-fluorescence lifetimes of normal, precancerous and cancerous tissues are different because of varying biochemical changes, enabling characterization of various lesions. Protoporphyrin IX (PX), an important chemical of heme synthesis, is one kind of natural fluorophores in human cells (13). One of the rate limiting steps in the heme biosynthesis pathway is the conversion of protoporphyrin IX (i.e. the photosensitizer) to heme, which is controlled by a rate-limiting enzyme, ferrochelatase (FC), through adding a ferrous iron to PX (14). Because of a lack of ferrochelatase, cancerous cells may accumulate more PX, presenting significantly longer fluorescence lifetime than that

of normal cells. So, till date no study was published on ferrochelatase and protoporphyrin IX levels in oral precancerous and cancerous lesions and their relation to VELscope imaging. Hence, the aim of this chapter was to find out the relationship between fluorescence imaging and protoporphyrin IX levels in tissues, serum and saliva of oral precancerous and cancerous patients. This chapter tried to validate a simple non-invasive approach used for the diagnosis of occult oral diseases.

Materials and methods

Subjects*:* Twenty-five squamous cell carcinoma (SCC), 30 leukloplakia (OLU, age 56-75 years; M:F;15:15) and 25 lichen planus patients (aged; 54-76 years; M:F;13:12) with biopsy-confirmed and 25 normal healthy (age 54-75 years, M:F; 13:12) were recruited, after taking informed consent based on the definition of oral cancer and precancerous lesions by the World Health Organization (9) . Ethical approval was taken from center under JBR society (Ethical approval number JBR#1238). An additional 100 patients presenting for routine dental care (aged; 45-75 years, M:F; 52:48) were selected to test sensitivity and specificity. Patients with history of chemotherapy, radiotherapy, oncological surgery, obesity, systemic diseases, bronchial asthma and drug allergies were excluded from the study.

Clinical and Laboratory investigations: All subjects underwent comprehensive clinical examinations, followed by fluorescence imaging and white light photography. Positive imaging areas were referred for confirmation by scalpel biopsy and further histopathological examinations. Photographs of oral cavity tissues were taken with a digital camera and VELscope (LED Med. Inc., White Rock, BC0) (8-12).

VELscope examination: Room lights were dimmed and the oral cavity was re-examined using the VELscope. Visual fluorescence loss (VFL) and visual fluorescence retention (VFR) were assessed and mapped on a data collection sheet. VFL was defined as mucosal sites which showed a decrease in the normal pale green auto fluorescence (appeared dark) when compared to adjacent tissues as an anatomic control on the contra lateral side. The examiner's clinical impression/clinical diagnosis of areas exhibiting VFL were recorded. VFL was considered a positive VELscope finding and, therefore, necessitated biopsy. The VELscope images and white digital images were compared.

Oral biopsy and tissue preparation: Oral biopsies of lesions as well as normal healthy tissues were taken for analysis of PX by one of the oral surgeon. Two-mm punch biopsies were trimmed to remove a large part, frozen in liquid nitrogen, and homogenized at 2,000 r.p.m.

with the Microdismembrator U (Merck KgaA, Darmstadt, Germany) for 2 min. Chloroform: methanol (2:1 vol/vol, Merck KgaA, Darmstadt, Germany) mixture was employed as an extraction medium. Mixtures were centrifuged for 15 min at 15,000 r.p.m., and supernatant was collected for further analysis. A second extraction cycle was performed by calculating the same amount of chloroform: methanol (2:1 vol/vol) mixture to the pellet. This mixture was vortexed for at least 5 min until a new suspension was created and centrifuged for 15 min at 15,000 r.p.m. Subsequently, the supernatant was collected and mixed with supernatant from the first extraction cycle. The PX extraction procedure was identical to the procedure used by previous study (15). All these steps were performed in a dark laboratory environment taking final concern to prevent exposure of the specimens to light.

***Serum and salivary protoporphyrin analysis**:* Approximately 5 ml of blood sample was drawn under aseptic precautions and centrifuged for 5 min to obtain serum which was stored at -65° C in sterile vials. During the examination, paraffin wax stimulated whole saliva was collected, and samples were stored at -20°C until analysis. Serum and salivary protoporphyrin levels were measured fluorometrically as in a previous study (16).

Statistical analysis

Kruskal-Wallis H-test with Bonferroni correction was used for comparing salivary and serum PX in controls and patients. Sensitivity and specificity corresponding were calculated for VELscope and salivary PX as well as combination of VELscope and salivary PX by biopsy examination. Furthermore, mean salivary and serum PX of the groups were correlated by using Pearson correlation test.

Results

Patient's demographic data are shown in Table 3.1 as mentioned below:

The oral cancerous and precancerous lesions showed deep purple to deep brown and dark green colour changes respectively, while normal tissues showed pale green colour. All precancerous and cancerous lesions exhibited some degree of VFL.

Table 3.1. Demographic characteristics of patients and controls

Variables	Oral leukoplakia	Oral lichen planus	Squamous cell carcinoma	Controls
Gender	30 (M:F; 15:15)	25 (M:F; 10:15)	25 (M:F; 13:12)	50 (M:F; 25:25)
Sites	30 buccal mucosa	15 buccal mucosa, 10 retromolar areas	25 buccal mucosa	
Smokers (cigarettes /day; mean [SD])	17 [4.7]	18 [6.5]	17 [5.8]	16 [4.6]
Alcoholic status (g of alcohol /day; mean [SD])	52.3 [23.4]	54.6 [19.8]	56.5 [34.1]	54.6 [34.5]

The protoporphyrin X levels in serum, saliva and tissues were significantly higher in precancerous and cancerous patients as compared to normal healthy individuals (P=0.01), furthermore, the levels were also significantly higher in cancerous as compared to precancerous patients (Table 3.2, P=0.05 as mentioned below).

Table 3.2. Mean (SD) tissue, serum and salivary protoporphyrin IX levels in oral precancerous and cancerous and normal healthy subjects

Subjects	Serum protoporphyrin IX levels (mg/l)	Salivary protoporphyrin IX levels (mg/l)	Tissue protoporphyrin IX levels (pmol per mL)
Squamous cell carcinoma	2.58 (1.59)*	0.45 (0.32)*	35.3 (4.8)*
Oral leukoplakia	1.99 (1.25)*	0.32 (0.27)*	28.5 (7.9)*
Oral lichen planus	1.77 (1.35)*	0.31 (0.22)*	20.8 (6.8)*
Normal healthy	0.76 (0.46)	0.12 (0.18)	10.2 (7.8)

*p<0.005 compared with normal healthy

Salivary and serum PX levels were highly correlated (R^2=0.72) in all groups. Salivary and tissues PX were moderately correlated (R^2=0.68) and high correlation was also found in serum and tissue PX (R^2=0.75) in all groups. Sensitivity and specificity values were also calculated to evaluate the validity of VELscope® system and salivary PX in oral precancerous and cancerous lesions. The cutoff value of salivary PX levels 0.39, 0.26, 0.24, 0.11 mg/ml for

squamous cell carcinoma, oral leukoplakia, oral lichen planus and normal healthy individuals were taken**.**

The sensitivity and specificity to the discrimination of precancerous and cancerous lesions & condition from the healthy tissues were higher by combination approach of salivary protoporphyrin X and VELscope® system as compared to individual approach (Table 3.3).

Table 3.3. Sensitivity and specificity of salivary protoporphyrin (SP), VELscope (VEL), combination of salivary protoporphyrin and VELscope (SPVEL) in oral precancerous and cancerous lesions.

Value %	Squamous cell carcinoma			Oral leukoplakia			Oral lichen planus			Normal healthy		
	SP	VEL	SP +VEL	SP	VEL	SP +VEL	SP	VEL	SP+VEL	SP	VEL	SP+VEL
Sensitivity	71	67	80	68	63	79	69	60	80	68	78	79
Specificity	70	62	79	65	53	77	65	61	78	68	74	79

Discussion

The endogenous fluorophores that are most important for optical screening and diagnosis of precancerous and cancerous lesions are those exciting in the spectrum from visible blue (400-450 nm) to UV-A (315-400 nm) with properties spectroscopically correlated to diseased tissues. The concept behind tissue auto-florescence is change in the structure, metabolism of the epithelium, and alteration of the subepithelial stroma which further alters their interaction with light (11-18). Endogenous porphyrins have been controversially argued in literature concerning their tumor-localizing properties (19, 20). A theory is that, the red fluorescence is a product of microbial porphyrin synthesis and therefore, its sharing is limited to the necrotic surface of necrotic tumors (20). The oral cancerous lesions appeared deep purple to deep brown and precancerous appeared dark green, while normal showed pale green color

changes. It might be due to higher accumulation of PX and a decrease of green auto fluorescence in the precancerous and cancerous lesions. This occurs due to relative lack of ferro-chelatase, an enzyme required to incorporate chelated iron into PP IX to form the heme molecule (11, 18). Higher concentration of PIX in serum, tissues and saliva was found in cancerous and pre-cancerous conditions as compared with normal healthy. Tissue, salivary and serum PX levels were highly correlated in all groups. Also, salivary and tissue PX were highly correlated. Therefore, salivary testing, a non-invasive alternative to serum and tissues testing can be considered as an effective modality for diagnosis and prognosis prediction of various diseases such as oral cancer and pre-cancerous lesions, as well as for monitoring the patient's post therapy status.

The VELscope is a portable clinical diagnostic tool permitting the direct visualization of the oral cavity and is sold for use in the screening of oral cancer. Oral cancerous and precancerous lesions show deep purple to deep brown and dark green color changes respectively, while normal shows pale green. VELscope achieved a sensitivity of 62.8% and specificity of 62.2% in discriminating clinically evident oral pre-cancerous and cancerous lesions from normal tissue, which is lower than previous reports (16, 17, 18, 21-23). The tonality for interpretation purpose is straightforward: pale green for healthy tissues and dark green, brown or black (loss of fluorescence) for a pathological situation. The VELscope is intended to be used by a dentist or physician as an adjunct to traditional oral examination by incandescent light to enhance the visualization of oral mucosal abnormalities that may not be apparent or visible to the naked eye, such as oral cancer or premalignant dysplasia (11, 17-23). VELscope is intended to be used by a surgeon to help identify diseased tissue around a clinically apparent lesion and thus aid in determining the appropriate margin for surgical excision. The sensitivity and specificity to the discrimination of precancerous and cancerous lesions from the healthy tissues were higher by combination approach of salivary protoporphyrin X and VELscope® system as compared to individual approach. Further studies are required to investigate large samples of oral precancerous and cancerous lesions to test sensitivity and specificity and thus validate the clinical applicability of combination approach of fluorescence imaging and salivary biomarkers in (pre)cancerous diagnostics.

