



Diagnostic Role of Saliva in Oral Cancers

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Abstract. *Saliva is a noninvasive diagnostic fluid alternative containing almost all the compounds found in blood, oral and tumoral cells. The aim of this study is to analyze the correlations occurred between different key biomarkers involved in different stages of oral cancer, such as inflammation markers - interleukin 6 (IL-6), extracellular collagen degradation markers - matrix metalloprotease 9 (MMP-9) and tissue inhibitors of metalloprotease 2 (TIMP-2). This study included a lot of 30 patients diagnosed with oral cancer at the Department of Oro Maxillo-Facial Surgery, University Hospital of Dental Medicine Prof. Dr. Dan Teodorescu. The patients were included in this study after informed consent was obtained. Salivary biomarker analysis was made using ELISA methods, biochemical or colorimetric assays. Uric acid (UA) levels were positively correlated with total antioxidant capacity (TAC) levels ($r = 0.78$). TAC levels were negatively correlated with IL-6 levels ($r = -0.66$). MMP-9 levels were positively correlated with the Ki-67 protein (also known as **MKI67**) levels ($r = 0.65$) and negatively correlated with TIMP-2 levels ($r = -0.71$). We can conclude upon the results that salivary research may lead to the usage of saliva as a quick and reliable screening and diagnostic tool for oral cancer.*

Keywords: *oral cancer, saliva, oxidative stress*

1. Introduction

Oral cancer is one of the most frequent types of cancers being responsible for almost 9% of deaths worldwide [1], head and neck squamous cell carcinoma being the most frequent type of cancer that affects cervical and oral regions [2]. This form of cancer is characterized by a very low five-year survival rate due to late stage diagnosis, high degree of invasiveness and development of therapeutic resistance [3].

Early diagnosis plays a crucial role in oral cancer therapy, clinical examination being still the most widely used screening method [4]. Alternative screening and prognosis methods such as tissue sample, serum or saliva analysis, using specific biomarkers could greatly improve early detection rates, prognosis assessment and could predict and detect recurrences after treatment. Tissue sample and serum analysis are invasive methods requiring trained medical personnel, tissue analysis taking longer time and it is usually being performed after surgical tumor removal [5].

Saliva is proving to be a noninvasive diagnostic fluid alternative [6], containing almost all the compounds found in blood and also oral cells and tumoral cells. It has constant contact with malignant and premalignant lesions [7, 8]. Saliva collection doesn't require trained medical personnel or special medical devices thus making it suitable for everyday screening usage in dental offices [9, 10].

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Oral cancer biomarkers are specific compounds with roles in every aspect of malignancy from triggering factors to markers of progression, inflammation or invasiveness. They can be classified in genomic, proteomic or metabolomic markers [11, 12].

The aim of this study is to analyze the correlations that occur between different key biomarkers involved in different stages of oral cancer development, such as markers of inflammation - interleukin 6 (IL-6), markers of extracellular collagen degradation and their inhibitors - matrix metalloprotease 9 (MMP-9) and tissue inhibitors of metalloprotease 2 (TIMP-2). Our aim is to select suitable biomarkers for oral cancer.

2. Materials and methods

Patients selection

This study included a lot of 30 patients diagnosed with oral cancer at the Department of Oro-Maxillofacial Surgery, University Hospital of Dental Medicine Prof. Dr. Dan Teodorescu. The patients were included in this study after informed consent was obtained. Patients' age varied between 45 and 60. The control group included 14 healthy volunteers with no associated oral or general diseases, ages ranging between 40 and 60 years old. For both control and patients group gender distribution showed an almost equal distribution between male and female subjects.

Patients group included 15 patients with parotid gland cancer, 8 patients with different jaw bones cancers, 4 lip cancer patients and 3 with tumors located elsewhere in the oral cavity.

Saliva collection

Unstimulated whole saliva was collected into sterile tubes between 9 and 10 am following a single mouth rinse with 5 mL distilled water for washing out food and exfoliated cells. All subjects were required to refrain from eating or drinking prior to saliva collection.

