Biomarkers of Oxidative Stress, Proliferation, Inflammation and Invasivity in Saliva from Oral Cancer Patients

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Abstract: Cancer represents the main cause of death in the economically developed countries and the second cause of death in developing ones. Head and neck squamous cell carcinomas are the sixth most common malignancies worldwide with oral cavity and pharynx cancers being the most common. Saliva qualifies as one of the most suitable diagnostic fluids due to the non-invasivity nature, simple handling procedures, easy collection and storage and good cooperation with patient groups such as children or persons with disabilities.

The aim of the present study is to assess the presence in saliva of several cancer biomarkers such as: tumor cells proliferation - Ki-67 Antigen and Squamous Cell Carcinoma Antigen (SCCA), inflammation - Interleukin-6 (IL-6), extracellular matrix collagen degradation - Matrix Metallo-proteinase-9 (MMP-9) and Tissue Inhibitor of Metalloproteinases 2 (TIMP-2), oxidative stress - total antioxidant capacity and uric acid. Both uric acid and total antioxidant capacity showed decreased levelsin the saliva of oral cancer patients. IL-6, Ki-67, SCCA and MMP-9 showed increased levels in the saliva of oral patients compared to the control group. Salivary TIMP-2 levels were also decreased in the patients group. We can conclude that salivary diagnosis has the potential of becoming a powerful tool in detecting and monitoring oral cancer patients.

Keywords: MMP-9, TIMP-2, IL-6, Ki-67, SCCA, saliva, oral cancer.

INTRODUCTION

Cancer represents the main cause of death in the economically developed contries and the second cause of death in developing ones. Head and neck squamous cell carcinomas are the sixth most common malignancies worldwide with oral cavity and pharynx cancers being the most common [1]. For the past three decades the 5-year survival rate has remained virtually unchanged to around 80% when the disease is diagnosed early and it drops to less than 20% when diagnosed in advanced clinical stages. Epidemiological data shows that oral cavity cancers tend to be twice more common in men than in women and have risk factors that include alcohol consumption, smoking or papilloma viruses infections. Generally the incidence of oral cancers varies with the geographical region: thus Central and Eastern Europe countries such as Romania are among the areas with the highest incidence rates [2]. In this respect new methods that can correctly detect and monitor oral malignancies are greatly needed.

A solid body of research demonstrates that saliva has the potential of being an alternative diagnostic fluid for a wide variety of oral and systemic affections [3-8]. Saliva fulfills a set of requirements that qualify it as one of the most suitable diagnostic fluids: non-invasivity, simple handling procedures, collection and storage, good cooperation with patient groups such as children or persons with disabilities [9].

The aim of the present study is to assess the presence in saliva of several biomarkers directly connected to processes with importance in cancer biology: Ki-67 Antigen and Squamous Cell Carcinoma Antigen (SCCA), inflammation - Interleukin-6 (IL-6), extracellular matrix collagen degradation - Matrix Metallo-proteinase-9 (MMP-9) and Tissue Inhibitor of Metalloproteinases 2 (TIMP-2), oxidative stress - total antioxidant capacity and uric acid.

MATERIAL AND METHODS

Patient Selection

The samples were obtained from patients diagnosed with oral cancer at the Department of Oro Maxillo Facial Surgery, University Hospital of Dental Medicine Prof. Dr. Dan Theodorescu. All samples were obtained following informed consent form each patient. Oral cancer diagnosis was done by one specialized examiner based on clearly defined criteria and histo-pathological examination following surgery. The study included 30 patients, ages between 40 and 65, diagnosed as follows: 15 with cancers of the parotid

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gland, 4 with lip cancer and 8 with tumors of the jaw bones and 3 with tumors locate elsewhere in the oral cavity. In this study we included patients with oral squamous cell carcinomas.Gender distribution showed an almost equal distribution of 14 men and 16 women with ages ranging between 45 and 60 years old. The included control group consisted of 14healthy volunteers with no associated oral or general diseases with ages ranging between 40 and 60 years old.

Saliva Collection

All subjects included in the study were asked to refrain from eating or drinking in the morning before collecting the samples. 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. Following collection of about 2mL of saliva, all samples were centrifuged at 3000rpm for 10 min in order to remove microorganisms and debris. All samples were afterwards kept at -80^{0}C until further analysis. Salivary parameters were made using ELISA method, biochemical or colorimetric assays.

