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Salivary biomarkers in premenopausal women with invasive ductal carcinoma before and after surgical removal of tumor mass

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Abstract : ***Background:***Despite the numerous advances made in breast cancer research, carcinoma of the breast is still the most common, disfiguring and deadliest cancer among women. Multiple molecules isolated from saliva can be used as cancer biomarkers for diagnosis, prognosis, and monitoring studies.

Objective:This study is designed to evaluate secretory status, total protein, CA15-3, and pH in the saliva of premenopausal women diagnosed with invasive ductal carcinoma before and after surgical removal of tumor mass in comparison with those in healthy corresponding women.

Methodology: Forty Iraqi premenopausal women with invasive ductal carcinoma (IDC) in different sites and hospitals for early detection of breast cancer and gynaecology were involved in this study along with 25 healthy women as control. Secretory status and the level of pH, CA15-3, and TP markers were detected in the saliva of all subjects.

Results: 72.5% of IDC patients are non-secretors, which is significantly higher than 32.5% of healthy women, and the salivary pH of IDC patients before surgical removal of tumor is significantly more acidic than that of healthy women and still acidic even after surgical removal. CA15-3 and TP levels are significantly elevated in the saliva of IDC patients whether before or after surgical removal in comparison with those in healthy women. Statistical analysis showed significant negative correlation between salivary levels of both CA15-3 and TP with pH value in IDC patients before surgery, and with both pH value and frequency of non-secretors after surgery.

Conclusion: These findings suggest that saliva possesses a considerable diagnostic and prognostic values in breast cancer, and secretory status may act as a risk factor associated with aggressiveness behavior of breast cancer that need further investigation.

Key words: Breast cancer, invasive ductal carcinoma, saliva, secretory status, CA15-3, pH, TP.

Introduction

Unlike other bodily fluids, salivary diagnostics offer an easy, inexpensive, safe, and non-invasive approach for disease detection, and possess a high potential to revolutionize the next generation of diagnostics[1,2]. Generally, saliva sampling involves a simple and non-invasive collection method that allows easy storage and transport[3]. In addition, sampling of saliva is technically easier than collection of blood, particularly when volunteers are invited for repeat sample collection[4,5]. Furthermore, saliva sampling does

not require specialised instruments or trained personnel with phlebotomy skills, it has minimal or no risk of cross contamination among patients and offers very low exposure of healthcare personnel to blood-borne pathogens such as HIV and hepatitis[2, 5]. Because its composition changes under different pathological conditions, multiple molecules isolated from saliva can be used as cancer biomarkers for diagnosis, prognosis, drug monitoring and pharmacogenetic studies[6,7-8]. Promising new technologies have unveiled large numbers of medically valuable salivary biomarkers that can potentially provide useful information for clinical diagnosis and prognosis of a variety of cancers (oral, pancreatic, lung, breast, and liver), and for different disease conditions including autoimmune, viral, bacterial, cardiovascular, and metabolic diseases[9,10-13].

Despite the numerous advances made in breast cancer research, carcinoma of the breast is still the most common, disfiguring and deadliest cancer among women. The understanding of BRCA1 and BRCA2 gene mutations has saved lives, but these findings account for only 5 to 10% of breast cancer cases, therefore, additional paradigms may be needed in order to increase early detection and treatment efficacy[14,15-16].A salivary proteins were reported to be useful for the early detection and measurement of breast cancer patients' response towards chemotherapy and/or surgical treatment of the disease[17,18-19]. CA15-3 is a tumor marker which is found on the surface of cancer cells and sheds into the blood stream. This tumor marker was found in the saliva of women diagnosed with breast cancer. It is used to monitor advanced and metastatic cases[17, 20].The positive correlation between serum and salivary concentrations of the protein CA 15-3 in patients with breast cancer suggests that saliva could be an alternative to blood sampling to help breast cancer monitoring[21].Moreover, salivary protein factors such as vascular endothelial growth factor, epidermal growth factor, and Carcinoembryonic Ag were elevated in breast cancer patients compared to healthy subjects, hence making it a possible biomarker for breast cancer detection[22].Recently, it has been observed that lung resistant protein (LRP) was present in significantly higher concentrations among the subjects with breast cancer as compared to healthy women, suggesting it is a novel prognostic indicator for carcinoma of the breast[23]. More recently, VEGF and the combination of VEGF and CA15-3 showed high diagnostic value in early breast cancer[24].