Conclusions

Combination of salivary protoporphyrin X and VELscope® system might be used for discrimination of precancerous and cancerous lesions from healthy tissues. Further studies are required on large samples of oral precancerous and cancerous lesions to test sensitivity and specificity and therefore validate the clinical applicability of fluorescence imaging in (pre)cancerous diagnostics.

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CHAPTER 4

Salivary apoptotic cells in oral (pre-) cancer as a potential diagnostic means

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Abstract

Apoptosis is a genetically programmed form of cell death which is indispensable for development and homeostasis of multi-cellular organism. The aim of this study was to find out the salivary apoptotic cells in oral precancerous and cancerous patients and furthermore to observe the potential diagnostic value of salivary apoptotic cells in detection of oral pre-cancer and cancer. Unstimulated saliva was collected from a group of 103 subjects diagnosed with oral (pre-)cancer and a control group of 30 healthy age- and gender-matched individuals. The test group diagnosed with (pre-)cancer was further subdivided in 4 lesion groups oral squamous cell carcinoma (OSCC), oral lichen planus lesions (n=26), oral leukoplakia (n=25), oral sub-mucous fibrosis (n=24). Apoptotic cells were morphologically studied using fluorescence microscopy (TUNEL technique). While the morphology of apoptotic cells in oral pre-cancer and cancer are morphological similar to the typical epithelial cells of oral cavity mucosa, the number of apoptotic cells was significantly less in OSCC as compared to precancerous and normal healthy tissues. It could therefore be concluded that salivary apoptotic epithelial cells might be used in early detection and diagnosis of oral pre-cancer and cancer. It could therefore be concluded that decreased number of salivary apoptotic cells might be used in early detection and diagnosis of oral pre-cancer and cancer.

Introduction

Oral cancer is the sixth leading cancer worldwide (1, 2). Eighty-five percent of head and neck cancer is associated to tobacco use (1, 2). Alcohol and tobacco have synergistic effect as compared to tobacco alone. Also, human papilloma virus is a risk factor for oral cancer (1). Most of the oral cancers develop from oral premalignant lesions such as leukoplakia, erythroplakia, and lichen planus (3). Oral leukoplakia, submucous fibrosis and lichen planus are major precursor lesions. Malignant transformation of oral pre-malignant lesions may develop in 1 to 18% of the cases into oral cancer; with certain clinical subtypes of leukoplakia being at higher risk (3). Early detection of oral cancer considerably increases survival rates and diminish other health effects (4). Despite of advancement in technologies, oral cancer cases are diagnosed at very late stage due to lack of awareness of the symptoms and risk factors between public as well as lack of prevention and early detection by oral physicians (5-7). Presently, diagnosis depends mostly on a thorough clinical oral examination and histo-pathological examination by taking a biopsy. A definite diagnosis is based on biopsy. Various technologies such Chemiluminescence, autofluorescence, Toluidine Blue, Brush biopsy, OralCDx Brush Test, ViziLite Plus with TBlue, VELscope Vx, Sapphire Plus, MicroLux(™) /DL, Identafietc have been proposed, but these technologies need strong evidences to be used in clinical setup (7-8). Chemiluminescence demonstrates good sensitivity for diagnosing any potentially malignant disorders and oral cancer. VELScope cannot precisely differentiate between potentially malignant disorders and erythematous lesions of benign inflammation, which might give false-positive results (7). Early detection is most valuable means to reduce death rate and for good prognosis as well as for better survival rate from diseases (7). It would be beneficial if it could be done through noninvasive, cost effective and easy to use technology for early detection of oral pre-cancer and cancer. Such screening could be extremely useful in rural areas with a high incidence of such (pre)cancerous lesions and a very low access to the healthcare system (8).

All normal cells require stimulation to undergo growth, differentiation and proliferations which are specific signals carried by different types of growth factors (9). Angiogenesis is critical in growth, invasion and metastasis of cancer or tumors (9, 10). Homeostasis of cell growth and cancer regression is controlled by apoptosis (9) which is further regulated by genes (11). There has been reported disturbance in gene regulation and apoptosis in oral carcinogenesis (12-14). Detection of apoptotic cells in cancer has been identified from different body fluids such as blood, serum and urine. Few studies have been conducted on

salivary apoptosis in oral pre-cancer and cancer (14). So, this chapter was aimed to find out the salivary apoptotic cells in oral pre-cancer and cancer and furthermore, to find the diagnostic value of salivary apoptotic cells in detection of oral cancer and pre-cancer.

Materials and Methods

A total of 103 patients with histopathologically confirmed oral (pre-) cancerous lesions were recruited from Baba Nidhan Singh, Punjab, India for the purpose of this study. Oral leukoplakia lesions, oral lichen planus, oral sub-mucous fibrosis and oral squamous cell carcinoma were identified based on the criteria as proposed by the World Health Organization (13). An age- and gender-matched group of normal healthy subjects was selected to serve as control. Demographic data of patient and control characteristics are shown in Table 4.1 below:

Table 4.1. Demographic characteristics of patients and controls

Variables	Oral leukoplakia	Oral lichen planus	Oral submucous fibrosis	Oral Squamous Cell Carcinoma	Controls
Patients (n, M:F)	25 (13:12)	26 (13:13)	24 (12: 12)	28 (15:13)	30 (15:15)
Age in range (years) Mean (SD)	43-60 (48.6 (9.8))	42-61 (48.7 (9.5))	41-61 (49. 2 (9.3))	43-62 (48.2 (9.6))	42-64 (48.3 (9.6))
Sites	23 buccal mucosa, 2 retromolar area	20 buccal mucosa 6 gingival area	24 buccal mucosa	28 buccal mucosa	
Smokers (cigarettes /day) Mean (SD)	15 (4.6)	16 (6.4)	14 (5.2)	15 (3.5)	13 (2.6)
Alcoholic status (g/day) Mean (SD)	61 (11)	62 (10)	60 (25)	61 (20)	60 (30)
Clinical classification	Flat (11) Corrugated (13) Verrucous (1)	Erosive (10) Plaque like (6) Reticular (6)			

Chronic intoxication refers here particularly to those individuals drinking alcohol continuously without any pause for 10 to 120 days, while being addicted to it. Alcohol-dependent individuals met the criteria for the alcohol and nicotine dependence according to ICD-10 and DSM-IV criteria respectively (15-16). Smokers with average of 18 cigarettes daily were taken as chronic smokers. All selected subjects had neither any oral diseases, gingival or periodontal inflammation, nor any oral lesions and symptoms and/ or signs of systematic infections and other diseases. Informed consents were obtained from all subjects. This study was approved by the ethical committee of Baba Nidhan Singh hospital, Punjab, India. Unstimulated whole saliva from subjects was collected over ice. The saliva samples were stored at -4 C for further analysis. The salivary apoptotic cells were detected by using TUNEL assay (Molecular Probes, Inc, Eugene, USA) as described in a previous study (14). Briefly, Saliva cells were incubated for the designated duration before being trypsinzed and washed by using phosphate-buffered saline, and fixed in 2% paraformaldehyde for 20 minutes. The cells were washed three times by using phosphate-buffered saline and stored at -20°C in 70% ethanol for 12-18 h prior to performing the TUNEL assay as per manufacturer's instructions. Total epithelial and apoptotic cells were measured by using bright fields and fluorescent microscope respectively as described as in previous study (14). Inter- and intra-observers difference were measured using Pearson correlation by selecting four samples from each group. The examiners were first author (Jasdeep Kaur) and colleague. A clinical criterion of examination was number of cells type in fixed area. Each cell type is measured using fraction of particular cell type in percentage of its number in total exfoliated epithelial cells. Pearson correlation between living, apoptotic and dead cells with gender, age, smoking and drinking habits was analyzed. Kruskal-Wallis and one-way analysis of variance (ANOVA) method were applied. Correlations between living cell, dead and apoptotic cell were estimated by using Spearman s rank test. P values less than 0.05 were taken as statistically significant. The data was analysed using SPSS. 11.0 version (SPSS Inc, Chicago, USA).

Results

The dead, apoptotic and living cells in oral pre-cancer and cancer are morphological similar to the typical epithelial cells of oral cavity mucosa. The living, apoptotic and dead cells were shown in Table 2 and 3. The dead cells were significantly higher in SCC as compared with pre-cancerous and normal healthy (Tables 4.2 and 4.3, $p<0.01$), also non-significantly higher

in oral precancerous cells as compared with normal healthy. Apoptotic and living cells were significantly lower in SCC as compared with precancerous and normal healthy (see Tables 4.2 and 4.3, $p<0.01$), and non-significantly lower in oral precancerous tissues in contrast to normal healthy oral mucosa on the other hand.