ELISA Method

Separate enzyme-linked immunosorbent assays were used for quantitative detection ofKi-67, Foxp3 and SCCA (squamous cell carcinoma antigens), TIMP-2, MMP-9. All this parameters were detected using kits from R&D Systems Inc. Mineapolis – USA.

The protocol followed the manufacturer instructions. In short for each used kit the standards, controls and samples were pipetted into their specific pre-coated microwells. In a next step detection antibodies were added in each well. Following several minutes of incubation with a specific peroxidase which binds to the detection antibodies, the substrate solution was pipetted into each well. Color development was followed and quantified as absorbance using a microplate reader set to a specific wavelength. Al parameters were normalized with the salivary concentration of albumin (Albumin Assay Kit, Barcelona, Spain)

IL-6

IL-6 detection used a immuno fluorescence kit from Siemens Healthcare Global – Germany. The protocol followed the manufacturer instructions. Results were analyzed using the IMMULITE 1000 automatic analyzer (Siemens Healthcare Global – Germany).

Uric Acid

Uric acid determinations were made using the colorimetric methods with a commercial kit form Biosystems Company (Barcelona, Spain). Results were analyzed using an automatic analyzer also from Biosystems Company (Barcelona, Spain). The method is based on the oxidation of uric acid to allantoin by uricase. Reading was done at 293 nm.

Total Antioxidant Capacity (TAC)

For the total antioxidant capacity we used the TEAC method (Trolox equivalent antioxidant capacity) using a kit from R&D Systems Inc. Mineapolis – USA. This method is based on the color change induced by the oxidants on ABTS from radicalic and cathionic form to nonradical form. Reading was done at 415 nm.

Statistical Analysis

Results were expressed as means and standard deviations were appropriate. The data was analyzed using StataIC 11 (StataCorp. 2009. Stata: Release 11. StatisticalSoftware. College Station, TX, USA). Statistical analysis was done using a Student's t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

The present study assessed saliva of patients with oral cancer in comparison with healthy controls. Salivary biomarkers were grouped as follows: uric acid and total antioxidant capacity for monitoring oxidative stress, IL-6 as inflammatory marker evaluation, MMP-9 and TIMP-2 as markers of invasivity and Ki-67 and SCCA as markers of cancer proliferation.

Following biochemical analysis of uric acid, we found an average level of 1.25 mg uric acid / mg albumin ± 0.2 mg uric acid / mg albumin ($p < 0.05$, Student's t test) in the saliva of oral cancer patients compared to an average salivary level of 2,9 mg uric acid / mg albumin ± 0.44 mg uric acid / mg albumin ($p < 0.05$, Student's t test) in the control group saliva (Figure **1**). Salivary total antioxidant capacity showed a significant decrease in the oral cancer group when compared to respective controls: 0.62 mmol / mg albumin ± 0.12 mmol / mg albumin vs. 1.31 mmol / mg albumin ± 0.8 mmol / mg albumin ($p < 0.05$, Student's t test). Our data demonstrates a statistically significant decrease of total antioxidant capacity levels in the oral cancer group as opposed to respective controls (Figure **2**). We observed a significant increment in IL-6 levels in

the patient group versus control group: 2.9 pg IL-6 / mg albumin ± 0.4 pg IL-6 / mg albumin vs. 0.8 pg IL-6 / mg albumin IL-6 ± 0.07 pg / mg albumin ($p < 0.05$, Student's t test) (Figure **3**). Matrix metalloproteinase-9 levels were increased in saliva from patient with oral cancer when compared with control group 21.2 pg MMP-9 / mg albumin ± 4.2 pg / mg albumin versus 12.5 pg MMP-9 / mg albumin ± 1.7 pg / mg albumin) ($p < 0.05$, Student's t test) (Figure **4**). A significant decrease could be observed in TIMP-2 salivary levels in oral cancer patients versus control group: 41.1 pg TIMP-2 / mg albumin ± 2.8 pg / mg albumin vs. 63 pg TIMP-2 / mg albumin ± 4.7 pg / mg albumin) ($p < 0.05$, Student's t test) (Figure **5**). Our results showed an increase in Ki-67 levels in oral cancer patient's saliva versus saliva from healthy subjects: 1.6 ng / mg albumin ± 0.2 Ki-67 mg albumin) versus 0.35 ng / mg albumin ± 0.05 ng / mg albumin) ($p < 0.05$, Student's t test) (Figure **6**). SCCAg was absent in saliva collected from healthy patients but was found increased in oral cancer patients thus biochemical determination of salivary SCCA levels showed an average value of 3.9 ng / mg albumin ± 0.6 ng SCCAg / mg albumin ($p < 0.05$, Student's t test) in the patients group. SCCA levels for the control group could were lower than the detection threshold (Figure **7**).

