The Value of Salivary Soluble CD44 Level Determination in Oral Malignant and Potentially Premalignant Lesions

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Abstract

Objective: order to determine the validity of sCD44 ELISA test on saliva and its effectiveness in diagnosing and predicting prognosis of oral cancer and premalignant lesions

Design: prospective comparative clinical trial

Patients & Methods: Patients with suspected lesions for cancer were subjected to sCD44 ELISA test on saliva then followed by biopsy and histopathological examination. Results were compared to healthy participants to check the difference

Results: Salivary sCD44 level was detected in saliva of all patients and in the cancer group salivary sCD44 level (48.05 ± 20.66 ng/ml) was much higher than that of the control group (12.37 ± 3.82 ng/ml) with a statistically significant difference. On the other hand, when the mean level of salivary sCD44 was compared between the premalignant without dysplasia and the premalignant with dysplasia groups, salivary sCD44 level in the premalignant with dysplasia group (34.98 ± 11.27 ng/ml) was found to be significantly higher than its level in the premalignant without dysplasia group (14.67 ± 7.27 ng/ml). sCD44 was found to predict recurrence in cases with high levels of this marker.

Conclusion: salivary sCD44 test may be an effective for diagnosis and as a prognostic marker for recurrence of oral cancer and malignant transformations in oral mucosal premalignant lesions. More detailed studies for using saliva on a wider scale are recommended for earlier diagnosis and prognosis determination in oral mucosal lesions.

INTRODUCTION

Invasive oral squamous cell carcinoma (OSCC) is often preceded by the presence of clinically identifiable premalignant changes of the oral mucosa. Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys to reducing the mortality, morbidity and cost of treatment associated with OSCC (1). A potential marker for HNSCC is CD44 which is one of the adhesion molecules. CD44 proteins are also released in soluble form (sCD44) via proteases and are detectable in normal circulation. Circulating levels of sCD44 correlate with metastases in some tumors (2).

The easy non-invasive nature of collection and the relationship between oral fluid and plasma levels make oral fluid a valuable clinical tool (3).In some malignant diseases, markers can be detected in the saliva, such as the presence of protein p53 antibodies in patients with oral squamous cell carcinoma, or high levels of defensin-1 positively correlated with the serum levels. The presence of the c-erbB-2 tumor marker in the saliva and blood serum of breast cancer patients and its absence in healthy women is a promising tool for the early detection of this disease. In ovarian cancer too, the CA 125 marker can be detected in the saliva with greater specificity and less sensitivity than in serum (4). Surprisingly, only few studies examined tumor markers in the saliva of OSCC patients. Consequently, the present investigation was carried out in order to determine the validity of sCD44 ELISA test on saliva and its effectiveness in detecting oral cancer and possibly early malignant transformations in some premalignant lesions.

PATIENTS AND METHODS

The present study was performed on a total of 40 individuals selected from the out patient clinic of Oral Medicine and Periodontology Department, Faculty of Oral

and Dental Medicine, Cairo University & Surgical Oncology Unit, Faculty of Medicine, Menofia University, Between April 2007 & May 2010. All patients gave consent to participate and the Institutional Board Approval was obtained.

All subjects were selected to be not taking any drugs inducing any changes that could affect the salivary flow. Participants were subdivided into four groups: **Group I:** included healthy control subjects, all were nonsmokers. **Groups II, III:** participants had mucosal lesions suspected to have premalignant tendency; (leukoplakia, speckled leukoplakia, lichen planus, smoker's keratosis). They were further subdivided according to biopsy results into patients with dysplastic lesions and others with no dysplastic changes; 10 patients into each group. **Group IV:** included patients suffering from oral malignancy (squamous cell carcinoma, mucoepidermoid carcinoma)

All participants were subjected to full history taking, careful clinical examination, Biopsy from lesions of the premalignant and malignant groups, salivary sample collection and finally detection of CD44 level in salivary samples

Salivary sample collection:

Collection of whole unstimulated saliva (WUS) using standard techniques was done as described by Navazesh (5). Briefly, subjects refrained from eating, drinking, using chewing gum etc., for at least 1 ½ h prior to the evaluation. Samples were obtained by requesting subjects to swallow first, tilt their head forward and expectorate all saliva in a tube for 5 minutes without swallowing. After collection, all samples were immediately stored at -80^{0}C until assayed.