Accordingly, the present study is designed to evaluate secretory status, total protein, CA15-3, and PH in the saliva of premenopausal women diagnosed with invasive ductal carcinoma before and after surgical removal of tumor mass in comparison with those in healthy corresponding women.

Materials & Methods

This study has been designed upon 70 Iraqi premenopausal women in different sites and hospitals for early detection of breast cancer and gynecology;30 of them with normal breast, and 40 with invasive ductal carcinoma (IDC). The diagnosis was confirmed according to the fine needle aspiration (FNA) technique carried out by specialists.Saliva was collected from all subjects (after rinsing their mouth 2 times with cold drinking water 5-10 minutes prior to collection); about 3-5 ml of saliva was transferred into test tubes and centrifuged for 5 min at 1000 rpm and supernatant was collected and dispended into three aliquots. The first aliquot was directly used to determine the PH value of saliva by using pH meter, while the second aliquot was placed in a boiling water bath for 10 min to denature the salivary enzymes, then cooled and centrifuged for 5 min at 1000 rpm and supernatant was collected and diluted with an equal volume of normal saline to detect the ABH secretor status by hemagglutination inhibition method. The test was provided by adding of one drop of carefully adjusted agglutinating reagent to 1:8 titer (Anti-A, Anti-B, or Anti- H from Biorex, UK) and one drop of clear saliva and to control tube one drop of saline were added, later mixed, and finally incubated at room temperature for minimum of 10 minutes. Then one drop of appropriate indicator erythrocytes was added to each tube, mixed and incubated at room temperature for 10 minutes. All control samples showed clumping as there was no antigen present. The test group was considered as positive if agglutination was not seen, indicating that antigen-antibody reaction had taken place between saliva and antisera, and there was no antibody left for RBCs to react, indicating the presence of blood group and vice versa was applied for negative test group samples[25, 26]. However, the third aliquot was used to determine salivary tumor marker CA15-3 and total protein. Tumor marker CA15-3 was estimated by using an automated quantitative Mini VIDAS test (from (BioMerieux, France), while total protein was estimated by using the Biuret method (Linear chemicals. S.L, Spain)[27].

Descriptive data were expressed as percentage values, whereas measurable data were expressed as mean ± standard error (M ± SE). Differences among groups were analyzed either by using Chi-square test for

descriptive values or by using one-way analysis of variance (ANOVA). The correlation coefficient (r) between two parameters was carried out by Pearson correlation coefficient test. The *P* values of difference < 0.05 were considered significant.

Results

Results showed non-significant difference in the average age between normal-breast women (44.4± 1.2 years) and patients with invasive ductal carcinoma (45.8 ± 0.76 years), however the frequency of non- secretors is significantly increased from 32% in normal breast group (control) up to 72.5% in patients group as illustrated in Table 1.

Table 1: Characterization of premenopausal women of control and patients groups

Character		Control n=25	IDC n=40	Significance
Age (years) (M±SE)		44.4± 1.2	45.8 ± 0.76	*P* = 0.307
Secretory status (n, %)	Secretors	17 (68%)	11 (27.5%)	X2 = 10.29
	Non-secretors	8 (32%)	29 (72.5%)	*P* = 0.0013

Table 2: Evaluation ofbiomarkers in saliva of control and patients groups

Parameter M±SE	Control	IDC Patients	
		Pre-operation	Post-operation
PH	7.22 ± 0.02 A	5.73 ± 0.05 B	5.96 ± 0.06 C
CA 15-3 (U/ml)	0.85 ± 0.10 A	10.86 ± 0.37 B	9.67 ± 0.35 B
TP (g/dl)	0.14 ± 0.007 A	1.02 ± 0.047 B	0.84 ± 0.028 C
Different letters represent significant difference between columns according to ANOVA & HSD test at *P*< 0.05			

Concerning with the evaluation of biomarkers in saliva of control and patients groups, PH value is significantly decreased from neutral (7.22 ± 0.02) in control group down to slightly acidic in patients group whether before surgical removal of tumor mass (5.73 ± 0.05) or after surgical removal (5.96 ± 0.06) which are also significantly differ (Table 2). Similarly, total protein (TP) level is significantly elevated from (0.14 ± 0.007 g/dl) in the saliva of control group up to (1.02 ± 0.047 g/dl) and (0.84 ± 0.028 g/dl) in pre- and post-operative patients respectively. However, tumor marker CA15-3 is significantly elevated in patients group before and after surgical removal (10.86 ± 0.37 U/ml, 9.67 ± 0.35 U/ml respectively) in comparison with (0.85 ± 0.10 U/ml) in control group. Relative to the normal level of CA15-3, and TP in control group, CA15-3 is increased about 12.7 times in patients before surgical removal of tumor and then decline to 11.3 times, while relative increment in TP is about 7.2 times before surgical removal then decline to 6 times after surgery (Figure 1).