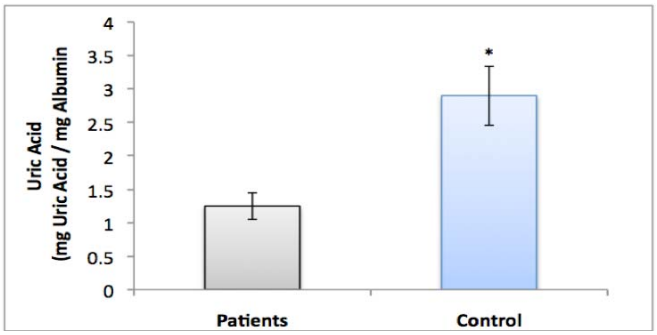

Figure 1: Uric acid levels in saliva (mg uric acid / mg albumin) of oral cancer patients versus control. (* - p value was set at <0.05).

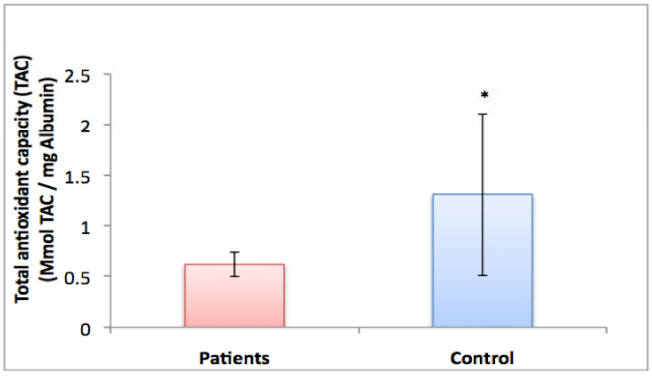

Figure 2: Total antioxidant capacity levels in saliva (mmol / mg albumin). (* - p value was set at <0.05).

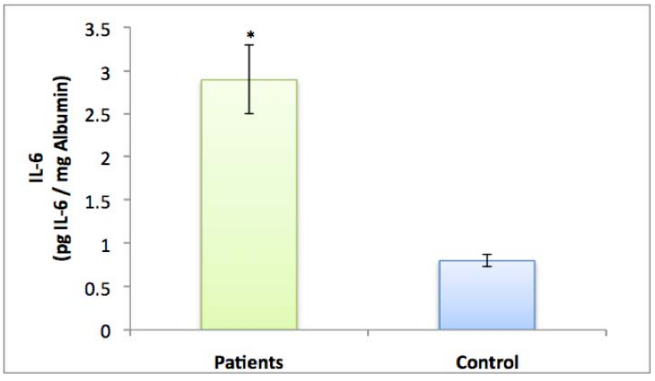

Figure 3: IL-6 levels in saliva (IL-6 pg / mg albumin). (* - p value was set at <0.05).

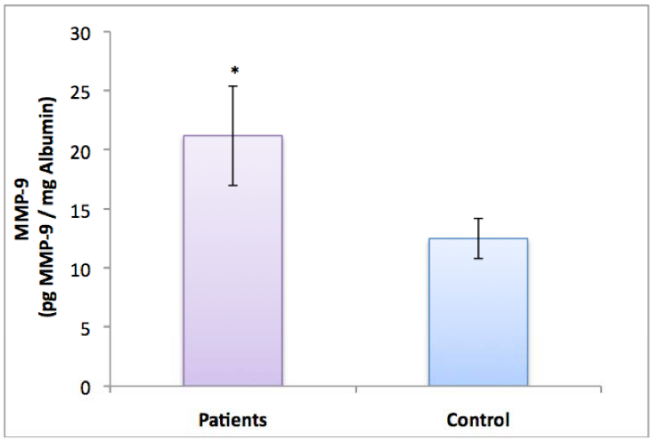

Figure 4: Salivary MMP-9 levels in oral cancer and healthy patients (MMP-9 pg / mg albumin). (* - p value was set at <0.05).