Biopsy:

Biopsies were taken from the lesions. Ring block anesthesia was performed. There was no direct infiltration of the anesthetic solution into the examined areas to avoid its direct damage. A surgical double wedge incisional biopsy was carried out to a depth of about 2mm .

Biopsy specimen were immediately fixed in 10% neutral buffered formalin and then processed in the routine way for preparing a paraffin block. Five microns thick sections were cut and stained with conventional Hematoxylin & Eosin (H&E) for histopathologic examination.

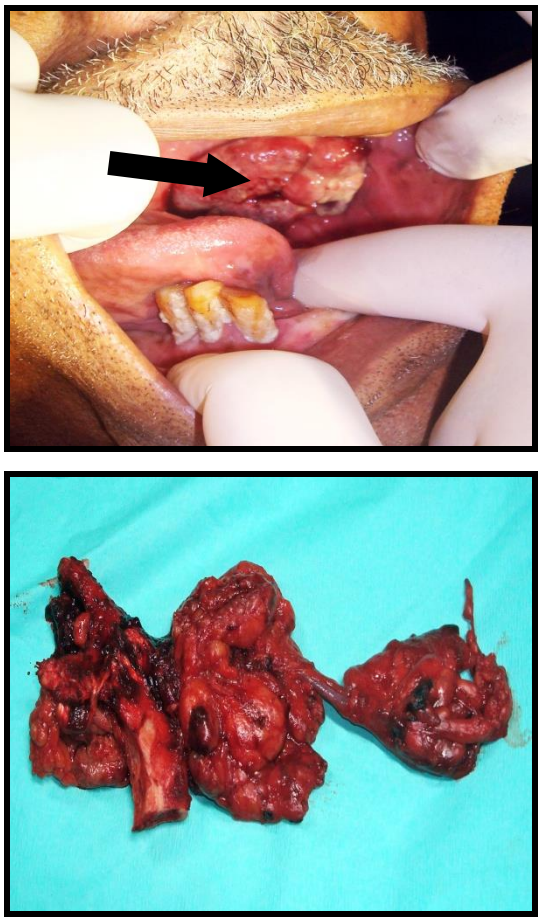

Figure (I): Surgical specimen after complete resection and neck dissection.

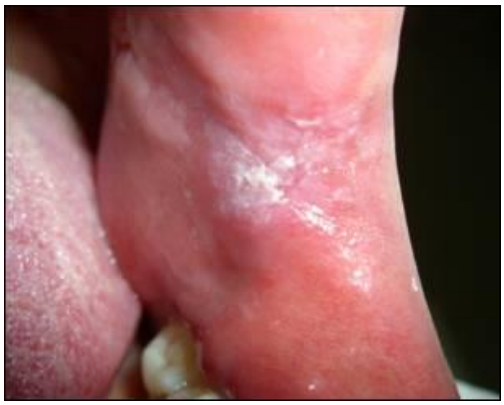

Figure (2): Clinical photograph showing a case with smoker's keratosis (male, age: 44, heavy smoker 20 cigarettes/day for 20 years).

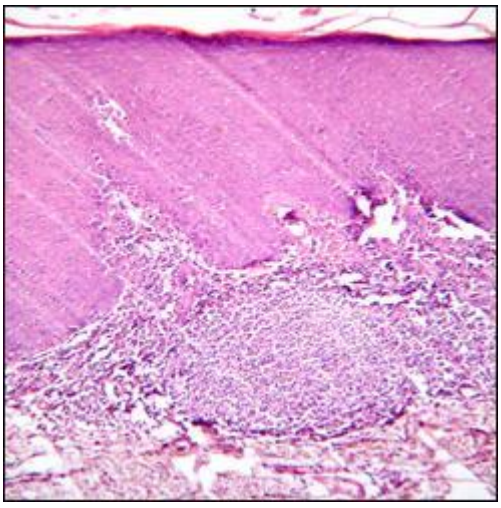

Figure (3): Photomicrograph of H&E stained biopsy specimen of one of the included lichen planus cases showing moderate dysplastic changes (SIN2).

