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The diagnostic and prognostic value of salivary sCD44 level determination in oral malignant and potentially premalignant lesions

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Dalia M. Ghalwash, Khaled El-Gaaly, Fat'heya M. Zahran, Olfat Shaker, Hossam A. El-Fol; The diagnostic and prognostic value of salivary sCD44 level determination in oral malignant and potentially premalignant lesions

ABSTRACT

A key factor in the lack of improvement in prognosis of oral squamous cell carcinoma (OSCC) lesions over the years is the fact that a significant proportion are not diagnosed or treated until they reach an advanced stage. A molecular marker for malignant transformation in innocent looking oral lesions and a monitor for the aggressiveness of malignant lesions might be of help. The present study included 40 subjects: 10 healthy control subjects, 10 patients with potentially premalignant oral lesions with dysplastic changes and 10 others without, in addition to 10 patients suffering from OSCC. Levels of soluble CD44 (sCD44) were measured in whole unstimulated saliva (WUS) using an enzyme linked immune- assay (ELISA). In patients suffering from malignant lesions the salivary sCD44 level was correlating well with the grading of the lesion. Also, most of the patients with the highest salivary sCD44 levels showed postoperative relapse. A highly significant difference was found 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, a non significant difference was found between the control and the premalignant without dysplasia group. Also, a highly significant difference was found between salivary sCD44 level in cancer patients and those with premalignant lesions without dysplasia, and non significant difference between the cancer patients and those with premalignant lesions with dysplasia. A ROC Curve was created to estimate salivary sCD44 level with the highest sensitivity and specificity which was 100% and 66.7% respectively. Results indicated 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.

Key words: salivary sCD44, oral malignant lesions, oral potentially premalignant lesions

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for more than 95% of all head and neck malignancies. Unfortunately, the majority of HNSCC patients present with advanced stage disease, requiring multimodality therapy. Even with combinations of intensive chemotherapy, radiotherapy, and surgery, cure rates are only 30% for advanced stage disease. Those cured often face serious morbidities including speech and swallowing problems, disfigurement and exorbitant healthcare costs [10,17]. Invasive 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 [24]. One candidate molecular 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 [25]. CD44 is known to be linked to various trans-membrane and intra-cytoplasmic signalling pathways, such as those involving epidermal growth factor receptor, mitogen-activated protein kinase, phosphoinositide 3-kinase, and others. The interaction of CD44 with these receptor-mediated

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signalling pathways has been shown to promote various tumor progression behaviors, including tumor cell growth, migration, invasion, and metastasis [25]. Studies of squamous cell carcinoma (SCC) report increased levels of CD44s and unusual expression of CD44 splice variants containing the V5, V7, V8 and, most notably, V6 exon products in pre-invasive and high risk pre-cancerous lesions [13]. Technological advances over the past decades have enabled the oral fluid to expand its usefulness in the diagnosis of disease, prediction of disease progression, monitoring of therapeutic drug levels and detection of illicit drugs. The easy non-invasive nature of collection and the relationship between oral fluid and plasma levels make oral fluid a valuable clinical tool [6]. In some malignant diseases, markers can be detected in the saliva [7]. Surprisingly, only few studies examined tumor markers in the saliva of OSCC patients. Such an examination might be of great benefit because of the direct contact between the oral cancer lesion and saliva. Moreover, these scarce salivary reports focused only on one commonly analyzed tumor marker, the CEA, which did not prove to be sensitive or specific enough [20]. More recently, Chang *et al*. [5] concluded that the possible roles of CD44 in the diagnosis of oral and maxillofacial malignancy deserve further elucidation and evaluation. Moreover, results of recent researches suggest a role for sCD44 as a prognostic marker [2] and a therapeutic target [9] in various malignancies. 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 as well as its value in prognosis evaluation for malignant oral lesions.

Materials and Methods

The present study was performed on 40 individuals from the out patient clinic of Oral Medicine and Periodontology Department, Faculty of Oral and Dental Medicine, Cairo University; and National Cancer Institute, Cairo. All subjects signed a consent form.

Subjects were subdivided into:

Group I: included 10 healthy control subjects, nonsmokers.

Groups II, III: 20 patients were chosen to have potentially premalignant lesions; according to previous literature [19,28]. They included cases with smoker's keratosis, speckled leukoplakia, leukoplakia and atrophic /ulcerative lichen planus Among these 20 patients, biopsy results enabled us to further subdivide them into 10 patients with dysplastic lesions and 10 others without dysplasia.

Group IV: included 10 patients suffering from OSCC.

Histopathologic evaluation:

Biopsy specimen [8] were obtained from all lesions to confirm the diagnosis and establish the degree of dysplasia in the potentially premalignant lesions. Evaluation was done according to the latest WHO classification system [12].

