

Evaluation of protein level's changes in saliva and sera of Oral Squamous Cell Carcinoma patients

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Abstract:

This study aimed to identify the changes in total protein in saliva and sera samples of patients with oral squamous cell carcinoma in comparison to those of healthy controls. These changes were followed using electrophoresis (PAGE). Meanwhile, determinations of albumin, globulin and albumin to globulin ratio were carried out on sera samples only.

Two groups were the participants in the present study, 18 patients with Oral Squamous Cell Carcinoma (OSCC), and 20 ages and gender matched healthy controls.

Key words: Oral cancer, Squamous cell carcinomas, Saliva, Albumin.

Introduction:

Oral cancer is one of the most common cancers in the world, with approximately 274300 new cases and 127500 deaths occurring each year [1]. Over 90% of oral cancers are Squamous Cell Carcinomas [2, 3]. In Iraq, according to Iraqi national cancer registry, oral cancers accounts for about 1.8 -2 % of all malignancies during the years 2000-2004 with an incidences of 0.77, 1.2, 0.88 and 1.14 per 100000 population for the years 2000, 2001, 2003 and 2004 respectively (Results of Iraqi cancer registry, 2000, 2001, 2003, 2004)[4]. (Kurdistan provinces are not included in this registry).

Proteins are major components of all cells, and present in all body fluids [5]. Over 100 individual proteins have a physiological function in serum. The proteins of the plasma are actually a complex mixture that includes not only simple proteins but also conjugated proteins such as glycoproteins and various types of lipoproteins [6, 7]. Quantitatively, the

single most important protein in the human body is albumin (3.5 to 5 g/dl), the other proteins are known collectively as globulins (2.5-3.5 g/dl) [8, 9]. So any Changes in the concentration of these individual proteins will lead to a change in total serum protein.

Saliva is a complex fluid produced by a number of specialized glands and secreted into the oral cavity of mammalian vertebrates. Most of the saliva is produced by the major salivary glands (parotid, submandibular and sublingual) but a small contribution is made by the numerous small labial, buccal and palatal glands which line the mouth [10]. Salivary proteins may be originated as an ultra-filtrate of plasma proteins or through salivary & mucosal secretions, so any violation to the integrity of capillary bed or mucosal lining as occurs in oral neoplasia may be associated with an increase in salivary proteins especially albumin.

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Materials and methods:

This study was carried out during the period of about a year and a half at the chemistry department college of science Baghdad University. The patients group was those who attended department of Oral and Maxillofacial Surgery (Al Wasity Hospital and Hospital of Specialized Surgeries of the Medical City in Baghdad).

Patients with Oral Squamous Cell Carcinoma (n=18) were included in the current study, as well as (n=20) controls matched for age and gender (Table1).

Provisional diagnosis was made by the clinical specialists. The final diagnosis was confirmed by Histopathological examination. The healthy individuals were nonsmoking and nondrinking volunteers who were not suffering from any disease and matched for age and gender were also included to be used as control.

Table 1: Distribution by age, sex, and histological classification for the study (Oral Squamous Cell Carcinoma & controls).

Group	N o.	Gender	Age (years) Mean±SD (Range)	Samples used	Type of tumors
Control	20	11 males 9 females	56.45±10.17 (46-79)	Blood&saliva	-
Malignant (OSCC)	18	10 males 8 females	59.24±11.63 (43-85)	Blood&saliva	Oral Squamous cell carcinoma

Saliva and blood samples were collected from each individual:

Unstimulated 5 to 10 mL whole, mixed-saliva samples, were collected and preserved in an ice container under resting conditions in a quiet room between 8.0-9.0 A.M. Patients and healthy individuals were asked to rinse their mouth with normal saline, then to generate saliva in their mouth and to

spit into a plastic container for 10 minutes. The saliva sample was immediately divided into two test tubes, the first one (total saliva) was stored frozen, while the remaining saliva in the second tube was centrifuged at (2000 xg) for 10 minutes at 4 °C to obtain the supernatant. The resulting supernatant and Pellet were stored frozen at - 20 °C in polyethylene tubes until they were used for the assay.

Ten milliliters of venous blood samples were collected by using plastic disposable syringes from overnight fasting patients (before surgery), and from the control groups, in plane polyethylene tube. Samples were allowed to clot for ten minutes at 37 °C in water bath, and then blood samples were centrifuged at (3000 x g) for 10 minutes. The obtained clear serum supernatant was stored frozen at -20 °C until it was assayed. Sediment of total saliva were extracted using phosphate buffer 0.2M; pH 7.0 containing 0.1% CTAB [11].