Histopathologic evaluation:

All the prepared sections were examined in order to determine the presence or absence of epithelial dysplasia as well as the degree of such dysplastic changes in the epithelium, if present. This was done according to the latest WHO classification system (6) that histologically categorizes precursor and related lesions (table-1).

Detection of Soluble CD44 level in salivary samples:

Levels of soluble CD44 (sCD44) were measured using an ELISA assay (Bender MedSystems, Vienna, Austria) that recognizes all sCD44 normal and variant isoforms (total sCD44). Saliva samples were centrifuged at 2000 Xg and the supernatants were separated and stored at -80˚C for quantitation of salivary sCD44 by ELISA.

Test Protocol:

Samples were diluted 1:60 with Sample Diluent according to the following

scheme:10 µl sample + 590 µl Sample Diluent. The microwell strips were washed twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Wash Buffer was allowed to sit in the wells for about 10 – 15 seconds before aspiration. After the last wash step, wells were emptied and tapped on absorbent pad or paper towe l to remove excess Wash Buffer. 100 µl of human salivary sCD44 diluted standard ranging from 4.0 to 0.13 ng/ml were added to appropriate wells. 100 µl of Sample Diluent were added to the blank wells. 100 µl of diluted Samples were added to the sample wells. 50 µl of HRP-Conjugate were added to all wells. Incubation was done at room temperature (18 to25°C) for 3 hours. Wells were emptied, washed 3 times. Then 100 µl of TMB Substrate Solution were added to all wells .and The microwell strips were incubated at room temperature (18° to 25°C) for about 10 minutes. 100 µl of Stop Solution were added into each well. Absorbance of each microwell was read on a spectro-photometer using 450 nm as the primary wave length.

A standard curve was created by plotting the mean absorbance for each standard concentration on the ordinate against the human salivary sCD44 concentration on the abscissa. The concentration read from the standard curve was multiplied by the dilution factor (x300). The limit of detection of human salivary sCD44 was determined to be 0.02 ng/ml (mean of 6 independent assays).

Statistical Analysis

Statistical analyses were performed on the results from all the samples. The salivary sCD44 concentrations for each sample were averaged and SD calculated. Student's t-test was used to compare between mean level of salivary sCD44 in ng/ml between normal volunteers, patients suffering from premalignant lesions without dysplasia, patients suffering from premalignant lesions with dysplasia and cancer patients.

A ROC Curve (receiver-operator characteristic curve) was created to estimate salivary sCD44 level with the highest sensitivity and specificity in an attempt to

determine a preliminary cut off point in salivary sCD44 level.

RESULTS:

The present study was carried out on 40 subjects, 10 patients suffering from frank oral malignancy, 10 patients with oral premalignant lesions without dysplasia, 10 patients with oral premalignant lesions with dysplasia and 10 controls. The control group consisted of 8 females (80%) and 2 males (20%). Their ages ranged from 32.00-51.00 years. The same ratio was found in the premalignant without dysplasia group which included 8 females (80%) and 2 males (20%) with an age range of 35.00-65.00 years. On the other hand, the premalignant with dysplasia group consisted of 4 females (40%) and 6 males (60%) and their ages ranged from 37.00-65.00 years, while the oral cancer group consisted of 6 females (60%) and 4 males (40%). Their ages ranged from 38.00-73.00 years.

Salivary sCD44 level was detected in saliva of all patients and in the cancer group salivary sCD44 level (48.05 ± 20.66 ng/ml) was much higher than that of the control group (12.37 ± 3.82 ng/ml) with a statistically significant difference. On the other hand, when the mean level of salivary sCD44 was compared between the premalignant without dysplasia and the premalignant with dysplasia groups, salivary sCD44 level in the premalignant with dysplasia group (34.98 ± 11.27 ng/ml) was found to be significantly higher than its level in the premalignant without dysplasia group (14.67 ± 7.27 ng/ml). (Table I) (Figure II)

There was significant difference in the mean value of salivary sCD44 level between the control group and the premalignant with dysplasia and the cancer groups, and on the other hand, the non significant difference between the control and the premalignant without dysplasia groups. When comparing salivary sCD44 level both in males and females, though higher in the formers, there was no statistically significant difference ($p < 0.159$). When comparing salivary sCD44

level both in smokers and non smokers there was no statistically significant difference ($p < 0.182$).