Salivary sample collection:

Collection of whole unstimulated saliva (WUS) was done as described by Navazesh [22].

Detection of Soluble salivary CD44 level:

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

Test Protocol:

- Samples were diluted 1:60 with Sample Diluent (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 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.
- 100 µl of TMB Substrate Solution were added to all wells.
- 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.

Calculation of Results:

1. 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.
2. The concentration read from the standard curve was multiplied by the dilution factor (x300).

Sensitivity of the test: 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 s.d. calculated. Student's t-test was used to compare between mean level of salivary sCD44 in ng/ml between all studied groups.

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:

Age, gender, clinical diagnosis, smoking status and salivary sCD44 level of all included subjects are shown in table (1).

Demographic data:

Group I (conrtols):

It consisted of 10 individuals; 8 females (80%) and 2 males (20%). Their ages ranged from 32.00-51.00 years with mean age of 36.10 ± 7.13 years.

Table 1: Age, gender, clinical diagnosis, smoking status and salivary sCD44 level of all included subjects.

Sr.	Age	Gender	Diagnosis	Smoking	CD44(ng/mL)
1	51	Male			8.3565
2	34	Female			8.6538
3	48	Female			14.8353
4	33	Female			8.2419
5	33	Female			16.4835
6	32	Male			8.2403
7	32	Female			15.2472
8	33	Female			17.7198
9	32	Female			14.817
10	33	Female			11.1264
11	60	Male	Lichen planus		11.1264
12	45	Female	Lichen planus		8.6538
13	65	Female	Lichen planus		16.4846
14	65	Male	Smoker's keratosis	Smoker	8.6538
15	55	Female	Lichen planus		13.599
16	55	Female	Lichen planus		20.1924
17	57	Female	Lichen planus		11.1264
18	60	Female	Leukoplakia		32.142
19	45	Female	Lichen planus		8.6538
20	35	Female	Leukoplakia		16.0713
21	50	Female	Lichen planus		25.9614
22	46	Male	Speckeled leukoplakia	Smoker	26.3736
23	50	Female	Lichen planus		26.4847
24	44	Male	Smoker's keratosis	Smoker	40.701
25	45	Male	Speckeled leukoplakia		51.678
26	65	Male	Lichen planus	Smoker	34.203
27	65	Male	Speckeled leukoplakia		17.3076
28	49	Female	Lichen planus		41.481
29	37	Male	Smoker's keratosis	Smoker	50.763
30	60	Female	Lichen planus		34.878
31	50	Female	squamous cell carcinoma		67.683
32	66	Male	squamous cell carcinoma		68.97
33	55	Female	squamous cell carcinoma		26.3736
34	70	Female	mucoepidermoid carcinoma		68.2419
35	73	Male	squamous cell carcinoma		61.281
36	38	Male	squamous cell carcinoma		20.604
37	55	Female	squamous cell carcinoma		25.9614
38	73	Male	mucoepidermoid carcinoma		68.91
39	45	Female	squamous cell carcinoma		34.203
40	55	Female	squamous cell carcinoma		38.2419

Group II (premalignant without dysplasia):

It consisted of 10 patients; 8 females (80%) and 2 males (20%). Their ages ranged from 35.00-65.00 years with mean age of 54.20 ± 9.70 years.

Group III (premalignant with dysplasia):

It consisted of 10 patients; 4 females (40%) and 6 males (60%). Their ages ranged from 37.00-65.00 years with mean age of 51.10 ± 9.34 years.

Group IV (cancer):

It consisted of 10 patients; 6 females (60%) and 4 males (40%). Their ages ranged from 38.00-73.00 years with mean age of 58.00 ± 12.10 years.

As shown by table (2), in patients suffering from malignant lesions the salivary sCD44 level was correlating well with the grading of the lesion. Also, most of the patients with the highest salivary sCD44 levels showed postoperative relapse and one of them (with level of 68.2419 ng/ml) died in less than 2 years.

The data displayed in table (3) and figure (1) demonstrates the highly 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 group. Also, the highly significant difference between salivary sCD44 level in cancer patients and those with premalignant lesions without dysplasia, and non significant difference between the cancer patients and those with premalignant lesions with dysplasia.

Table 2: Cases with oral malignant lesions.

No	Diagnosis	Site	Pathological presentation	CD44 ng/ml	Treatment	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	Hemi-mandibulectomy , 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	Hemi-mandibulectomy & 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	Hemi-mandibulectomy, RND, RT & ChT	_____
9	SCC	Lower Alveolar margin	Grade II , no LN involvement	34.203	Hemi-mandibulectomy & RND	_____
10	SCC	Lower Alveolar margin	Grade II , LN involvement (2 LN)	38.2419	Hemi-mandibulectomy, RND & RT	_____

RND: radical neck dissection
FND: functional neck dissection
RT : radiotherapy ChT: chemotherapy

Table 3: Comparison between salivary sCD44 in the studied groups.