A modified Lowry method by Hartree [12] was used to determine total serum, saliva and sediment proteins concentration using Bovine Serum Albumin (BSA) as a standard. Total Protein concentrations of serum, saliva and Pellet were expressed in g/L. Serum albumin was determined by dye-binding method [13] using kit manufactured by BioLabo. The concentration of globulin in serum samples of control and all patient groups was calculated, using the following equation: $C_{glob} (g/L.) = C_{TP} - C_{alb}$

$C_{glob.}$ = concentration of globulin
 C_{TP} = concentration of Total Protein
 C_{alb} = concentration of albumin

Throughout the present study conventional polyacrylamide gel (7.5%) electrophoresis (PAGE) was

carried out on sera and saliva samples of control and patients group to detect the differences in total proteins and glycoproteins present in the studied samples. Tris - glycine buffer (0.075 M, pH 8.9) was used as the electrodes buffer solution. Pre-electrophoresis conditions were 50 mA as a constant current for 30 minutes, with a voltage of 15 v/cm, and at 4°C. Electrophoresis was carried out for 10 minutes at 20mA, then the process was continued for 3.5 hours at 4°C by using 40 mA as a constant current and voltage of 15 v/cm (LKB note). The separation of different proteins is based on the differences in both molecular size and the charge of these proteins. The polyacrylamide gel was stained for protein, using silver stain [14], while Schiff's reagent was used for glycoprotein detection [15].

Statistical analysis was carried out using the program Statistical Package for the Social Science (SPSS for windows, version 16.0). The differences between groups were tested by the student t-test, and P-value was considered significant if it is < 0.05. (SPSS version 16 software packages used).

Results and discussion:

Many researchers pointed out to the possibility of using saliva rather than blood as a diagnostic non-invasive body fluid [16, 17, and 18]. Several studies determined proteins concentration in the supernatant of saliva in patients with various diseases such as different types of cancer: breast cancer, ovarian cancer, oral cancer, and leukoplakia [19, 20, 21, and 22].

Therefore in the current study the changes in salivary proteins and sera proteins were followed using electrophoresis. These changes were examined in different samples: blood

and whole saliva. The results are shown in Figure 1 where the electrozymogram shows distinct differences in salivary protein profile in the studied group.

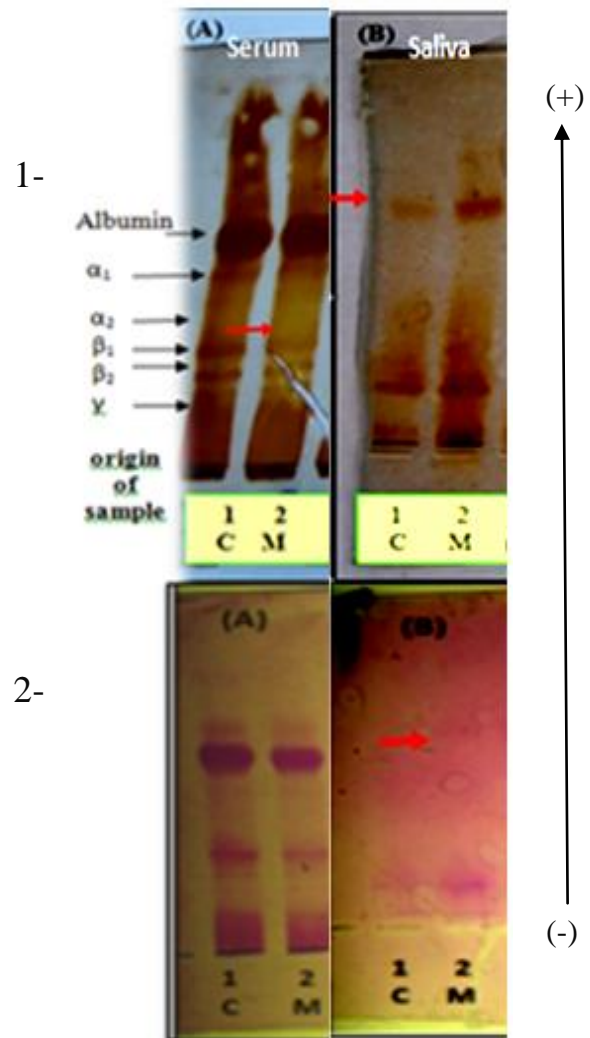


Fig 1: Electrozymogram showing protein and glycoprotein profile using 7.5% acrylamide gel.