Table 4.2. Living, apoptotic and dead cells (Mean [SD], CI %) in precancerous and cancerous lesions

	Dead cells Mean [SD], CI %	Apoptotic cells Mean [SD], CI %	Living cells Mean [SD], CI %
Control	60.78 [9.34], 3.34	7.13 [3.28], 1.17	30.12 [7.26], 2.6
Oral leukoplakia	69.21 [7.33], 2.87	5.23 [2.09], 0.82	24.36 [8.48], 3.32
Oral lichen planus	71.03 [11.3], 4.34	4.98 [1.89], 0.73	24.41 [7.93], 3.05
Oral submucous fibrosis	69.8 [10.2], 4.08	5.13 [2.34], 0.94	26.57 [9.84], 3.94
Oral squamous cell carcinoma	89.3 [15.3]*,5.67	2.14 [1.23]*, 0.46	17.43 [5.34]*, 1.98

*$p<0.01$ as compared to control

Table 4.3. Living, apoptotic and dead cells (Mean [SD], CI %) in precancerous and cancerous lesions

	Dead cells Mean [SD], CI %	Apoptotic cells Mean [SD], CI %	Living cells Mean [SD], CI %
Control	60.78 [9.34], 3.34	7.13 [3.28], 1.17	30.12 [7.26], 2.6
Oral precancerous	70.12 [14.1], 3.19	5.16 [2.12], 0.48	24.4 [8.45], 1.91
Oral squamous cell carcinoma	89.3 [15.3]*,5.67	2.14 [1.23]*, 0.46	17.43 [5.34]*, 1.98

*p<0.01 as compared with control

Intra and inter-observer reliability (Cohen's kappa) was found to be 95% and 92% respectively (Tables 4.4 and 4.5) as shown below:

Table 4.4. Inter observer correlation (Cohen's Kappa)

Two examiners	Observer 1	Observer 2
Observer 1	1	0.95
Observer 2	0.95	1

Table 4.5. Inter observer correlation (Cohen's Kappa)

Two examiners	A2	B2
A1 Pearson correlation	1	0.92
B1 Pearson correlation	0.92	1

A1 and B1: first reading by observers 1, 2;
A2 and B2: second reading by examiners

We reported non-significant positive correlation between dead & apoptotic cells and smoking and drinking (Table 4.6) as shown below:

Table 4.6. Correlation of living, apoptotic and dead cells with gender, age, smoking and drinking habits

	Dead cells (R2)	Apoptotic cells (R2)	Living cells (R2)
Gender	0.25	0.24	0.20
Age	0.35	0.34	-0.30
Smoking	0.45	0.56	-0.45
Drinking	0.46	0.53	-0.51

Discussion

Apoptosis is a genetically programmed form of cell death which is vital for development and homeostasis in human (9). Apoptosis is observed in normal healthy individuals as well as in different diseases. Disturbances in apoptotic mechanisms have been associated to an extensive range of pathologies such as oral diseases including oral pre-cancer and cancer (14). Thus, it might be implicated in clinical application for early detection, diagnosis, prognosis and monitoring oral pre-cancer and cancer. Numerous studies have been published on detection of apoptotic cells in pre-cancer and oral cancer using TUNEL method (14). The main advantages of this method are less time consumption, high specificity & sensitivity and early detection of apoptosis (14). The inter- and intra-observer reliability was evaluated to be excellent. Our study supported the previous study (14), which showed dead cells were significantly higher in SCC as compared with normal healthy, and also non-significantly higher in oral precancerous cells as compared with normal healthy. Living cells were significantly lower in SCC as compared with precancerous and normal healthy which supports previous study (14). This method can be considered reliable in detection of apoptotic cells (14). Apoptotic activity was significantly lowered in SCC as compared with normal healthy, which supported previous studies (14, 17). However, it was contradicted with some other studies (18, 19). Furthermore, it was non-significantly higher in oral leukoplakia and oral submucosa fibrosis. It could be due to low resistance to apoptosis. The rise in number of neoplastic cells can occur during enhanced proliferation, diminished cell turnover, as well as a combination of proliferation & diminished cell turnover. Suppression of apoptosis during carcinogenesis was considered to play an important role in the development and progression of different types of cancers (20). Down-regulation in cancer cell apoptosis was due to

somatic and non-somatic mutation and loss of expression of pro-apoptotic molecules and over-expression of apoptosis inhibitory molecules (21). Many studies have been conducted on saliva based diagnosis of oral cancer and pre-cancer (22-25), but there is very little research published on its cellular components. The dead, apoptotic and living cells in oral pre-cancer and cancer were morphological similar to typical epithelial cells of oral cavity mucosa as supported by a previous study (14). It might be due to inactivate salivary epithelial cells (26). Positive correlation between dead & apoptotic cells and smoking and drinking were found as supported by previous studies (27, 28).

Salivary apoptotic cells were significantly less in oral cancer as compared to normal oral tissues. However, salivary apoptotic cells were significantly less in oral cancer than both precancerous and normal oral tissues. Furthermore, salivary apoptotic cells were non-significantly lower in precancerous than in normal oral tissue. So, salivary apoptotic cells might be potentially useful as a biomarker for distinguishing between cancerous, precancerous and normal healthy oral soft tissues. Further studies are required to find out underlying molecular mechanism between oral epithelium, saliva and apoptosis.

Conclusions

Salivary apoptotic cells might have potential diagnostic values for detection of oral cancer.

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CHAPTER 5

Salivary and Serum leptin levels in patients with squamous cell carcinoma of buccal mucosa

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Abstract

Leptin level is linked to energy expenditure and appetite in healthy individuals. The present chapter was aimed to evaluate serum and salivary leptin concentrations in patients with squamous cell carcinoma of buccal mucosa. Forty-one patients with squamous cell carcinoma of buccal mucosa and 40 healthy controls were enrolled. Serum leptin levels (ng/ml) were measured by using enzyme linked immuno-sorbent assay (ELISA) method. Salivary leptin levels were measured by a highly sensitive and specific non-equilibrium version of a dedicated custom radioimmunoassay. A significant reduction in salivary and serum leptin levels in patients with squamous cell carcinoma of buccal mucosa was observed as compared with the controls. Highly significant positive correlation of leptin levels in serum and saliva and TNM (Tumor Nodes Metastasis) staging variants of squamous cell carcinoma of buccal mucosa was observed. The results of this chapter demonstrated that possible role of salivary and serum leptin in squamous cell carcinoma of the buccal mucosa. Salivary leptin might play role in squamous cell carcinoma of the buccal mucosa.

Introduction

Oral squamous cell carcinoma (OSC) is the most common head and neck malignancy. As the sixth most common cancer in the world, it is a serious public health problem. Early detection of OSC is essential to achieve good results. It has been reported that half of the cancer patients are affected by cachexia characterized by anorexia, loss of adipose tissue and wasting of skeletal muscles, contributing significantly to the mortality (1). Cachexia is more common in children and elderly patients and becomes more pronounced as the disease progresses. The prevalence of cachexia increases from 50% to more than 80% before death, being the main cause of death in 20% of the patients (2). Cachexia occurs secondarily as a result of a functional inability to ingest or use nutrients. This can be related to mechanical interference in the gastrointestinal tract such as obstruction or mal-absorption, surgical interventions or treatment-related toxicity, in patients receiving chemotherapy or radiation therapy, nausea, vomiting, taste changes, stomatitis and diarrhoea can contribute to weight loss (3). Several hypotheses like cytokines, circulating hormones, neuropeptides, neurotransmitters and tumour-derived factors have been proposed (4-9). An emerging view is that the anorexia in cachexia is mainly caused by the cytokines produced by the tumour mass or by the immune system in response to the presence of malignancy that induce profound cytolysis and protein degradation (1).

Leptin, a hormone secreted by adipose tissue is now known to be an integral component of the homeostatic loop of body weight regulation (1-5). Low leptin levels in the brain increase the activity of the hypothalamic orexigenic signals that stimulate feeding, suppress energy expenditure and decrease the activity of anorexigenic signals that suppress appetite and increase energy expenditure (6-7). Most of the orexigenic signals are known to be up-regulated through fasting in experimental animals. This suggests these signals play an important role in facilitating the recovery of lost weight. Cancer-induced anorexia may result from circulating factors produced by the tumour or by the host in its response. Several cytokines have been proposed as mediators of the cachectic process, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (8). Our study indicated that high serum and salivary levels of IL-8, IL-6, TNF-α have been found in oral precancerous and cancerous patients (Chapter 6). Chronic administrations of these cytokines either alone or in combination are capable of reducing food intake and reproducing the distinct features of the cancer anorexia-cachexia syndrome (9). These cytokines may

produce long-term inhibition of feeding by stimulating the expression and release of leptin and/or by mimicking the hypothalamic effect of excessive negative feedback signalling from leptin, leading to the prevention of the normal compensatory mechanisms in the face of both decreased food intake and body weight. Various studies have been reported that there is decrease in serum leptin in cancer patients compared to the controls (10), but only one study has been reported on serum leptin levels in oral squamous cell carcinoma patients. A significant reduction in leptin levels of oral squamous cell carcinoma patients was observed (11). Squamous cell carcinoma of the buccal mucosa is highly prevalent in North part of India. There is still no study published on salivary and serum leptin levels in squamous cell carcinoma of buccal mucosa. Hence, the aim of this chapter was to assess the salivary and serum leptin levels in squamous cell carcinoma of buccal mucosa and to compare it to levels in controls and furthermore those levels were correlated to the factors like histopathological gradings, TNM (Tumor Nodes Metastasis) staging of the tumour and body mass index (BMI).

Materials and Methods

The forty one patients (20:21; M:F) with histopathological confirmation of squamous cell carcinoma of buccal mucosa and 40 normal subjects (20:20; M:F) without any systemic disease aged 40-65 years from north part of India were selected after obtaining informed consent. All patients were staged by TNM classification (12). Patients with history of chemotherapy, radiotherapy, oncological surgery, malignancy, obesity, systemic diseases, bronchial asthma, drug allergies alcoholic and smokers include chewing tobacco users were excluded from the study. Different parameters were studied such as BMI, TNM staging, histo-pathology, age and demographical parameters, salivary and serum leptin levels. BMI of each individual was calculated by using the following formula: $BMI=m/l^2$ (kg/m2); m=mass in kg, l=height in m (13). This study was approved by the ethical committee of Baba Nidhan Singh hospital, Punjab, India. Approximately 5 ml of fasting blood sample was drawn under aseptic precautions and centrifuged for 5 min to obtain serum which was stored at -65°C in sterile vials. During the examination paraffin wax stimulated whole saliva was collected, and samples were stored at -20°C until analysis. Saliva was centrifuged at 8000 g for 9 minutes. Samples were frozen immediately and stored at -25°C until measurement.

Serum leptin was determined with a double antibody radioimmunoassay (Linco Res., St. Louis, MO, USA). Intra-batch coefficients of variation were between 3-5%, and inter-assay coefficient of variation was 6-12%. To measure salivary leptin, a highly sensitive and specific non-equilibrium version of an in-house radioimmunoassay was used (13). The sensitivity (accuracy) of this modification was 2 pg/mL. Inter-assay and intra-assay coefficients of variation were 15.2% and 9.1%, respectively. Student's t test was used for comparing salivary and serum leptin levels in controls and patients of squamous cells carcinoma of buccal mucosa. Gender effects and the influence of age and BMI were checked as well. Furthermore, analysis of variance (ANOVA) was used to compare salivary and seum leptin levels and BMI in various histopathological gradings and TNM stagings. Mean BMI, leptin, mean serum and salivary leptin of both the groups were correlated by using Pearson's correlation. Correlation between BMI, serum and salivary leptin levels for each histopathological grading and TNM stage was also performed.