Group	CD44 range	CD44 Mean± SD	Student-t test results		
			Vs control	Vs premalignant without dysplagia	Vs premalignant with dysplagia
Control	8.24-17.72	12.37 ± 3.82			
Premalignant without dysplagia	8.65-32.14	14.67 ± 7.27	0.885		
Premalignant with dysplagia	17.31-51.68	34.98 ± 11.27	6.008*	4.789*	
Malignant	20.60-68.97	48.05 ± 20.66	5.370*	4.820*	1.756

* : Statistically significant at $p < 0.05$

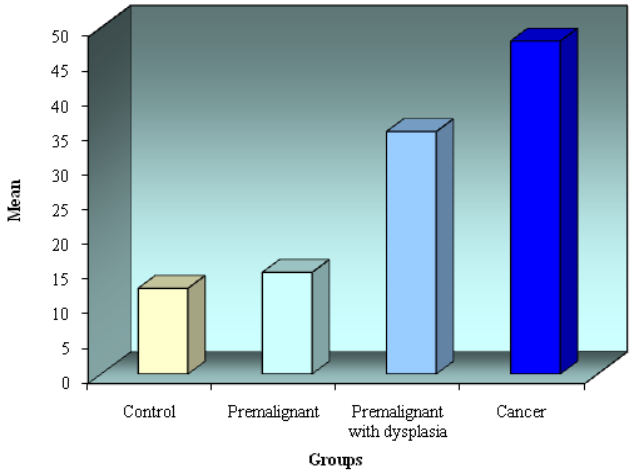

Fig. 1: Comparison between the studied groups according to salivary sCD 44 levels.

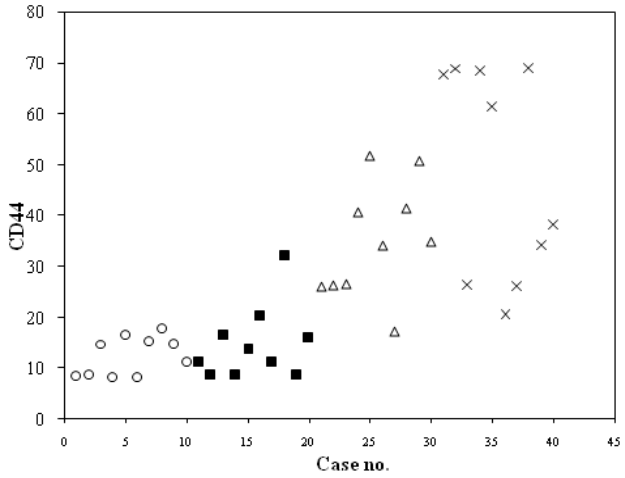

Fig. 2: Distribution chart for s CD44 level for 40 cases.

According to the distribution chart shown in figure (2), the range of values of salivary sCD44 level among the control individuals hangs within a very narrow range (from 8.24 to 17.72 ng/ml). The range widens with broader distribution among patients with premalignant lesions without dysplasia (8.65-32.14 ng/ml). However, in patients suffering from premalignant lesions with dysplasia and cancer the distribution is very wide and there is a shift in the range of values to occupy the area between (17.31-51.68 ng/ml) and (20.60-68.97 ng/ml) respectively.

Cut off point determination:

A ROC 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 when the four groups were considered:

the first three groups acting as control compared to the cancer group as shown in figure (3). However, 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 as illustrated in figure (4).

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.

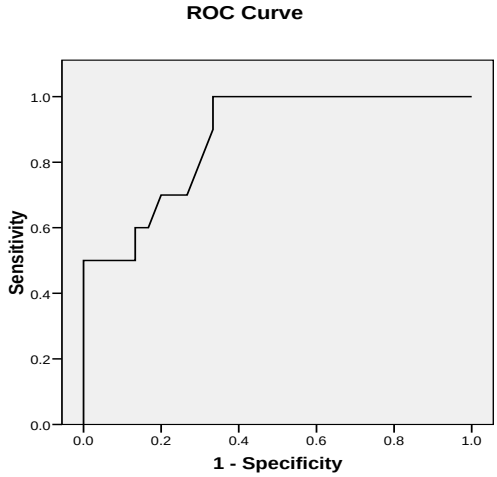

Fig. 3: ROC curve; considering the 4 groups (the first three groups as control).