Electrogram A 1&2 refer to: 1 pooled crude sera (control) (C) & 2 pooled crude sera (malignant) (M) (OSCC). While electrogram B 1&2 refer to: 1 pooled crude saliva (control) (C) & 2 pooled crude saliva (malignant) (M) (OSCC). The red arrows refer to the extra bands appears in the indicated samples.

As it is clear there is a visible increase in the density in the region of the γ -globulins bands associated with

an increase in albumin in saliva samples of patients with Oral Squamous Cell Carcinoma. As well as there is a slight increase in the region belongs to α_2 -globulins bands, in contrast a slight decrease in α_1 -globulins and β -globulin bands were observed. Moreover, there is a slight reduction in the intensity of bands that belong to γ region. It is clearly obvious from the zymogram additional band (the red arrow) appeared in saliva samples of patients with oral squamous cell carcinoma in comparison to that of the control samples), and this band moved further toward the anode.

The differences are also clear among the studied group when the gel was stained for glycoproteins where the above mentioned additional band appeared to be glycoproteins. Also the change in glycoproteins profile is clear particularly in the globulins region. This may be related to the changes in cell surface glycoproteins during malignancy and as a result of synthesis of the acute phase proteins during inflammation processes [23].

To measure the observed change quantitatively the concentration of protein was measured in whole mixed saliva, supernatant and the pellet fraction. The results of measurements are illustrated in Table 2.

Table 2: Comparison of total protein in saliva samples of patients with Oral squamous cell carcinoma.

Group	Age (years) Mean \pm SD (Range)	Total protein (g/L)	Total saliva	Supernatant of Saliva	Pellet of saliva
Control (n=20)	56.45 \pm 10.17 (46-79)	Mean \pm SD (Range)	2.69 \pm .961 (1.58-4.71)	2.136 \pm .737 (1.28-3.4)	.223 \pm .101 (.083-.51)
Malignant (OSCC) (n=18)	59.24 \pm 11.63 (43-85)	Mean \pm SD (Range)	4.37 \pm 1.34* (2.45-6.0)	2.711 \pm .822* (1.5-4.1)	.461 \pm .336* (.137-1)
		P-value	.000	.037	.005

* Significant difference in comparison to control at (P < 0.05).

The results in this table reveal the presence of a significant increase (p < 0.05) in whole, supernatant and pellet saliva proteins of patients with malignant tumors (OSCC) in comparison to that of the control, where an increase of 35.82% were observed in the patients group. Shpitzer et al reported that there was a 26% increase of total saliva protein in patients with oral squamous cell carcinoma in comparison with healthy controls. This increase is in agreement with other studies carried on (OSCC) patients whereas an increase in salivary total protein concentration in total saliva was also recorded in [24, 25, 26, 27, and 28] studies.

On measuring the percentage of total protein that is present in the saliva supernatant and saliva pellet in relation to the concentration protein in total saliva, it was found: - That in the patients group, 79.41% and 8.29% of total protein was measured in the supernatant and pellet of the saliva respectively. In the controls group, 62.04% and 10.55% of total saliva proteins was detected in the supernatant and pellet saliva samples respectively.

The saliva pellet's proteins is significantly higher in patients with OSCC than that of the control group (table 2), and this may be explained by the presence of dead cells in the saliva pellet of the patients. The proteins

concentration of the saliva supernatant and pellet collectively is less than that of whole saliva, which may be due to denaturation of some of the proteins during preparation and separation.

Concentration of total protein, albumin and globulin were measured in sera sample and the results are presented in Table 3.

Table 3: Comparison of total protein, Albumin, Globulins & Albumin/Globulin Ratio in sera samples of patients with Oral squamous cell carcinoma.

Group	Age (years) Mean±SD (Range)	Mean±SD (Range)			
		P-value			
		Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	A/G Ratio
Control (n=20)	56.45±10.17 (46-79)	75.09±5.35 (62.33-84.37)	44.01±2.78 (40-48)	31.32±4.95 (20.33-38)	1.44±.25 (1.13-2)
Malignant (OSCC) (n=18)	59.24±11.63 (43-85)	74.05±8.53 (60.19-86)	38.05±3.87* (33-43)	35.04±5.66* (25.43-45.5)	1.04±.19* (.77-1.45)
		.657	.000	.040	.000

* Significant difference in comparison to control at ($P < 0.05$).