Results

A significant reduction in salivary and serum leptin levels in patients with squamous cell carcinoma of buccal mucosa was observed as compared to the controls (Table 5.1) as shown below:

Table 5.1. Mean (SD) of BMI, serum and salivary leptin in patients and control group (ANOVA)

Parameters	Control group (n=41)	Study group (n=40)	P-value
Range and mean (SD) age (years)	45-60, 46.45 (10.45)	46-60, 42.67 (16.02)	0.578
Mean serum leptin (ng/ml)	6.03 (2.76) Male=5.06 (2.34) Female=7.78 (2.72)	3.54 (1.77) Male=3.03 (1.08) Female=4.92 (2.12)	0.0001
Mean salivary leptin (pg/ml)	180 (101) Male=178 (89) Female=189 (100)	60 (21) Male=55 (23) Female=65 (20)	0.0001
BMI (kg/m2)	26.46 (3.48)	17.52 (3.23)	0.00001

By the analysis of Pearson's correlations, it was observed that salivary and serum leptin levels showed a significant correlation with BMI in both groups. Furthermore, it was found that serum leptin (r=0.71) was highly correlated with BMI as compared to salivary leptin (r=0.62).

Table 5.2. Correlation between mean serum and salivary leptin in categories of histological grading

Histological variants	Mean serum leptin (ng/ml)	Mean salivary leptin (pg/ml)	r	p	Inference
Well-differentiated oral squamous cell carcinoma	2.98 (1.85)	54 (35)	0.86	0.007	significant
Moderately differentiated oral squamous cell carcinoma	3.54 (2.45)	65 (23)	0.78	0.005	significant
Poorly differentiated oral squamous cell carcinoma	3.84 (1.45)	67 (24)	0.45	0.879	not significant

Mean salivary and serum leptin levels were compared with various histopathological gradings (Table 5.2) and significant differences were observed between mean serum leptin levels of well-differentiated and moderately differentiated squamous cell carcinoma of buccal mucosa. with advancement of histological tumor grading (r=0.56) (p=0.01).

Discussion

The overall aim of the present study was to assess the serum and salivary leptin concentrations in squamous cell carcinoma of buccal mucosa patients. We found a significant decrease in concentrations of salivary and serum leptin in cancer group when compared to controls. While other studies reported decrease in serum leptin concentrations in controls as compared to lung and colorectal cancer (10-17). The decrease in serum leptin concentration may be related to decreased body fat mass in these subjects (15-16). Weight loss was noticed in our cancer patients. Consequently, decrease in leptin concentration might be linked to decreased body fat mass which is secondary to weight loss in our patients. It has been

accounted that normal women have more adipose tissue and as a result have higher leptin concentrations as compared to men with equivalent BMI (15). Similarly, in the present study, serum and salivary leptin concentrations were higher in women in contrast to men in controls. Furthermore, a significant difference in serum and salivary leptin levels of various histopathological grading of squamous cell carcinoma of buccal mucosa was observed. This might be due to possible relationship between aggressiveness of tumour - serum and salivary leptin which can also reveal mitogenic and angiogenic effects of leptin (17). It might be due to leptin and leptin receptor dysregulation in cancer cells including squamous cell carcinoma of buccal mucosa (18). Leptin signaling leads to the metabolic features associated with cancer malignancy, such as switching the cells' energy balance from mitochondrial β-oxidation to the aerobic glycolytic pathway. Leptin rapidly induced activation of JAK2, STAT3, and MAPK (ERK1/2) signaling cascades; it may also induce HER2 transactivation via leptin-induced phospho-JAK2 (19). Leptin provides the tumor microenvironment, mainly through its ability to potentiate both migration of endothelial cells and angiogenesis, and to sustain the recruitment of macrophages and monocytes, which in turn secrete vascular endothelial growth factor and proinflammatory cytokines (20). In this study, we observed a positive correlation between leptin and BMI in well-differentiated and moderately differentiated squamous cell carcinoma of buccal mucosa which may indicate an increased activity in the tumour mass in relation to the cytokines inducing anorexia (3). Significant differences were observed in salivary and serum leptin levels, BMI and various TNM staging of tumour between the extent of the tumour and leptin, it might be explained by an enhanced production of cytokines developing negative feedback signalling from leptin on the hypothalamus (5). Another study was observed on type IV staging of tumour only (11), it could be due to small sample size. High positive correlation was observed between serum and salivary leptin levels in both groups. This study established the analysis of salivary and serum leptin levels which could early detect the squamous cell carcinoma of buccal mucosa.

Conclusion

Salivary and serum leptin can be used as diagnostic biomarker for early detection of squamous cell carcinoma of the buccal mucosa.

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CHAPTER 6

Proinflammatory cytokine levels in oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis and oral cancer

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Abstract

Head and neck is a common cancer form with relatively low 5-year survival rates, due to detection in very advanced stage and little knowledge of molecular markers for diagnostic and treatment. The objective of this chapter was to identify salivary and serum concentrations of interleukin-8 (IL-8), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in patients with oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis, oral cancer and healthy controls. Confirmed diagnosed cases of oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis, oral cancer and healthy controls were selected. Salivary and serum cytokine concentrations were measured using enzyme-linked immunoassay kits in all subjects. Analysis showed that there were significant correlations between serum and salivary cytokines. Salivary and serum IL-8, IL-6, TNF-α were significantly higher in patients with precancerous oral lesions than in controls group. Furthermore, salivary and serum IL-8, IL-6, TNF-α levels were increased more in oral cancer and advanced stages as compared to lower precancerous staging. Regression equations were proposed to differentiate the patients and controls. Salivary and serum IL-8, IL-6, TNF-α might act as diagnostic tools for detection of oral pre-cancer and cancer.

Introduction

Oral cancer is the sixth commonest cancer in the world (1). Its incidence is predominantly high in India especially north India, some other countries in Asia, and in certain places in the western hemisphere. It has been reported that 90% of oral cancers in India among men were attributable to chewing and smoking habits. In India, the age-standardized incidence rate of oral cancer is 12.6 per 100 000 population (2). Oral squamous cell carcinoma develops through a multi-step process of genetic, epigenetic and metabolic changes resulting from exposure to carcinogens (3). The initial presence of a precancerous lesion, later developing into cancer is well known in oral cancer (4). Oral leukoplakia, submucous fibrosis, lichen planus are major known precursor lesions. The prevalence of malignant transformation of oral lichen planus is around 0.5% and for leukoplakia around 1% (5). Ability to clinically predict malignant transformation is limited and routine histopathological diagnosis has limited its prognostic value. Despite of advances of technologies in early detection of oral precancerous and cancerous lesions, there are limitations for its use such as: the diagnosis is essentially subjective, all lesions showing dysplasia do not ultimately become malignant and several may even revert, and carcinoma can develop from lesions in which epithelial dysplasia was not detected in previous biopsies (6,7). Hence, it is essential to develop other methods for detecting the malignant potential of pre-malignant lesions and preventive measures (7).

Recently, strong evidences have suggested that the nuclear factor-κB (NF-κB) signaling pathway plays a critical role in carcinogenesis, protection from apoptosis and chemo resistance in a number of cancer types, including head and neck cancer, breast cancer, hepatocellular carcinoma and gastric cancer (8-12). Recently, accumulating evidence suggested that the NF-κB dependent cytokine levels in saliva and tissue specimens of patients with oral premalignant lesions are elevated (13). Furthermore, different cytokines can act as diagnostic tools for detecting oral cancerous and precancerous lesions and conditions. IL-6 levels are significantly increased in oral cancer patients (14-17). It has been evident that different cytokines are expressed by cancerous cells, but most commonly found are expression of tumor necrosis factor (TNF)-α, interleukines (IL)-1, -6 and -8 (15-17). Limited studies were conducted on role of TNF-α, IL -6 and -8 in oral pre-cancer and cancer (15-17). In this study, we hypothesized that salivary and serum TNF-α, IL -6 and -8 levels could be elevated in the oral pre-cancerous lesions and conditions. To test this hypothesis, this chapter

was conducted to find concentrations of TNF-α, IL-6 and -8 in oral pre-cancer by using saliva and serum samples.

Materials and methods

Consecutive patients clinically and histopathologically confirmed as having oral lichen planus lesions, oral leukoplakia and oral sub-mucous fibrosis were recruited from Baba Nidhan Singh, Punjab, India for this study based on the definition of oral cancer and precancerous lesions by the World Health Organization (9). Healthy normal served as the control group. Demographic characteristics of patient and controls characteristics were mentioned in table-1. All subjects neither had smoking history and detectable gingival and/or periodontal inflammation, nor any visible oral lesions under careful examination. Moreover, they had not received any treatments for the oral lesions within 90 days prior to the specimen collection, and had no history, symptoms and/or signs of systematic infections and other diseases. The conventional histopathological diagnosis was made by one oral pathologist. This study was approved by the ethical committee of Baba Nidhan Singh hospital, Punjab, India and informed consents were obtained from all subjects as per Declaration of Helsinki. For each sample, 1.0 ml of supernatant was used for the enzyme-linked immunoassay (ELISA), cytokine assays by using the human ELISA kit for TNF-α, IL-6, and IL-8 (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. Blood and serum samples were drawn between 9:00-10:00AM from all subjects. Serum was separated from blood cells by centrifugation at 1000g for 5 min. The whole unstimulated saliva was collected between 9:00-10:00AM. The subjects were abstained from eating and drinking for at least 2 h prior to the sampling. All subjects were requested to swallow first, tilt their head forward at more than 45°, and then expectorate saliva (10 ml) into a sterile centrifuge tube without swallowing for 4-5 min. The saliva was centrifuged for 25 min at 3500g, and the clarified supernatants were separated into 2.0 ml aliquots. All samples were immediately frozen at -60°C for the further uses. Earlier to the assays, the serum or saliva supernatants were held on for 2 hours to unfreeze completely at the room temperature. For each sample, 1.0 ml of supernatant was used for the enzyme-linked immunoassay (ELISA) cytokine assays, using the human ELISA kit for TNF-α, IL-6, and IL-8 (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance of the samples at 492 nm for TNF-α, IL-6, and IL-8 were measured with a spectrophotometer (Sirio, Seac, Florence, Italy). A standard curve was organized by plotting the absorbance value of

the standards versus corresponding concentrations. The concentrations of the cytokines in the sample were determined by extrapolating from the standard curve. Tests were always duplicated to check reliability. Protein contents were expressed in pg/ml. The inter-assay coefficient of variation was 3.0-4.5% and the intra-assay coefficient of variation was 2.5-4.2%. All data were statistically analysed using the SPSS statistical package (version.11.5, IL, USA).