About 2mL of whole saliva was collected. The samples were centrifuged for 10 min at 3000 rpm and stored at -80°C.

Salivary biomarker analysis was made using ELISA methods, biochemical or colorimetric assays.

ELISA methods

Salivary biomarkers analysed in this study using ELISA technique were Ki-67, SCCAg, MMP-9 and TIMP-2. ELISA kits from R&D Systems Inc. Minneapolis – USA were used for all these biomarkers and manufacturer instructions were followed.

Salivary albumin levels were also determined using an Albumin Assay Kit (Barcelona, Spain) in order to normalize the results due to the lack of standardization of oral biomarker levels.

Biochemical methods

Immuno-fluorescence method was used for IL-6 analysis using an immuno-fluorescence kit from Siemens Healthcare Global – Germany. Results were analyzed using the IMMULITE 1000 automatic analyser (Siemens Healthcare Global – Germany).

Colorimetric methods

Salivary uric acid levels were determined using a commercial from Biosystems Company (Barcelona, Spain), kit based upon the oxidation of uric acid to allantoin catalyzed by uricase. Results were analyzed at 293 nm, using an automatic analyzer from Biosystems Company (Barcelona, Spain).

Total antioxidant capacity was determined using the TEAC method (Trolox equivalent antioxidant capacity) based upon the color change induced by the oxidants on ABTS from radical and cationic form to non-radical form. The kit used was from R&D Systems Inc. Minneapolis – USA. This method is. Reading was done at 415 nm using an automatic analyzer from Biosystems Company (Barcelona, Spain).

Statistical Analysis

Results were expressed as means and standard deviations were appropriated. The data was analyzed using StataIC11 (StataCorp. 2009. Stata: Release 11. Statistical Software. College Station, TX, USA). Statistical analysis was done using a Student's t-test. A p-value < 0.05 was considered statistically significant. Correlations between biomarkers were analyzed, a r - value < -0.4 or r > 0.4 was considered statistically significant.

3.Results and discussions

Our experimental results are shown in the next tables (Table 1, 2 and 3):

Table 1. Mean values of the salivary antioxidant parameters in the oral cancer patients group *versus* controls

Parameter	Oral cancer patients group	Controls	p
TAC (mmol/mg albumin)	0.62±0.1	1.31±0.7	< 0.05
Uric acid (mg/mg albumin)	1.25±0.4	2.9±0.48	< 0.05

Our results analysis showed significant correlations between several parameters:

1.UA levels were positively correlated with TAC levels (r = 0.78) (Figure 1).

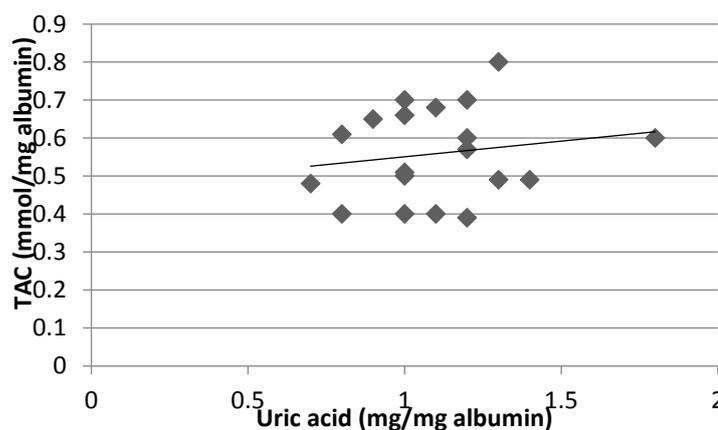


Figure 1. Positive correlation between uric acid and total antioxidant capacity levels

Table 2. Mean values of inflammation and tumoral invasiveness salivary parameters in the oral cancer patients group *versus* controls

Parameter	Oral cancer patients group	Controls	p
IL-6 (pg/mg albumin)	2.9±0.8	0.8±0.04	< 0.05
Ki67 (ng/mg albumin)	1.6±0.3	0.35±0.06	< 0.05

2.TAC levels were negatively correlated with IL-6 levels (r = -0.66) (Figure 2).