Cut off point determination:

In an attempt to determine a preliminary cut off point in salivary sCD44 level, a ROC Curve (receiver-operator characteristic curve) was created to estimate salivary sCD44 level with the highest sensitivity and specificity which was 100% and 66.7% respectively.

The most probable cut off point was estimated to be 20.4 ng/ml where the four groups were considered where the first three groups were considered as control and were compared to the cancer group. On the other hand, when only two groups were considered, the control group and the cancer group the best cut off point was estimated to be 19.2 ng/ml. These results indicate that a level of salivary sCD44 lying within the range of 19.2 to 20.4 ng/ml could indicate malignant transformation within oral mucosal lesions.

After adequate surgery of cancer patients, relapse was found in cases with high levels of CD44 indicating its value as a prognostic marker for recurrence.

Table (1): Comparison between control group, potentially premalignant without dysplasia and those with dysplasia and cancer groups according to salivary sCD44 level.

	Control	**Cancer**	**Premalignant without dysplasia**	**Premalignant with dysplasia**
CD 44	8.24-17.72	20.60-68.97*	8.65-32.14	17.31-51.68*
Mean± SD	12.37 ± 3.82	48.05 ± 20.66	14.67 ± 7.27	34.98 ± 11.27

t: Student t-test

* : Statistically significant at $p \leq 0.05$

Table (2): Salivary CD 44 as a prognostic marker for recurrence of cancer after surgery

No	Diagnosis	Site	Pathological presentation	CD44 ng/mL	TTT	Relapse
1	SCC	Ant 2/3 of tongue	High grade undifferentiated SCC with LN involvement,	67.683	hemiglossectomy, FND & Postop. RT	Local recurrence after 13 months.
2	SCC	Ant. 2/3 of tongue	Grade III (high grade) , LN involvement	68.97	Hemiglossectomy, FND & postop. RT	_____
3	SCC	Post. 1/3 of tongue	Grade II , no LN involvement	26.3736	Hemiglossectomy, RND & postop. RT	_____
4	MC	Lower alveolar margin	High grade undifferentiated SCC with LN involvement.	68.2419	Hemimandibulectomy, RND & postop. RT	Extensive Local recurrence & brain metastasis after 15 months & died 2 months later.
5	SCC	Buccal mucosa	Grade III (high grade) , LN involvement (4 LN)	61.281	Excision, RND, Pectoralis major reconstruction & RT	Local recurrence after 1 year
6	SCC	Retro-molar	Grade II , no LN involvement	20.604	Hemimandibulectomy & FND	_____
7	SCC	Floor of mouth	Grade II , no LN involvement	25.9614	Excision & FND	_____

8	MC	Retro-molar	Grade III (high grade) , LN involvement (5 LN)	68.91	Hemimandibulectomy, RND, RT & ChT	_____
9	SCC	Lower Alveolar margin	Grade II , no LN involvement	34.203	Hemimandibulectomy & RND	_____
10	SCC	Lower Alveolar margin	Grade II , LN involvement (2 LN)	38.2419	Hemimandibulectomy, RND & RT	_____

DISCUSSION:

The fact that a saliva-based diagnostic and screening test for cancer is a simple and attractive concept in addition to the fact that conventional diagnostic cancer tests tend to be imperfect give value to the present results.(7) In the present study, comparison between salivary sCD44 level in the potentially premalignant lesions with dysplasia and the control group revealed that salivary sCD44 level in the premalignant with dysplasia (34.98 ± 11.27 ng/ml) was significantly higher than its level in the control group (12.37 ± 3.82 ng/ml). On the other hand, in the premalignant without dysplasia group salivary sCD44 level (14.67 ± 7.27 ng/ml) was higher but not significantly different than that of the control group (12.37 ± 3.82 ng/ml). Our results were in accordance with Franzmann et al. (8) who reported that salivary sCD44 levels were significantly elevated in HNSCC patients compared with normal controls and that the salivary sCD44 ELISA seems to effectively detect HNSCC at all stages.