Results

Table 6.1. Demographic characteristics of patients and controls

Variables	Gender (F:M)	Vegetarian: Non-vegetarian	Median Age (in years)	Smokers (cigarettes/day); mean (SD))	Periodontal status	Alcoholic status (g /day; mean (SD))
Leuko-plakia	25:25	19:31	56.71	18 (5.6)	Bleeding on probing (%) 21.3 (2.5) Probing depth (mm)=2.2 (0.3) Clinical loss of attach. (mm) 0.5 (0.2)	61.2 (11.2)
Lichen planus	24:30	29:25	54.81	17 (9.2)	Bleeding on probing (%) 22.2 (2.2) Probing depth (mm)=2.3 (0.3) Clinical loss of attachment (mm) 0.5 (0.3)	62.4 (14.5)
Cancer	23:27	23:27	55.31	23 (4.5)	Bleeding on probing (%) 22.4 (2.2) Probing depth (mm)=2.3 (0.2) Clinical loss of attachment (mm) 0.6 (0.3)	76.2 (14.3)
Fibrosis	24:27	21:30	57.92	19 (7.8)	Bleeding on probing (%) 22.4 (1.9) Probing depth (mm)=2.3 (0.4) Clinical loss of attachment (mm) 0.5 (0.2)	64.3 (14.5)
Control	23:27	24:26	51.06	15 (2.6)	Bleeding on probing (%) 22.6 (4.6) Probing depth (mm)=2.3 (0.4) Clinical loss of attachment (mm) 0.5 (0.3)	64.2 (14.6)

Demographic characteristics of patient and controls were mentioned in Table 6.1. There was no significant difference in periodontal parameters in different groups. The levels of serum and salivary TNF-α, IL-6, and IL-8 were statistically significantly higher in oral cancer as compared to precancerous lesions (Figures 6.1-3, P<0.05) as shown below.

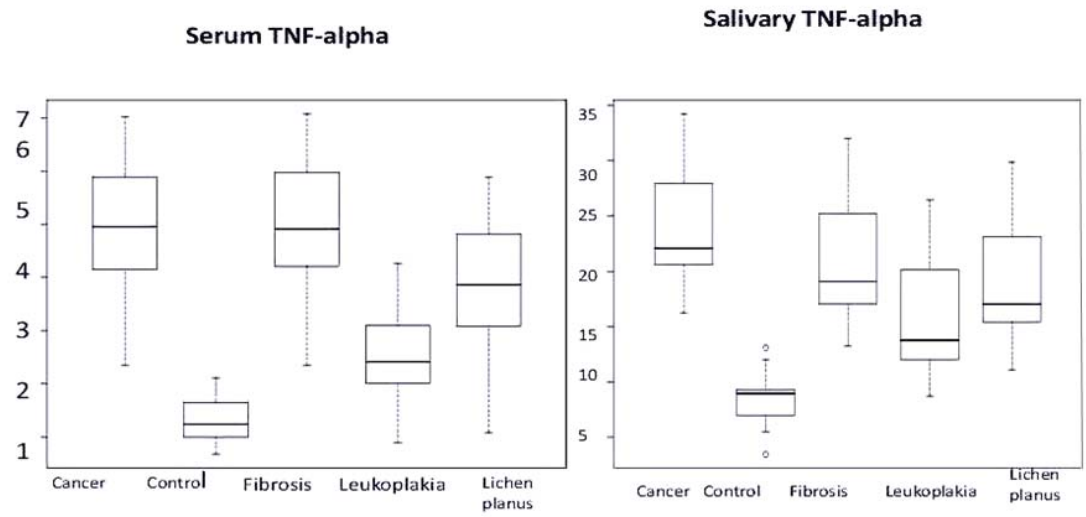

Figure 6.1. Serum and salivary TNF-alpha levels in patients and controls

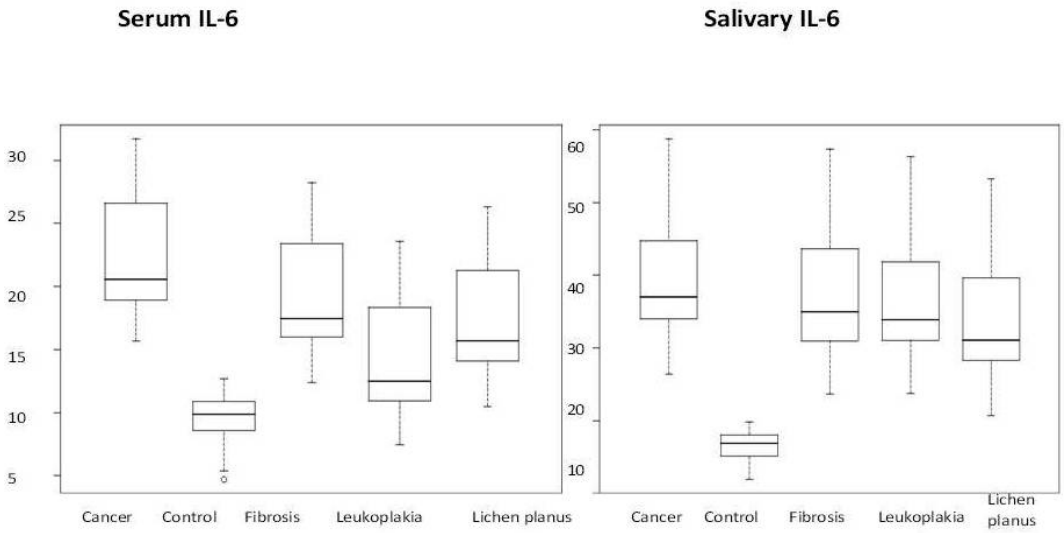

Figure 6.2. Serum and salivary IL-6 levels in patients and controls

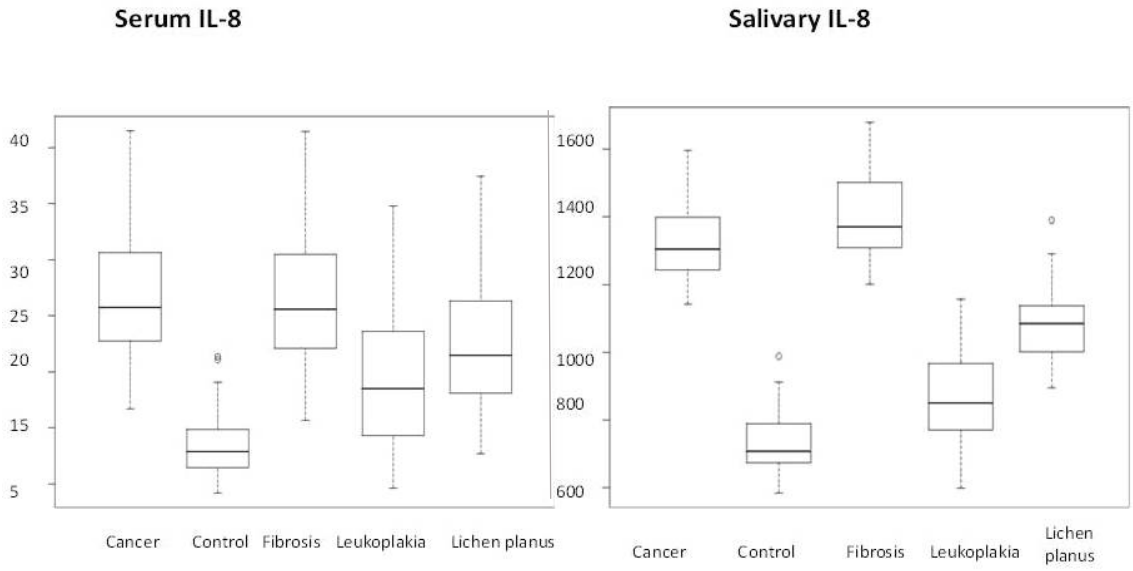

Figure 6.3. Serum and salivary IL-8 levels in patients and controls

The levels of serum and salivary TNF-α, IL-6, and IL-8 were statistically significantly increased in oral leukoplakia, submucous fibrosis, lichen-planus in contrast to normal healthy subjects (Figure 6.1-3, $p<0.05$). Salivary IL-8 has high sensitivity and specificity for diagnosis of oral leukoplakia, submucous fibrosis, lichen-planus and cancer from controls (Table 6.2).

Table 6.2. ROC analysis (threshold, sensitivity and specificity) for discriminating diseases from others

Variables	Biomarker	Threshold value	Specificity	Sensitivity
Leukoplakia	Saliva TNF	14.7	67.8	66
	Serum IL-6	13.8	67.3	62
	Saliva IL-8	993.8	72.2	88
Lichen planus	Saliva TNF	17.2	50.7	57.4
	Saliva IL-6	31.5	55.7	53.7
	Saliva IL-8	1198.5	49.3	81.5

Table 6.2 continued

Variables	Biomarker	Threshold value	Specificity	Sensitivity
Cancer	Saliva TNF	19.8	74.1	80
	Serum IL-6	17.8	71.2	86
	Saliva IL-8	1201.2	71.2	92
Control	Serum TNF	2.1	90.7	100
	Saliva IL-6	20.3	100	100
	Saliva IL-8	918.5	85.9	98
Fibrosis	Serum TNF	4.1	71.6	78.4
	Serum IL-6	15.9	55.9	76.5
	Saliva IL-8	1267.6	84.3	96.1

Linear and constant coefficients for differentiation of diseases from other groups are shown in Tables 6.3 and 6.4 below:

Table 6.3. Linear coefficients for discriminating diseases from others

	Cancer	Control	Oral Submucous Fibrosis	Oral Leukoplakia	Oral lichen planus
Age	0.38	0.54	0.34	0.61	0.47
Sex	0.45	1.14	0.68	0.70	0.77
Vegetarian	0.72	1.62	0.21	0.02	0.98
Serum TNF alpha	1.23	-0.66	1.53	-1.11	0.59
Saliva TNF alpha	1.01	0.05	1.08	0.09	0.79
Serum IL-6	0.29	0.63	-0.33	-0.69	-0.11
Saliva IL-6	-0.56	-0.13	-0.45	1.14	-0.13
Serum IL-8	-1.16	-0.86	-1.18	-1.35	-1.05
Saliva IL-8	0.14	0.07	0.15	0.07	0.11

Table 6.4. Constant coefficients for discriminating diseases from others

Cancer	Control	Oral submucous fibrosis	Oral leukoplakia	Oral lichen planus
-96.91	-39.75	-106.29	-53.16	-68.38

Discussion

Salivary IL-8 has high sensitivity and specificity for diagnosis of pre-cancer and oral cancer. Serum and salivary TNF-α, IL-6, and IL-8 were higher in oral cancer and advanced stages as compared to early stages of precancerous lesions and condition which support previous studies (15-24). TNF-α, IL-6, and IL-8 are potent angiogenic meditators with significant effects on tumour growth, as well as are associated with increased tumour vessel density and cancer prognosis (15-18), they might act as surrogate biomarkers of angiogenesis and prognosis. It has been reported that excessive cell proliferation and activation of cellular actions can be led by chronic inflammation, which leads to induction of irreversible DNA damage (25). TNF-α, IL-6, and IL-8 released through inflammatory response would promote tumour growth, while tumour growth further stimulates the inflammatory response (26). In present study, serum and salivary TNF-α, IL-6, and IL-8 levels were up-regulated in oral cancer and precancerous lesions and condition. This finding is in line with previous conducted studies (14-17), while there is one study which oppose our study, in which salivary TNF-α and IL-6 were down regulated (18, 19). It could be due to small sample size and not taking matched age and gender samples. Increased levels of NF-κB mediators might be associated with the development of oral pre-cancerous and cancerous lesions. In the normal cell, stimulation of cytokines causes growth inhibition while in oral cancer cells, stimulation of cytokines leads to up regulation of positive cell cycle regulators including NF-κB, signal transducer and activator of transcription and mitogen-activated protein kinase/extracellular signal-regulated pathway (16). Upregulation of TNF-α, IL-6, and IL-8 might be protective in action. Although smoking is considered a major risk factor of oral precancerous and cancerous lesions (2), smokers were excluded from the present study. TNF might act as an endogenous tumour promoter as well as inducing tissue remodeling required for tumour growth and spread (22). The IL-6 signaling has also been implicated in tumorigenesis (23). TNF-α, IL-6, and IL-8 were also elevated in periodontitis patients (21), but in present study,

subjects with periodontitis were excluded. Thus confounding variables such as inflammatory conditions such as periodontitis were avoided. The latter may imply that elevation of TNF-α, IL-6, and IL-8 might be rather related to inflammatory reaction in precancerous and cancerous lesions. Elevation of cytokines in saliva might indicate precancerous development into oral cancer (22). Salivary Interleukin-6 and -8 levels in patients with oral cancer were higher as compared to patients with chronic oral inflammatory diseases (25). Furthermore, these cytokines are improperly produced in precancerous which could lead to induced growth, invasion, disruption of tumour suppression and immune status. NF-κB activation leads to up-regulation of anti-apoptotic genes by cell survival mechanism by physiological stress which triggered the inflammatory response. In addition, NF-κB induces cytokines that regulate TNF-α, IL-6 and IL-8, which lead to the recruitment of leukocytes to the sites of inflammation (27). There were good correlations between salivary and serum TNF-α, IL-6, and IL-8 in all groups. Since saliva can be easily collected; measurement of biomarkers of diseases may prove useful in early detection of oral cancer risks. The salivary analysis for oral diagnosis may prove a cost effective method for screening large populations (24). Hence, salivary levels NF-κB mediators such as TNF-α, IL-6, and IL-8 were significantly higher in patients with oral (pre-) cancerous lesions, assuming the diagnostic and prognostic utility of those biomarkers for detection of precancerous and cancerous lesions. This study did not take into account factors such as diet, alcoholic history and environmental factor. So further studies are required on large samples and taking into account cofounding variables as such to determine the relationship between salivary biomarkers and oral cancer and to further clarify the mechanism of action.

Conclusions

It can be concluded that patients with oral precancerous lesions and cancer have elevated salivary and serum cytokines compared to healthy controls and the level of salivary cytokines increase with the advancement of staging of cancer.

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CHAPTER 7

Salivary 8-hydroxy-2-deoxyguanosine, malondialdehyde, Vitamin C and E in oral precancer and cancer: diagnostic value and free radical mechanism

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Abstract

In order to determine the value of salivary biomarkers in diagnosis of oral pre-cancer and cancer patients, the oxidative DNA and lipid damage were analyzed by using salivary 8-hydroxy-2-deoxyguanosine (8-OHdG), MDA (Malondialdehyde), Vitamin C and E in oral lichen planus lesions, oral leukoplakia, oral submucous fibrosis, oral squamous cell carcinoma (SCC) and controls. Unstimulated saliva was collected from a group of patients diagnosed with 30 oral squamous cell carcinoma (OSCC), 31 oral lichen planus lesions, 30 oral leukoplakia, 32 oral submucous fibrosis and from a control group of healthy age- and gender-matched individuals. Salivary 8-hydroxy-2-deoxyguanosine (8-OHdG), MDA, Vitamin C and E were measured. Squamous cell carcinoma and pre-cancer patients showed significantly higher levels of salivary 8-OHdG, MDA while lower levels of vitamin C and E as compared with healthy normal subjects. The specificity and sensitivity of combination of 8-OHdG, MDA, Vitamin C and E is high for diagnosis of oral pre-cancer and SCC as compared to the individual biomarker approach of 8-OHdG, MDA, Vitamin C and E. This chapter illustrates the presence of oxidative DNA and lipid damage in the precancerous and SCC patients. This mechanism might have a significant link to carcinogenesis in oral cancer. The salivary 8-OHdG, MDA, Vitamin C and E could act as diagnostic biomarkers of oral pre-cancer and cancer. Salivary 8-OHdG, MDA, Vitamin C and E might play significant role in oral cancer and precancer patients.

Introduction

Saliva-based diagnostics are non-invasive, non-infectious, cost effective screening tool substitute to blood and serum based diagnostic technologies. This can be useful technology for early detection, diagnosis, prognosis and prediction of oral pre-cancer and cancer (1-4). Oral cancers originating from head or neck area predominately (95%) consisting of squamous cell carcinoma (5-8). Squamous cell carcinoma of head and neck is among the sixth most common cancer worldwide (7). Major risk factors for oral cancer are smoking and other tobacco usage. Together with alcohol consumption there is a synergistic effect (8). Oral leukoplakia, oral submucous fibrosis and lichen planus are generally precursors of oral cancer, but very few of these lesions are reported to be malignant (9). Different technologies in early detection of oral pre-cancer and cancer have been proposed, but there are limitations for their use (10-11). Furthermore, treatment and management offered in oral cancer patients is based on TNM criteria and histopathological grading. So, it might be unreliable because rating and proposing same treatment modality for different types of cancers in similar staging and grading act very differently, i.e., one tumor responds to treatment while other might not. So, there is urgent need to understand the basic mechanism underling the oral cancer biology by using biomarker indicators for early detection, diagnosis, prognosis and treatment. Few studies have examined salivary biomarkers of oral cancer patients and reported significant changes caused by local connection between oral cancer and local fluid i.e. saliva (11-12). Various salivary IL-6, IL-8, IL-1β, basic fibroblast growth factor and oxidative stress related biomarkers are known biomarkers of oral pre-cancer and cancer (2, 14-21), but they need to be validated for prediction of early detection, malignant possibility of pre-malignant lesions and preventive measures in a clinical setting. Hence, this chapter was planned to find out the role of oxidative stress mechanism in oral pre-cancer and cancer and its usefulness in clinical setup for diagnosis. Furthermore, this study tried to validate a combination approach for the diagnosis of oral cancer and pre-cancer.

Materials and Methods

Patients confirmed by the clinical and histopathological evidence with oral lichen planus lesions, oral leukoplakia, oral sub-mucous fibrosis and oral squamous cell carcinoma patients were selected from Baba Nidhan Singh, Punjab, India for this study based on criteria by the World Health Organization (22). Age and gender matched healthy subjects were selected as control group. The demographic characteristics of patients and controls were shown in Table 7.1 below:

Table 7.1. Demographic characteristics of patients and controls

Variables	Oral leukoplakia	Oral lichen planus	Oral submucous fibrosis	Oral Squamous Cell Carcinoma	Controls
Gender	30 (M:F; 15:15)	31 (M:F; 15:16)	32 (M:F; 15: 17)	30 (M:F; 15:15)	30 (M:F; 15:15)
Age in range (years (SD))	41-60 [49 (9.2)]	41-63 [49 (9.4)]	41-60 [49 (9.3)]	43-64 [51 (10.1)]	41-63 [49 (9.4)]
Smokers (mean n° cigarettes /day (SD))	17 (5.7)	18 (6.3)	16 (3.7)	16 (4.7)	17 (4.5)
Alcoholic status (mean g /day (SD))	61 (11)	61 (12)	71 (12)	62 (14)	64 (21)
Periodontal status	Bleeding on probing (%) 22.4 (3.1); Probing depth (mm)=2.3 (0.4); Clinical loss of attachment (mm) 0.4 (0.3)	Bleeding on probing (%) 21.8 (2.9); Probing depth (mm)=2.1 (0.5); Clinical loss of attachment (mm) 0.5 (0.2)	Bleeding on probing (%) 23 (4.1); Probing depth (mm)=2.2 (0.5); Clinical loss of attachment (mm) 0.5 (0.2)	Bleeding on probing (%) 21.3 (3.2); Probing depth (mm)=2.2 (0.5); Clinical loss of attachment (mm) 0.5 (0.2)	Bleeding on probing (%) 21.8 (2.5); Probing depth (mm)=2.1 (0.2); Clinical loss of attachment (mm) 0.5 (0.2)

None of the subjects showed signs or symptoms of other oral diseases, oral lesions and systematic infections. Periodontal parameters were measured in different groups. The conventional histopathological diagnosis was made by one of oral pathologist. This study was approved by the ethical committee of Baba Nidhan Singh hospital, Punjab, India and informed consent was obtained from all subjects. Demographic data of patient and control

characteristics are shown in table-1. The chronic alcohol usage ranged from 20 to 130 days [87 (67)]; and consumed alcohol 34 [54] (20-80) g of alcohol daily without any pause. To check sensitivity and specificity, randomized 132 subjects were selected blindly and saliva samples and biopsy were taken. Histopathological diagnosis was taken as gold standard. The threshold value of salivary biomarkers was selected by using receiver operating characteristic analysis. Unstimulated whole saliva from subjects was collected over ice. The saliva was centrifuged for 25 min at 3500g, and samples were immediately frozen at -20°C for the further analysis. Lipid peroxidation products MDA were analyzed by thiobarbituric acid (TBA) reaction (23). Salivary levels of 8-OHdG in supernatant were determined using a competitive ELISA kit (Bioxytech 8-OhdG-EIA kit quantitative assay for 8-hydroxy -2-deoyguanosine, USA) and the determination range was 0.125 to 200 ng/ml). Vitamin C and vitamin E were estimated by HPLC as described in a previous study (2). The significance of association for definite variables was estimated by Fisher's exact test. The differences in significance between continuous variables were compared by using t test and Mann-Whitney u test. Data was analyzed by using SPSS (Version 11.5, Chicago, USA).