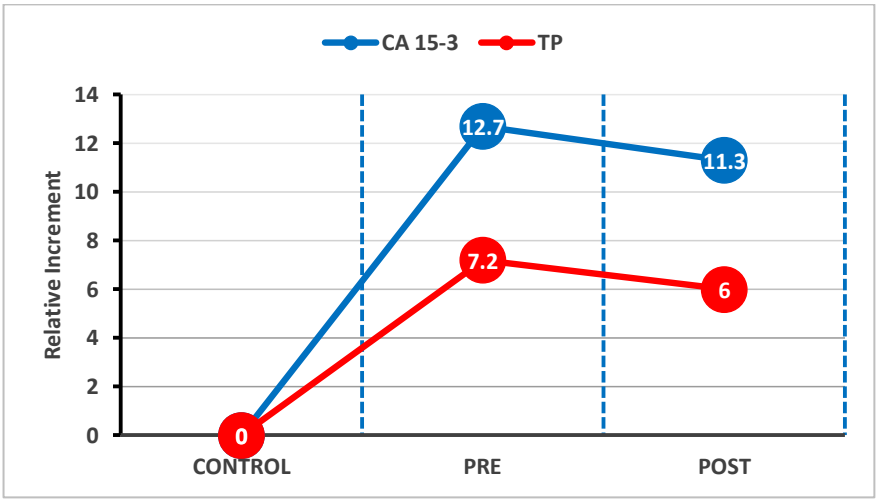

Figure 1: Relative increment of salivary CA 15-3 and TP before (pre) and after (post) surgical removal of tumor mass.

Table 3: Correlation (r) between different salivary biomarkers in control group

Parameter	secretory status	CA 15-3	TP
PH value	r= 0.0874 [NS]	r= -0.3336 [NS]	r= 0.1304 [NS]
secretory status		r= 0.2381 [NS]	r= 0.0195 [NS]
CA 15-3			r= -0.0156 [NS]
[NS] non-significant correlation at P < 0.05			

Table 4: Correlation (r) between different salivary biomarkers in IDC Patients before surgical removal of tumor mass.

Parameter	secretory status	CA 15-3	TP
PH value	r= 0.0913	r= -0.8255 *	r= -0.6215 *
secretory status		r= -0.1924	r= -0.3011
CA 15-3			r= 0.8379 *
* Significant correlation at P < 0.05			

Table 5: Correlation (r) between different salivary biomarkers in IDC Patients after surgical removal of tumor mass.

Parameter	secretory status	CA 15-3	TP
PH value	r= 0.1102	r= -0.7763*	r= -0.4832 *
secretory status		r= -0.3288 *	r= -0.3494*
CA 15-3			r= 0.7839*
* Significant correlation at P < 0.05			

On the other hand, statistical analysis of correlation (r) between different salivary biomarkers (secretory status, PH, CA15-3, and TP) in control group revealed non-significant association (Table-3). While in patients group before surgical removal of tumor mass, PH value is significantly reverse correlated with both CA15-3 (r= - 0.8255) and TP (r= - 0.6215), but secretory status showed non-significant association with other markers. Additionally, significant positive correlation (r= 0.8379) is recorded between CA15-3 and TP (Table-4). In

similar manner, PH value after surgery showed significant negative association with CA15-3 (r= - 0.7763) and TP (r= - 0.4832), as well as significant positive correlation between CA15-3 and TP (r= 0.7839), but salivary concentration of both is significantly decreased in secretors with correlation of about r= - 0.3288, and r= - 0.3494 respectively (Table 5).