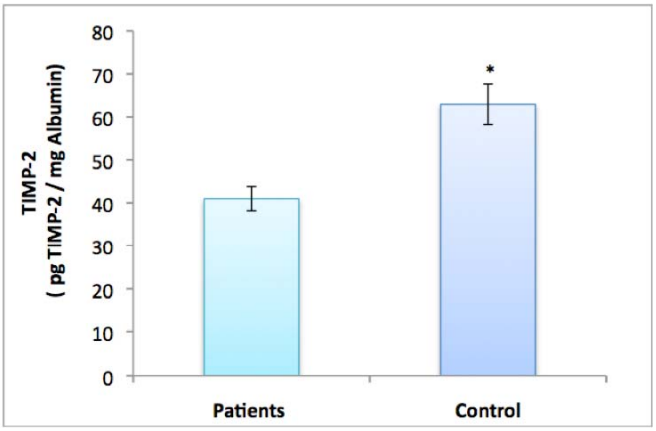

Figure 5: TIMP-2 salivary levels (TIMP-2 pg / mg albumin). (* - p value was set at <0.05).

DISCUSSION

Due to the anatomic proximity with the oral tissue intense efforts have been undertaken in the past years in order to indentify biomarkers of saliva than can mirror malignancies such a head and neck oral cancers. While there is no general consensus

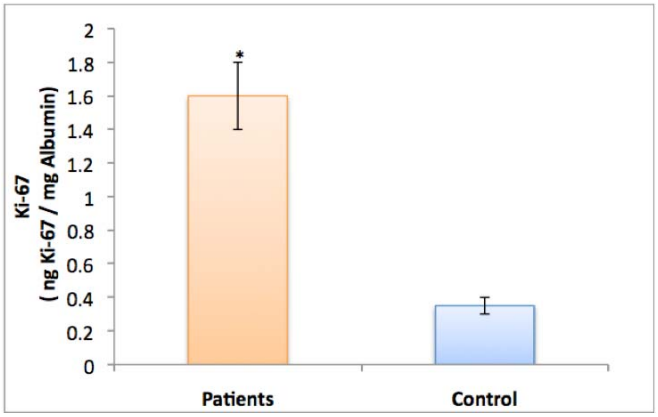

Figure 6: Ki-67 salivary levels (Ki-67 ng / mg albumin). (* - p value was set at <0.05).

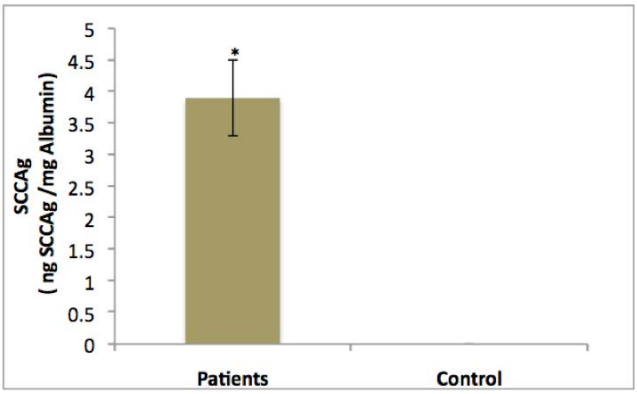

Figure 7: Salivary SCCAg (SCCAgpg / mg albumin).(* - p value was set at <0.05).

Table 1: Statistical Correlations between Different Salivary Biomarkers Analyzed in the Study. Statistical Significance was Set at r< -0.4; r>0.4

Parameter	Correlated with:	r
Uric Acid	TAC	0.78
IL-6	TAC	-0.66
MMP-9	Ki-67	0.65
TIMP-2	MMP-9	-0.71

regarding the definition of a biomarker, it can be described as "a cellular, biochemical, molecular or genetic alteration by which a normal, abnormal or simply biologic process can be recognized or monitored".

The present study analyzed several biomarkers in total saliva collected from patients with oral cancer.

We focused on different processes highly important for the development of oral cancers such as: markers that quantify oxidative stress such as uric acid and total antioxidant capacity; biomarkers of inflammation such as IL-6; molecules that monitor collagen degradation of the extracellular matrix and indirectly local invasiveness of the tumor - MMP-9 and TIMP-2 and cancer cells proliferation markers i.e Ki-67 and SCCA.

Inflammation is both a consequence and a possible cause of neoplastic processes development. Any tumor lesion is accompanied by a local and systemic inflammatory response triggered by the existence of tumoral mass. The role of this inflammatory process is to control and eradicate the tumoral growth. Interleukins are part of inflammatory mechanisms acting as signaling molecules. They are secreted by the immune cells located in and around the inflammatory process [10]. There are over 20 types of interleukins each having distinct roles in the inflammatory processes. IL-6 is a powerful interleukin that can induce chronic inflammation and thus can promote cancer development [11]. It regulates cellular growth, proliferation and cancer cell differentiation [12]. High levels of IL-6 induce cachexia alongside IL-1 and TNF-α [13].