Results

Patient's demographic data are shown in Table 7.1. The levels of periodontal parameters were not significantly different among the groups. The levels of salivary 8-OHdG and MDA levels were significantly higher, while levels of vitamin C and E were lower in squamous cell carcinoma as compared to pre-cancer (Tables 7.2-7.5).

The levels of salivary 8-OHdG and MDA were statistically and significantly higher while levels of vitamin C and E were lower in oral leukoplakia, submucous fibrosis and lichen planus in contrast to normal healthy subjects. There were reported significant correlations of 8-OHdG with MDA, Vitamin C and E in all groups (Table 7.3). The threshold value for 8-OHdG (ng/ml), MDA (µmol /l), Vitamin E (µg/l) and Vitamin C (µg/l) were 0.09, 0.12, 0.91 and 0.92 for control, while 0.92, 0.91, 0.23 and 0.21 for squamous cell carcinoma respectively. Threshold values for 8-OHdG (ng/ml), MDA (µmol /l), Vitamin E (µg/l) and Vitamin C (µg/l) were 0.34, 0.31, 0.43 and 0.42 for precancerous lesions. The sensitivity and specificity of 8-OHdG, MDA, Vit. C and Vit. E for distinguishing pre-cancer and cancer lesions from normal healthy tissues is shown in Table 4. The sensitivity and specificity of combination approach of 8-OHdG, MDA, Vit. C and E for distinguishing pre-cancer and

cancer lesions from normal healthy tissues were significantly higher when compared to the individual biomarker approach (Table 7.5).

Table 7.2. Salivary levels of oxidative stress markers in patients and healthy controls

Mean (SD)	8-OHdG (ng/ml)	MDA (µmol /l)	Vitamin E (µg/l)	Vitamin C (µg/l)
Control	0.11 (0.09)	0.12 (0.12)	0.91 (0.32)	0.92 (0.25)
Oral leukoplakia	0.37 (0.12)[a]	0.36 (0.21)[a]	0.65 (0.21)[a]	0.66 (0.19)[a]
Oral lichen planus	0.47 (0.14)[b]	0.41 (0.12)[b]	0.63 (0.22)[b]	0.64 (0.21)[b]
Oral submucous fibrosis	0.49 (0.13)[c]	0.42 (0.11)[c]	0.61 (0.12)[c]	0.59 (0.24)[c]
Oral squamous cell carcinoma	1.13 (0.23)[d]	1.02 (0.21)[d]	0.31 (0.12)[d]	0.27 (0.18)[d]

a,b,c,d: significant difference as compared to control (student t-test multiple comparisons)

Table 7.3. Correlations among salivary biomarkers (8-OHdG, MDA, Vit. C and Vit. E) estimates

Parameter	r	p-value significant at
8-OHdG & MDA	0.78	<0.001
8-OHdG & Vit.E	-0.79	<0.001
8-OHdG & Vit.C	-0.76	<0.001
MDA &Vit.C	-0.68	<0.001
MDA & Vit.E	-0.66	<0.001
Vit.E & Vit.C	-0.68	<0.001

Table 7.4. Diagnostic values of salivary 8-OHdG (A), MDA (B), Vit. C (C) and E (D) determination in distinguishing oral pre-cancerous, cancer patients from healthy individuals

Diagnostic values	Oral squamous cell carcinoma versus (vs) normal healthy				Oral precancerous vs normal healthy				Oral Squamous cell carcinoma vs precancerous lesions			
	A	B	C	D	A	B	C	D	A	B	C	D
Sensitivity (%)	79	61	58	57	77	55	55	56	75	45	43	42
Specificity (%)	77	57	55	54	75	53	52	54	76	45	42	41

Table 7.5. Diagnostic values of a combination of salivary 8-OHdG (A), MDA (B), Vit. C (C) and E (D) determination in distinguishing oral pre-cancerous and cancer patients from healthy individuals

Diagnostic values	Oral squamous cell carcinoma versus (vs) normal healthy			Oral precancerous vs normal healthy			Oral Squamous cell carcinoma vs precancerous lesions		
	AB	ABC	ABCD	AB	ABC	ABCD	AB	ABC	ABCD
Sensitivity (%)	82	83	84	81	82	83	80	80	81
Specificity (%)	81	81	82	80	81	81	79	80	80

AB= combination of salivary 8-OHdG and MDA
ABC= combination of salivary 8-OHdG, MDA and Vit. C
ABCD= combination of salivary 8-OHdG, MDA, Vit. C and E

Discussion

The development of cancer in humans is a multistep and complex mechanism. It includes molecular and cellular alterations which in turn contribute to cancer development and is mediated by different types of endogenous and exogenous stimuli such as generation of reactive oxygen species (ROS) (24). It has been reported that ROS not only initiates, but also promotes multistep carcinogenesis (3, 11, 25). Subsequently, lowered antioxidant capacity can lead to oxidative damage to cellular macromolecules further leading to cancer including oral cancer and pre-cancer (3, 11). Salivary MDA levels were significantly increased in SCC followed by oral pre-cancer patients as compared to controls as reported in previous study (3, 11). Lipid peroxidation leads to changes in function and structural integrity of the cell membrane. So, it suggests that lipid peroxidation products are elevated in precancer and oral cancer (3, 11, 26-27). Increased levels of salivary oxidation DNA adduct (8-OHdG) in oral pre-cancer and cancer along with other oxidative stress markers supports the increased DNA oxidation by free radical mechanism of cancer (2, 3, 17). The evaluation of 8-OHdG is an important factor in analysis of oxidative DNA damage. The hydroxyl radicals, singlet oxygen, and direct photodynamic action generate 8-hydroxylation of the guanine base (28, 29). The vitamin C and E levels were decreased in oral pre-cancer and cancer as compared to healthy subjects as reported by previous studies (2, 29). Vitamin C is consumed, followed by vitamin E in quenching the free radicals. Furthermore, Vitamin C regenerates vitamin E by non-enzymatic mechanism (2). Consequently, a decrease in antioxidant levels is observed in this study, which supports the concept that free radical mediated lipid peroxidation and related antioxidant consumption might be involved in the pathophysiological mechanisms of

oral pre-cancer and cancer (2, 29). Free radical mechanisms are involved in various pathologies such as ageing, cardiovascular diseases, neurodegeneration disorders, cancer, pain syndrome, complex regional pain syndrome and others (2-10, 30). Nonetheless, in the present study, salivary free radical mechanisms are proven by changes in salivary free radical related biomarkers in oral pre-cancer and cancer as other diseases and conditions were excluded.

In the present study, a good correlation between salivary 8-OHdG and MDA, vitamin C & E was noted in all groups. The combination approach of 8-OHdG, MDA, vitamin C and E biomarkers had high sensitivity and specificity for diagnosis of oral pre-cancer and SCC as compared with each individual approach. Hence, salivary 8-OHdG, MDA, Vitamin C and E might be useful in the clinical setup for diagnosis of oral pre-cancer and cancer. Since saliva can be easily collected, measurement of biomarkers in diseases may prove useful in early detection of oral cancer risks. Moreover, the salivary analysis for oral diagnosis might prove to be a cost effective method for screening large populations (2, 3, 17).

Thus, combination approach of salivary 8-OHdG, MDA, Vitamin C and E did not significantly improve the sensitivity and specificity. Therefore, they might have diagnostic and prognostic utility as useful biomarkers for detection of pre-cancer and cancer. This study did not take into account factors such as diet, alcoholic history and environmental factors. The further studies are required on larger samples to exclude other factors and to further clarify the mechanism of action and to determine the relationship between salivary 8-OHdG, MDA, Vitamin C, E and oral cancer.

Conclusions

Salivary 8-OHdG, MDA, vitamin C and E might play imperative role in oral cancer and pre-cancer patients.