According to a study performed by Kawano et al. (9) serum levels of sCD44st, sCD44v5, and sCD44v6 were markedly associated with TNM staging in patients with head and neck cancer. These results agree well with the present investigation. Another group of investigators explained the increase in salivary sCD44 levels in

oral lichen planus cases by the presence of a chronic inflammatory process in the lesions.(10) However, the findings of the present study point out to a different reasoning. As shown by the results, lichen planus cases in the present study did not all show the same increase in salivary sCD44 level, instead it was quite evident that lichen planus lesions with dysplastic changes registered levels that were all above the cut off point for salivary sCD44, while those lesions without dysplastic changes registered values, all below the cut off point. Perhaps these lower values could be the result of inflammatory reaction. Such results point obviously to the strong correlation between dysplastic changes in lichen planus lesions and salivary sCD44 levels above 20.4 ng/ml. (figure III)

In the present study when salivary sCD44 level was correlated with smoking status the mean value of salivary sCD44 level in smokers was found to be higher than the mean value of salivary sCD44 level in nonsmokers, but there was no statistically significant difference. This was in accordance with Scott et al. (11) who reported that serum sCD44 has been shown to be elevated in the blood of smokers, compared to non-smokers. Ioachim et al. (12) examined the expression of CD44 in a series of 34 squamous cell carcinomas, 13 in situ carcinomas, 35 cases with various degrees of epithelial dysplasia, 10 papillomas and 17 cases of keratosis. There was no significant difference of CD44 expression between in situ and invasive carcinomas. On the other hand, CD44 expression was statistically higher in dysplastic lesions than the cases of keratosis and papillomas suggesting that CD44 expression may be involved in the multiple mechanisms of the development and progression of these lesions and may help to predict the risk of transformation of the benign or precancerous lesions to cancer.

Through previous studies we do not have enough data to determine the appropriate cut off point for the salivary sCD44 test. In an attempt to determine a preliminary cut off point in salivary sCD44 level a ROC Curve has been used in the present investigation to estimate salivary sCD44 level with the highest sensitivity and

specificity which was 100% and 66.7% respectively. The best cut off point was estimated to be 20.4 ng/ml when the four groups were considered with the first three groups being considered as control and compared to the cancer group. On the other hand, when only two groups were considered, the control group and the cancer group, the best cut off point was estimated to be 19.2 ng/ml, both cut off points resulted in sensitivity of 100% and specificity of 66.7%.

While, in the study conducted by other investigators (13) a cutoff point estimated to be 12 ng/mL resulted in a sensitivity of 62% and specificity of 88% and a cutoff point at 10.5 ng/mL resulted in sensitivity of 70% and specificity of 75%. The difference in the cut off points between his study and the present study may be attributed to the methodological differences particularly in the criteria of choice of the control group in addition to the differences in the study population.

Thus, our preliminary estimates of sensitivity (sensitivity 100% and specificity 66.7%) compare favorably with other widely used screening tests such as prostate-specific antigen for prostate cancer (sensitivity 60-80%, specificity 90%) and the Papanicolaou test for cervical cancer (sensitivity 30-87%, specificity 86-100%) (14).

All patients were treated with curative intent according to a universally standard surgical protocol of histologically confirmed oral squamous cell carcinoma & Mucoepidermoid carcinoma. The protocol includes surgical control of the primary tumor and management of the neck for regional control. The significant increase in salivary sCD44 level in oral cancer patients makes it a potential molecular marker for oral cancer. Thus, it may be used as a diagnostic tool. However, an interesting finding in the present study is that high levels of CD44 were associated with high susceptibility of recurrence. This means that this marker can also be used as a prognostic marker for evaluation of prognosis of cases after surgery. Its perfect correlation with the clinical grading and aggressiveness of malignant lesions ,their tendency to recur and their fatality, furnishes a sound basis for its use

as an indicator for the prognosis and a monitor for the degree of aggressiveness of the treatment to be applied.

CONCLUSION:

Salivary sCD44 test may be an effective for early detection of oral cancer and malignant transformations in oral mucosal premalignant lesions. More detailed studies for using saliva on a wider scale are recommended for earlier diagnosis and prognosis determination in oral mucosal lesions.

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