Several studies linked high levels of IL-6 with poor cell differentiation and high dysplasia [14, 15]. Other studies linked high IL-6 levels with increased growth and invasiveness of squamous cell carcinomas [16]. Our study also found high salivary levels of IL-6 in oral cancer patients, making it a useful marker in assessing inflammatory levels in oral cancers.

There are four classes of tissue inhibitors of metalloproteinases (TIMP). TIMP-1 and TIMP-2 are the most important ones. The inhibition role is achieved by the interaction between TIMP's active N-terminal regions and the matrix metalloproteinases. This interaction maintains the normal tissular structure [17, 18]. Other roles include cell growth and differentiation inhibition. They also limit apoptosis and angiogenesis [19, 20]. Low MMP levels coupled with high TIMP levels give a good prognosis, the opposite situation, with low TIMP and high MMP levels are markers for increased invasiveness and metastasis [21, 22]. In our study we found low levels of TIMP-2 coupled with high MMP-9 levels in oral cancer patients saliva. We also found a negative correlation (r=-0,71) between these two parameters. We can conclude that the association between TIMP and MMP levels could be used as markers of oral cancer.

It is well established that uric acid represents the major salivary AO, accounting for more that 80% of the

total antioxidant potential. There is currently not a solid consensus regarding salivary uric acid concentrations; depending on the study it ranges between 40 and 240 μM. Its main action mechanism relies on its high reactivity with iron and copper ions and with HO• . Our previous reports show that uric acid levels are decreased in chronic periodontitis or in heaving smokers [23]. At the same time significant lower concentrations of uric acid were found in patients with oral lichen planus when compared with healthy controls. This inverse correlation between decreased concentrations or uric acid and the development of oral affections was also observed in the present study. Thus our data shows that oral cancer patients present a significant lower concentrations of uric acid when compared to healthy counterparts. We can conclude that chronic inflammation as well as premalignant or malignant lesions can decrease the salivary antioxidant potential, thus making the oral environment more vulnerable to oxidative stress.

The past decades have proved that matrix-metalloproteinases (MMPs) are host enzymes that play paramount roles in tissue destruction. The proteinase is involved in degradation of collagen IV and Vas well as other types of extracellular matrix proteins. MMP-9 is involved in normal physiological processes as well as in pathological ones. Thus MMP-9 breaks down extracellular matrix proteins in wound healing, cell migration, alveolar bone development or pathological events such as inflammation, arthritis or malignancy. The present study analyzed MMP-9 levels in patients with oral cancer. The results show that MMP-9 was significantly elevated in total saliva from the patients with oral cancer when compared to normal saliva. At the same time statistical analysis showed a direct correlation with Ki-67.

Total antioxidant capacity encompasses all salivary AO mechanisms and has an important significance in clinical settings when the AO status is evaluated in different pathological conditions. TAC concentrations vary depending on the analyzed oral fluid having different values for gingival crevicular fluid or for saliva. Our previous results [23] have shown that chronic periodontitis induces a significant decrease in the AO compartment together with a decrease in TAC levels when compared to healthy controls. Same results were observed for heavy smokers. The present study shows that malignancy is also associated with lower TAC which suggests the conclusion that in oral cancer the tissue homeostasis is altered and oral compartments are more vulnerable to exo or endo-genous OS.

World Health Organization refers to Ki-67 or MKI67 as potential indicators of aggressive progression of different cancers. Ki-67 is a marker of cellular division being present during active phases of the cell cycle and absent for cells that are quiescent, Ki-67 has a prognostic significance in many types of neoplasms through the assessment of Ki-67 labeling index. Previuous studies found high leveles of Ki-67 in oral cancer patient's saliva or in serum of leukemia patiens [24, 25]. In this study we have analyzed Ki-67 salivary levels. The results show that Ki-67 is absent in saliva form healthy patients but is significantly expressed in saliva from patients with oral cancer. Our data shows that this biomarker can be very valuable in detecting oral cancer by using a non-invasive method such as salivary diagnosis.

To sum up the results of the present study show that salivary diagnosis has the potential of becoming a powerful tool in detecting and monitoring oral cancer patients. However more studies on larger numbers of patients are needed in order to validate the data obtained so far.

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