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CHAPTER 8

General discussion and conclusions

General discussion and conclusions

The present thesis addresses the role of salivary biomarkers as an adjunct tool for diagnosis of oral pre-cancer and cancer. Salivary based tools are focused on measuring changes of specific salivary biomarkers such as proteins or nucleic acids, genomic or proteomic, vitamins, etc. (1-6). Salivary biomarkers are non-invasive, easy to use, cost effective, non-infectious, alternative to blood testing and an efficient modality for diagnosis and for prognosis prediction of oral pre -cancer and cancer (1-3). Imaging techniques and saliva based biomarkers provided early detection or diagnosis of pre-cancer and cancer (***Chapter 1***). It has been reported that a combination of different methods may improve early detection of oral pre-cancer and cancer, but few studies have been conducted on combination of salivary biomarkers with other existing methods of oral cancer and cancer. Despite the existence of different studies conducted on salivary biomarkers in detection of oral pre-cancer and cancer, various issues still need to be further addressed. Factors to enhance the use of saliva-based biomarkers for diagnosis of oral pre-cancer and cancer technology (***Chapter 2***) as proposed by different studies (7-8) were addressed, such as: type of saliva to be collected, saliva collection and handling process, analysis techniques, single biomarker and combination of biomarkers for validation. Combination of autofluorescence imaging and salivary protoporphyrin (PX) in detection of oral precancerous and cancerous lesions were studied (***Chapter 3***). The PX levels in serum, salivary and tissues were significantly higher in precancerous and cancerous lesions as compared with normal healthy tissues (9). The sensitivity and specificity to differentiate precancerous and cancerous lesions from the healthy tissues were higher by combination approaches of salivary protoporphyrin X and VELscope® system as compared with individual approach. To the best of our knowledge, only this study was conducted on the combination of salivary protoporphyrin X and VELscope® for diagnosis of oral pre-cancer and cancer. So, we cannot compare our studies with others. This study was conducted on small sample size and the comparison of non-cancerous diseases with cancerous diseases was not done. Thus, further studies are needed on large sample of oral precancerous and cancerous lesions to test sensitivity and specificity and also to further validate the clinical applicability of fluorescence imaging and salivary biomarkers in (pre)cancerous diagnostics. Salivary biomarkers were selected based on proposed pathological pathways of oral pre-cancer and cancer. Deregulation of cellular homeostasis pathway of oral pre-cancer and cancer by measuring the salivary apoptotic cells

in oral (pre-cancer) cancer (***Chapter 4***). The number of apoptotic cells were significantly less in OSCC as compared to precancerous and normal healthy tissues (10) as supported by previous study (11). This study was conducted on small samples size without taking into account dietary and other factors. So, further study is required on large sample size incuding these factors. Endocrine cancer pathway was explored by measuring leptin level in patients with Squamous Cell Carcinoma. A significant reduction in salivary and serum leptin level in patients with squamous cell carcinoma of the buccal mucosa was observed in comparison to control subjects (12) (***Chapter 5***). Serum leptin also increased in different types of cancer (13). It might be possible that the salivary leptin levels change in other cancers and other diseases as well, so further study is required to find out diagnostic value of salivary leptin in oral cancer and other cancers. Inflammatory pathway was explored by using estimation of salivary and serum proinflammatory cytokine levels in oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis and oral cancer (14). Salivary and serum IL-8, IL-6, TNF-α were significantly higher in patients with oral cancer and pre-cancer than in controls group. Furthermore, salivary IL-8 biomarker showed high sensitivity and specificity for diagnosis of oral lichen planus, oral leukoplakia, oral sub-mucous fibrosis and oral cancer (***Chapter 6***). It has been reported that combination of interleukin-8, choline, pipecolinic acid, l-phenylalanine, and S-carboxymethyl-l-cysteine demonstrated excellent diagnostic test accuracy in oral cancer (15). Regression equations were derived for detection of oral cancer and pre-cancer. This study did not include different factors, so further studies are required on large sample including factors such as diet, family history and environmental factors to determine the relationship between salivary biomarkers, oral cancer and pre-cancer. Free radical pathway of oral cancer was investigated by estimating 8-hydroxy-2-deoxyguanosine, malondialdehyde, vitamin C and E (16) (***Chapter 7***). Squamous cell carcinoma and precancerous patients showed significant higher levels of salivary 8-OHdG and MDA, while lower levels of vitamin C and E as compared to healthy normal as supported by previous published studies (17, 18). The specificity and sensitivity of salivary 8-OHdG was significantly higher. This study did not include other non-cancerous diseases. So, further studies are required to compare salivary 8-OHdG in oral cancer and other cancers as well as in other non-cancerous diseases. The present results conclude that the techniques for saliva collection and handling process and analysis need to be standardized. Furthermore, combination of salivary biomarkers (salivary biomarkers of different cancer pathways) can be used as an adjunct tool for diagnosis of oral pre-cancer and cancer and are required to be

validated. After validation, salivary-based biomarkers can possibly be integrated with different platforms such as biosensor, lab on chip and other smart technologies. Finally, this thesis contributed towards the growing field of in vitro diagnostics using saliva based biomarkers, which could improve human oral health care especially in remote areas or developing countries with huge populations and high risk factors. Such salivary sampling could help by increasing the availability and decreasing the threshold for access to screening and diagnostic tests as such to enhance patient care by early diagnosis and prevention.

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SUMMARY

This research aimed at contributing to the possible development of saliva based biomarkers for early detection of oral pre-cancer and cancer. In the present PhD thesis, salivary biomarkers were selected based on proposed pathological pathways of oral pre-and cancer. Therefore, existing information on the diagnostic potential of various techniques used for oral precancer and cancer were reviewed. (Pre)cancer diagnostic techniques were dealt with as a state-of-the art introduction (***Chapter 1***) while (pre)cancer-specific biomarkers were more systematically approached in a review article ***(Chapter 2***). A lot of variation also derived from the variables involved in the process of selection and analysis, such as saliva collection and handling process, analysis techniques, single biomarker and combination of biomarkers for validation. All these variables tended to have an impact on the diagnostic outcome of using salivary-based biomarkers for diagnosis of oral pre-cancer and cancer technology (***Chapters 1 & 2***). The present thesis resulted to identify an increased sensitivity and specificity for the detection of precancerous and cancerous lesions by combining two diagnostic approaches (salivary protoporphyrin X (PX) and VELscope® system) as compared with using only one approach. The PX levels in serum, salivary and tissues were significantly higher in precancerous and cancerous lesions as compared to those measured in normal healthy tissues (***Chapter 3***). The number of apoptotic cells was significantly less in oral cancer patients as compared with precancerous and normal healthy tissues (***Chapter 4***). Furthermore, one study explored the endocrine- cancer pathway by using measuring leptin levels in patients with squamous cell carcinoma, noting a significant reduction in salivary and serum leptin levels in patients with squamous cell carcinoma of the buccal mucosa (***Chapter 5***). Salivary and serum IL-8, IL-6, TNF-α were significantly higher in patients with precancerous oral lesions than in controls group. Moreover, salivary and serum IL-8, IL-6, TNF-α levels were increased more in advanced stages as compared with lower precancerous staging (***Chapter 6***). The free radical pathway of oral cancer was explored by assessing 8-hydroxy-2-deoxyguanosine (8-OhdG), malondialdehyde (MDA), vitamin C and E levels in oral precancer and cancer. Squamous cell carcinoma and precancerous patients showed significantly higher levels of salivary 8-OhdG and MDA, while lower levels of vitamin C and E as compared with healthy normal. The specificity and sensitivity of salivary 8-OHdG was significantly higher than for the other markers (***Chapter 7***). Finally, it should be stressed that the process of salivary sampling is also critical with influencing variables during all steps

from collection (type of saliva, sampling and handling process) towards analysis. The selection of the biomarker or a combination of biomarkers would also influence the diagnostic validity for oral pre-cancer and cancer detection (***Chapter 8***). The results obtained in the present thesis should form the basis of further validation studies, in which the biomarkers with the most promising diagnostic value, should be tested on larger patient samples following a standardized method for collection and analysis.

SAMENVATTING

Dit onderzoek heeft tot doel biomarkers in het speeksel te ontwikkelen die kunnen bijdragen tot een vroegtijdige opsporing van orale kankerletsels of precancereuze lesies. In het huidige doctoraatsonderzoek wordt eerst een stand van zaken opgemaakt inzake de klinisch gebruikte diagnostische methoden voor detectie van orale (pre)cancereuze lesies. Hierbij worden ook een aantal biomarkers in het speeksel geëvalueerd, op basis van hun relatie met de ontwikkelings- en/of ziekteprocessen van orale (pre-)kankerletsels (***hoofdstuk 1***). Vervolgens worden in een overzichtsartikel alle reeds gerapporteerde bevindingen omtrent de diagnostische rol van diverse speeksel-gebaseerde biomarkers in kankerdetectie, zoals beschreven in de aanwezige literatuur, samengevat (***hoofdstuk 2***). Daarbij valt op dat er geen gestandaardiseerde methode bestaat om speeksel te analyseren, waarbij diverse variabelen in het analyseproces een invloed blijken te hebben op het eindresultaat en de diagnostische waarde van de speekseltesten (collectie, manipulatie van speeksel, analyse technieken, gebruik van enkelvoudige biomarkers of analyse op basis van diverse biomarkers). In de huidige thesis wordt aangetoond dat de sensitiviteit en specificiteit van speeksel biomarkers voor diagnose van precancereuze lesies alsook orale kankerletsels verhoogd kan worden indien deze gecombineerd worden met een andere diagnostische methode (bijvoorbeeld: speeksel protoporfyrine X (PX) in combinatie met VELscope® systeem) (***hoofdstuk 3***). De PX waarden blijken daarbij significant verhoogd in serum, speeksel alsook in de weefsels van (pre)kankerletsels in de mond in vergelijking tot de gemeten waarden bij gezonde proefpersonen. Het aantal apoptotische cellen is significant lager bij patiënten met orale kankerlesies dan bij gezonde proefpersonen (***hoofdstuk 4***). In een volgende studie worden leptine waarden vergeleken in zowel serum als speeksel, waarbij een significante verlaging wordt vastgesteld bij patiënten met spinocellulair carcinoma van de buccale mucosa (***hoofdstuk 5***). Speeksel en serum waarden van IL-8, IL-6, TNF-α blijken significant hoger bij prekanker lesies en nog sterker verhoogd bij meer geavanceerde orale kankerlesies (***hoofdstuk 6***). Spinocellulair carcinomas alsook precancereuse letsels blijken te resulteren in significante verhogingen van 8-OhdG en MDA, met verlaagde niveaus van vitamines C en E indien vergeleken met de waarden in een gezonde controlegroep (***hoofdstuk 7***). Verder blijkt de sensitiviteit en specificiteit van speeksel 8-OHdG significant hoger dan voor andere biomarkers in het speeksel. Finaal moet het duidelijk zijn dat zowel het proces alsook het eindresultaat zeer gevoelig zijn aan tal van variabelen. De selectie van een of meerdere

biomarkers in het speeksel speelt uiteraard ook een belangrijke rol. Het resultaat bekomen in de huidige thesis kan bijzonder nuttig zijn bij verdere validatiestudies, als opstap naar het klinisch gebruik van speekselbiomarkers voor kankerdetectie (***hoofdstuk 8***). Daarbij is het belangrijk om de diagnostisch meest succesvolle biomarkers beschreven in de huidige thesis te testen op een grotere steekproef in een gecontroleerde prospectieve set-up, nadat eerst een gestandaardiseerde methode voor speekselcollectie en speekselstaalanalyse is neergeschreven.

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