Discussion

Several facts can be extracted from the present results, the first one is the acidity of saliva which acts as diagnostic marker in the saliva of breast cancer patients and can be used for monitoring purpose because pH value is significantly rises toward neutral PH after surgical removal of tumor in comparison with its lower value before surgery (Table 2). This acidity of saliva may be due to increase salivary concentration of tumor marker CA15-3 and TP several times (Figure 1), because as their concentrations decrease, the PH value increased (Table 4, Table 5). In agreement to our result, it was found that saliva in patients with breast cancer during the course of therapy present a significant reduction in pH[28]. This reduction in salivary pH in patients with breast cancer may be due to composition changes of saliva under different pathological conditions that shift its pH value to more acidity, for instances; Sugimoto *et al.*[29] identified 28 metabolites in a sample cohort of 30 saliva samples from patients with breast cancer. In a recent study, a comprehensive metabolite analysis of saliva samples obtained from breast cancer patients and healthy controls demonstrated significantly higher concentrations in breast cancer patients comparing with healthy individuals, especially in patients with IDC before treatments tended to be higher concentrations than those obtained after treatment[30]. Moreover, Cheng *et al.* reported that there were significant differences in salivary free amino acids (SFAAs) between early-stage breast cancer (Stage I and II), late-stage breast cancer (Stage III and IV) and healthy controls[31]. More recent, Takayama *et al.*[32] found that numerous polyamines were present in significantly higher concentrations in the cancer group with respect to the control group.

The second fact obtained from this study is the applicable of secretory status to be used as risk factor since there is significant higher frequency of non-secretors among BC patients (72.5%) in comparison with (32%) in healthy women (Table 1). Furthermore, although secretory status showed non-significant correlation with other salivary markers (pH, CA15-3, and TP) in both healthy women (Table 3) and preoperative BC women (Table 4), CA15-3 and TP levels are still elevated in non-secretor patients after surgical removal (Table 5). In our previous study, we found that among several non-preventable risk factors, secretor status was the strongest risk factor for getting breast cancer particularly when combined with other risk factors[33], also in our unpublished data we found that all patients (100%) with triple negative TN subtype of invasive ductal carcinoma are non-secretors in comparison with 63.6% of luminal subtype[34]. Thus non-secretor feature can be acts as a predictive marker that the BC patients probably having more aggressive subtype of breast cancer.

The third fact extracted from the present result is the usefulness of saliva as alternative sample for diagnosis, prognosis, and monitoring of breast cancer because both CA15-3 and TP as biomarker for breast cancer appear to be detectable in saliva with significant difference not only from healthy women, but even between BC women before and after surgery particularly for TP marker (Table 2), and both markers still positively correlated with each other even after surgical removal of tumor in comparison with their levels in healthy women (Table 3, Table 4, and Table 5). In agreement with our results, significant positive correlation between serum and saliva CA15-3 concentrations is reported[20], and this positive correlation between serum and salivary concentrations of the protein CA 15-3 in patients with breast cancer suggests that saliva could be an alternative to blood sampling to help breast cancer monitoring[7, 35]. In recent study, Zajkowska*et al.*[36] observed significantly high sensitivity and specificity of VEGF, macrophage colony-stimulating factor (M-CSF) and CA15-3 for detecting breast cancer, especially in early-stage cancer, and the combination of VEGF and CA15-3 showed high diagnostic value in early breast cancer[24].

Since in patients with breast cancer and other tumors there is over expression in several proteins, thus salivary levels of TP can acts as a good biomarker. It has been found that the salivary analyses of three pooled whole saliva specimens yielded approximately forty-nine proteins which were differentially expressed between the healthy control pool and the benign and breast cancer patient groups[37].Furthermore,the saliva of the BC patients had significantly higher levels of another proteins such as c-erbB-2, VEGF, EGF, CA 125, and lung resistant protein (LRP)[17, 38, 22, 39, 40, 23]. In contrary to our findings, Emikli-Alturfan *et al.*[28] found that saliva in patients with breast cancer during the course of therapy present a significant reduction in total protein.

Furthermore, no significant difference in salivary HER2 protein expression was found between the case and control groups, thus salivary expression of the HER2 receptor may not be a reliable alternative to tissue assessment[35].This controversial results may be due to the time at which salivary analysis performed, our results obtained from BC women before starting therapy, or due to different expression of certain proteins because in a study involving patients with breast cancer, Cao *et al.*[41]analysed the salivary proteome and found nine proteins with 1.5-fold upregulation or downregulation were associated with cancer with respect to the healthy controls.

In conclusion, the present study found significant differences in salivary markers including pH, CA15-3, TP not only between healthy and breast cancer patients but also between pre- and post-operative tumor. Also the frequency of non-secretors among BC patients is significantly higher than in healthy women. Therefore, saliva has potential for diagnosis, prognosis, and monitoring of BC patients.

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