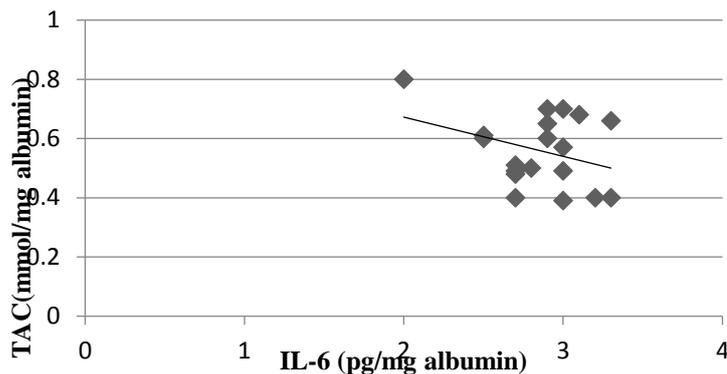


Figure 2. Negative correlation between total antioxidant capacity and IL-6 levels

Table 3. Mean values of proliferation salivary parameters (MMP-9 and TIMP-2) in the oral cancer patients group *versus* controls

Parameter	Oral cancer patients group	Controls	<i>p</i>
MMP9 (pg/mg albumin)	21.2±5	12.5±1.5	< 0.05
TIMP2 (pg/mg albumin)	41.1±3.1	63±4.1	< 0.05

3.MMP-9 levels were positively correlated with Ki-67 levels ($r = 0.65$) (Figure 3).

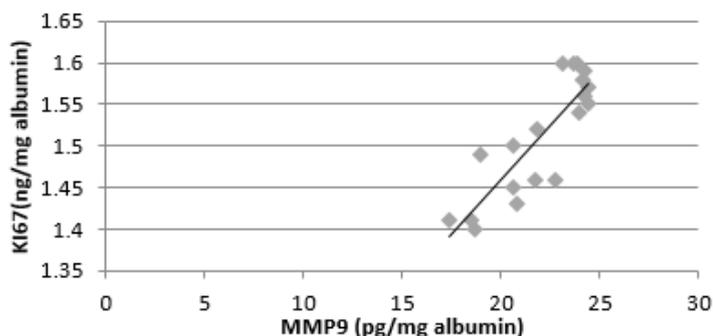


Figure 3. Positive correlation between MMP-9 and Ki-67 levels

4.MMP-9 levels were negatively correlated with TIMP-2 levels ($r = -0.71$) (Figure 4).

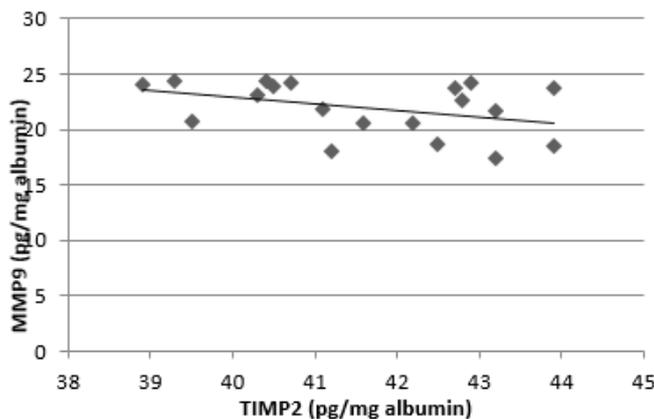


Figure 4. Negative correlation between MMP-9 and TIMP-2 level



Oxidative stress (OS) is defined as metabolic state in which there is an imbalance between free radicals such as hydrogen peroxide, superoxide anion radical, singlet oxygen and the antioxidant defences, free radicals levels exceeding defensive mechanisms [13-15].

Oxidative stress is an important factor in the development of a multitude of diseases including oral cancer by generating a chronic state of oxidation and a consequent state of chronic inflammation [13, 16]. It plays a crucial role in cancer development stages: initiation, promotion, and progression, inducing DNA mutations, lipid peroxidation or transcription factor activation such as mitogen-activated protein (MAP) kinase pathway or JAK-STAT3 pathway [17].