The results showed a decrease in total serum protein concentration in sera samples of patients with Oral Squamous Cell Carcinoma in comparison to that of the control samples in the study but the decrease was non-significant ($p > 0.05$).

The results of the albumin concentration in sera samples of patients and control group are presented in Table 3. A significant decrease (15.66%) in serum albumin was observed in patients' samples in comparison with that of the healthy control samples. Such result was confirmed by the electrozomogram Figures 1.

Albumin, which is produced in the liver, is a major plasma protein [29]; Since albumin is the major protein present in blood (about 60% of total serum protein), so any condition causing lowering of serum albumin will lead to a reduced total serum protein (hypoproteinemia), but hypoproteinemia may be due to hypogammaglobulinemia as well [30, 31]. In malignant neoplasia there will be a catabolic state mediated by many cytokines, this catabolic state is necessary to supply the malignant cells

with the primary materials necessary for cell division and growth as long as albumin acts as a source of nutrition to cells where it is hydrolysed to supply the raw materials [32]. In addition to this malignancy is known to be associated with high oxidative stress and as albumin may play a role as a sacrificial anti oxidant (it is destroyed by reactive oxygen species when it functions as an *antioxidant*), this may have an effect on the level of albumin in serum [33, 29, 34].

Globulin concentration in sera samples of the studied group was calculated and the result (Table 3) indicated that there is a significant increase in globulin concentration in patients samples compared with that in healthy controls in the study (which observe in Figures 1). Globulins are another type of protein, larger in size than albumin. They are divided into three main groups: alpha, beta, and gamma [9]. The observed increase in S.globulins may be explained as follows: an increase in serum globulins may be a compensatory reaction to the decrease in serum albumin to maintain osmotic pressure of plasma [7].

Also different components of globulins increase as a result of synthesis of positive acute phase reactants. The impact of increase of S.globulins is less than the impact of decrease in S.albumin on T.S.P. so T.S.P decreases in significant hypo albuminemia despite the increase in serum globulins.

The ratio of serum albumin to globulin in patients and healthy controls of the study group was determined mathematically and the results were presented in Table 3. A significant decrease in this value was found in patients samples compared with healthy controls in the studied group. In our study a decrease in this value is due to decrease serum albumin and increase in serum globulins, but the decrease in serum albumin was greater so the net level of total serum protein was slightly decreased

The current study is a pilot one which pointed out to presence of an alteration in glycoproteins profile in sera & saliva samples of the oral squamous cell carcinoma patients. Further studies are carrying out in our laboratory which concentrates on the additional band which was detected in saliva samples to find out the possibility of using this band to diagnose the disease. Since early detection of oral squamous carcinoma is the corner stone in effective management of these devastating tumors. This trial may represent a step in the efforts of early detection of this condition.

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تقييم التغيرات في مستوى بروتين اللعاب والأمصال في المرضى المصابين بسرطان الخلايا الحرشفية

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الخلاصة:

هدفت هذه الدراسة إلى التعرف على التغيرات في البروتين الكلي في عينات لعاب وأمصال المرضى المصابين بسرطان الخلايا الحرشفية في الفم ($n = 18$) وذلك بالمقارنة مع الأصحاء المتطابقة ($n = 20$). وتم متابعة تغيرات هذه البروتينات باستخدام تقنية الترحيل الكهربائي الهلامي (PAGE) لعينات اللعاب والأمصال المرضى مقارنة مع مجموعة الأصحاء و في الوقت نفسه تم متابعة الألبومين والجلوبيولين و نسبة الألبومين إلى الجلوبيولين على عينات الأمصال فقط. وأظهرت النتائج انخفاض غير معنوي ($p < 0.05$) في تركيز البروتين الكلي في عينات أمصال المرضى المصابين بسرطان الخلايا الحرشفية في الفم في حين كان هناك انخفاض معنوي ($P > 0.05$) في الألبومين في عينات أمصال المرضى و زيادة معنوية ($P > 0.05$) في عينات أمصال المرضى لتركيز الجلوبيولين. كما وجد انخفاض معنوي ($P > 0.05$) في نسبة الألبومين إلى الجلوبيولين في مجموعة المرضى مقارنة مع ذلك مع مجموعة الأصحاء.

تضمنت الدراسة مجموعتين من المشاركين 18 مريضاً بسرطان الخلايا الحرشفية (OSCC)، و 20 من الأشخاص الأصحاء من كلا الجنسين.