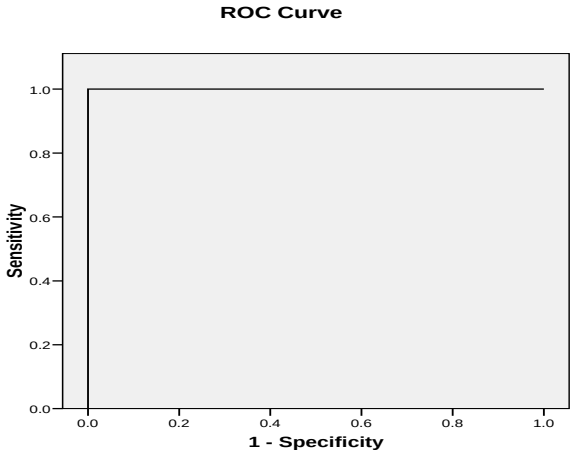

Fig. 4: ROC curve; considering the 2 groups, control and cancer.

Discussion:

In recent decades, there has been a dramatic switch from histopathological to molecular methods of disease diagnosis as changes occur at the molecular level before they are seen under the microscope and before clinical changes occur. 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 [26,27]. Clinically, it is important to note that the therapeutic modality currently offered to patients is based on traditional stage-predicting indices (based mostly on the tumor-node-metastasis criteria) and on histologic grading. Unfortunately, these predictors are subjective and relatively unreliable, as often two tumors with identical staging and grading behave in totally different fashions, and although one responds to therapy, the other is lethal. Accordingly, there has been an over-growing effort dedicated to the basic research of oral cancer, focusing on the identification of biological indicators for the diagnosis of its biological nature and aggressiveness [20]. Surprisingly, only few studies examined tumor markers in the saliva of OSCC patients. Such an examination might be of great benefit because of the direct contact between the oral cancer lesion and saliva, particularly as salivary analysis is a useful diagnostic tool for other distant malignancies, such as breast carcinoma [1,23,3,20].

In the present study, the mean salivary sCD44 level in the cancer group (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. When the mean level of salivary sCD44 was compared between the premalignant without dysplasia and the premalignant with dysplasia groups, the level in the premalignant with dysplasia group (34.98 ± 11.27 ng/ml) was found to be significantly higher than in the premalignant without dysplasia group (14.67 ± 7.27 ng/ml). Also, when premalignant without dysplasia group and cancer groups were compared, there was a statistically significant difference as salivary sCD44 level in the cancer group was significantly higher. While, comparison between premalignant with dysplasia group and cancer group revealed no statistically significant difference. On the other hand, the potentially premalignant lesions with dysplasia had significantly higher levels than the control group. On the other hand, in the premalignant without dysplasia group salivary sCD44 level was higher but not significantly different than that of the control group. Not only that, but also when the level of salivary sCD44 was matched in each cancer patient with his/her clinical grading, it correlated well. Our results were in accordance with Franzmann *et al*. [10] 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. Franzmann *et al***.** [11] evaluated the sCD44 ELISA test for HNSCC in a larger group of patients and his results showed that the mean solCD44 level was 24.4 ± 32.0 ng/mL for HNSCC patients and 9.9 ± 16.1 ng/mL for the patients with benign disease ($P < 0.0001$). However, levels did not correlate significantly with tumor stage, tumor size, presence of lymph nodes metastasis, history of previous HNSCC (recurrence or another primary), or history of prior radiation. On the other hand, according to a study performed by Kawano *et al*. [15] 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.

Chaiyarit *et al*. [4] investigated the expression of CD44 in biopsy specimens and saliva from oral lichen planus patients and demonstrated that levels of salivary sCD44 were significantly elevated in saliva of oral lichen planus patients than those in controls. This was also in accordance with our finding that the premalignant groups, which included cases with lichen planus, showed a higher salivary sCD44 level than did the control group. The authors explained the increase in salivary sCD44 levels in oral lichen planus cases by the presence of a chronic inflammatory process in the lesions. 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, 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 below the cut off point. Such results point obviously to the strong correlation between dysplastic changes in lichen planus lesions and salivary sCD44 levels above 20.4 ng/ml. Mignogna *et al*. [18] reported that patients with OLP and subsequent development of dysplasia/ oral squamous cell carcinoma are at risk of having multiple and multifocal neoplastic events of the oral cavity. If detected at an early stage, these neoplasias can be managed with superficial and complete resection.

Ioachim *et al*. [14] 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. While, in the study conducted by Franzmann *et al*. [11] 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 their study and ours 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. 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%) [29] and the Papanicolaou test for cervical cancer (sensitivity 30-87%, specificity 86-100%) [21]. 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.

Conclusion:

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. Also 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. Moreover, as salivary harvesting is noninvasive, it may provide an attractive, effective alternative to serum testing, and makes the development of home testing and chair-side kits possible.

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