The most important salivary antioxidant is UA, being responsible for about 70% of all AO defence, followed by ascorbic acid [18]. UA is the end product of purine degradation and acts like a scavenger molecule for ROS neutralizing hydroxyl radicals and hypochlorous acid [13, 19].

Total antioxidant capacity encompasses all salivary AO, and it is used to evaluate the efficiency of the whole antioxidant system [20]. The positive correlation found in this present study between UA and TAC confirms the importance of UA concentration as the main salivary AO.

Inflammation plays a crucial role in cancer development and progression. Molecular messengers such interleukins are produced either by normal cells as a response to malignancy or by cancer cells as a mechanism for influencing local tumoral environment [13, 21]. In our study we found a negative correlation with low TAC levels and high IL-6 levels in oral cancer patients group suggesting the possible link of high oxidative stress conditions and chronic inflammation that accompanies cancerous lesions via the JAK-STAT3 and PI3K-Akt common pathways [21].

Cellular proliferation is a fundamental characteristic of cancer [22]. Ki-67 protein is a cellular proliferation and prognostic marker [23]. It is encoded by Ki67 gene and it is expressed in all active stages of cell cycle (G1, S, G2 and mitosis) and absent in the resting phase (G0). This characteristic makes this marker a great tool for monitoring the growth rate of a cellular population both in tumoral and in potentially malignant lesions such as leukoplakia [24]. In our study, the high levels of Ki-67 found in oral cancer patient group confirmed the active proliferation characteristic for this type of cancer.

Local invasiveness is another key mechanism in oral cancer progression. Matrix metalloproteinases (MMPs) are a group of zinc-dependent endo-proteases with roles in degradation and remodeling of extracellular matrix (ECM) and are secreted by both normal and tumoral cells [13]. They act on all types and collagen and elastin in the ECM, hemopexin domain conferring substrate specificity for different collagen types and degrade the non-collagen constituents of the ECM [25]. MMPs secretion is mediated via JAK-STAT3 and MAPK pathways and can be induced by high levels of IL-6 levels, linking inflammation and local invasiveness, key processes in oral cancer development [26]. In our study high levels of Ki-67 and MMP-9 coupled with a positive correlation ($r = 0,65$) between these two biomarkers indicates that oral cancers have high cellular proliferation rates and are actively inducing MMP-9 secretion leading to a poor prognostic for metastasis, a known trait of this types of cancers [27].

Tissue inhibitor of metalloproteinases are a group of four classes of proteins that block MMPs action. TIMPs N-terminal domain is structurally similar with MMPs substrate, their interaction with the MMPs inhibit their catalytic domain [28]. TIMPs are secreted by various cells and are regulated by various factors such as hormones, growth factors and several cytokines such as IL-6 [29]. In normal tissues there is a balance between MMPs and TIMPs level, this balance regulating the ECM formation and degradation. In malignant tumors the over expression of MMPs coupled with lower levels of TIMPs plays a crucial role in local invasiveness and tumoral progression and are associated with a higher metastatic rate and poor prognosis [30-32]. In our study based upon the high level of MMP-9 coupled with a low level of TIMP-2 and the negative correlation ($r = -0,71$) between these two biomarkers support the assumption that ECM degradation plays a crucial role in cancer progression, a key aspect in the highly invasive oral cancers evolution. This process is achieved by upregulating MMP production and down regulating protection factors such as TIMPs.



4. Conclusions

Our data highlighted the idea that the antioxidant (TAC and uric acid) and inflammation (IL-6) salivary parameters correlated with invasiveness (MMPs TIMPs) and proliferation (Ki67) salivary biomarkers outlines a constellation of parameters that may become a very valuable candidate on the oral cancer diagnosis and monitoring scene.

Acknowledgements. This article was supported by a grant of “Creșterea performanței instituționale și a competențelor CDI în domeniul sănătății ale Universității de Medicină și Farmacie “Carol Davila” București”, project number GI-CEX-UMFCD-SAN-CDCP-16.

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Manuscript received: 12.02.2020