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Original Article

Diagnostic Accuracy of Salivary Gamma Synuclein in Oral Malignant and Potentially Malignant Lesions

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Abstract

Background: Oro-pharyngeal cancers are of the most common malignancies worldwide, they have poor prognosis and significant morbidity and mortality. Biomarkers can be helpful in cancer patients screening, to diagnose, and predict the course of disease. Saliva-based analysis can be used in diagnosis and prognosis of cancer, so it is of paramount importance to find a valid salivary diagnostic tool. The present study aimed to determine the diagnostic accuracy of gamma synuclein in differentiating between oral malignant and potentially malignant lesions.

Subjects and Methods: This prospective phase II diagnostic test accuracy study included 36 patients divided into three groups: 12 oral lichen planus patients, 12 oral cancer patients and 12 healthy controls. Saliva samples were collected from all patients to measure gamma synuclein using ELISA. Tissue biopsies and histopathological examination were done for the lesions to confirm the diagnosis.

Results: Salivary gamma synuclein showed high sensitivity (100%) and specificity (83.3%) in differentiating between potentially malignant lesions and oral cancer at a cut off point of (>2.01 ng/mL). Furthermore, it showed a statistically significant difference between dysplastic and non-dysplastic lesions (p=0.03). However, gamma synuclein levels had no diagnostic value in differentiating between different grades of cancer (p=0.220).

Conclusion: Salivary gamma synuclein is a useful diagnostic marker for the early cancer detection. It has potential as a prognostic tool to detect the presence of dysplasia in oral premalignant lesions.

Keywords: Gamma Synuclein, Oral lichen planus, Oral potentially malignant lesions, Diagnostic accuracy

1. Introduction

Globally, cancers of the head and neck are considered the sixth most common cancers worldwide. In high-risk countries, like India and Asian countries, oral cancer is the most commonly reported malignancy with more than 25% each year (Sarode et al., 2020; Warnakulasuriya, 2009). Oral cancer has an overall survival rate of less than 40%. It is essential to diagnose oral cancer in its early stages to raise the survival rates to about 80%. In most cases, patients are asymptomatic in the early stages and when the diagnosis is delayed, there is a higher risk for lymphatic spread (Abati et al., 2020).

Oral potentially malignant lesions (OPML) are lesions where epithelial dysplasia or cells atypia may be found in the histopathology indicating the ability to transform into oral cancer (Müller, 2018). Each type of OPMLs has a different rate of the malignant transformation thus, some lesions are more liable to transform to carcinomas than others (Iocca & Maio, 2020). Oral lichen planus is a common chronic oral disease that affects the oral cavity and other mucous membranes. It causes significant pain and affects the patient's quality of life as it rarely undergoes remission (Carrozzo et al., 2019). In Egypt, OLP was found to have a prevalence of 1.43%, the atrophic and erosive types form 59.37% of all OLP and most of the patients were females among the fourth to sixth decades of life (Mostafa & Ahmed, 2015).

A large percentage of OSCCs was found to be preceded by an OPML in the oral cavity (Abadeh et al., 2019). Thus, oral cancer is – theoretically - a preventable disease (Johnson et al., 2011) with a thorough oral examination and oral cancer screening for high-risk individuals, survival rates can be improved (Warnakulasuriya & Kerr, 2021). A second preventive strategy is the proper management of OPMLs (Abadeh et al., 2019). This explains the importance of early detection, immediate attention and regular follow-up of OPMLs to reduce the morbidity of oral cancer and monitor their potential transition to malignancy (Ho et al., 2019).

Proteomics is the branch of salivaomics that is related to the proteins found in the saliva as histatins, amylases, cystatins, and mucins (Goldoni et al., 2021). The family of proteins, called synucleins, contains three individual proteins that structurally have a lot of similarities but each one of the family has some significant distinct role from the other.

Gamma synuclein - also called the breast cancer-specific gene-1- was found to have a major role in cancer progression and the proliferation of cancer cells in many cellular pathways and different mechanisms. It has a role in obstructing apoptosis and fostering cancer progression and metastases (Guo et al., 2007; Pan et al., 2002; Surguchov, 2016). It was detected in different widely ranged cancer types, such as gastric cancer, liver, colon, lung, prostate, medulloblastomas, glial tumors, cervical cancers, and pancreatic cancers. Furthermore, overexpression of Gamma synuclein was associated with poorer prognosis, the proliferation of mammary cells, and ovarian cancer progression (Guo et al., 2007; Surguchov, 2016).

Therefore, the aim of the present study was to evaluate the diagnostic accuracy of salivary Gamma synuclein in differentiating between malignant and premalignant lesions.

2. Subjects and methods:

The present work is a prospective phase II diagnostic test accuracy study. The protocol for this study is registered on clinicaltrials.gov under identifier NCT04732741.

The study was approved by the Research Ethics Committee of Faculty of Dentistry, Cairo University, approval number 4-3-21 and was

conducted according to the declaration of Helsinki. Each patient was informed about the nature of the study and an informed consent was obtained from each patient.

Patients were recruited from March 2021 to December 2021. Oral cancer patients were recruited from the National Cancer Institute while patients with potentially malignant lesions and healthy controls were recruited from the Outpatient Clinic at the Faculty of Dentistry - Cairo University. The participants of this study were recruited consecutively and formed a convenience sample.

Sample size was calculated using the mean values of gamma synuclein reported by Wang et al. for oral cancer and oral potentially malignant disorders (OPMDs) and the estimated Pooled standard deviation. The sample size was calculated with an effect size of 0.5462 one-way , with a level of significance of 0.05 and a power of 80%, giving a sample size of 36 patients, 12 subjects per group.(Wang et al., 2020).

Pregnant subjects or those with systemic disease were excluded, as hormonal changes and systemic diseases may manipulate the gamma synuclein levels.

Patients were divided into three groups of 12; The first group included subjects diagnosed with OLP clinically and histopathologically using the modified WHO diagnostic criteria (van der Meij & van der Waal, 2003). Patients had to yet receive any treatment or had a month wash-out period had they received any previous treatment.

Subjects with oral cancer were all diagnosed with squamous cell carcinoma by clinical examination and histopathological examination of a tissue biopsy performed by specialists at the National Cancer Institute. All included patients had yet to receive any treatment.

The third group was the control group. It consisted of healthy individuals who were examined with the visual and tactile examination (according to the National Institute of Dental and Craniofacial Research, 2013). They had clinically normal oral mucosa, no detectable oral lesions, were medically free and had no history of any oral habits or risk factors.

2.1 Test methods:

History and Screening: All the participants of this study underwent basic charting of their demographic data, medical history, and oral habits. For participants of the first and second groups, their lesions' appearance was documented photographically as well as duration and associated symptoms. OLP was classified into either papular, which represents asymptomatic forms such as reticular, annular and plaque forms, or ulcerative which included symptomatic forms of OLP such as atrophic and erosive types (Oliveira Alves et al., 2010; Karbach et al., 2014).

A thorough clinical examination was performed on each subject in the three groups. Extra oral examination of the head and neck was firstly performed, the face, eyes, and neck, all were carefully visually inspected for any lesions, swellings, or asymmetry. Intra-oral examination of all the oral cavity was done in the three groups, and to assure the absence of any oral lesions in the control (Lewis, 2004).

2.2 Reference test for the study groups:

The reference standard was histopathological examination of a tissue biopsy (Williams et al., 2008). The first group's (OLP) tissue biopsies were performed at the Faculty of Dentistry – Cairo University. Specialists at the National Cancer Institute performed the second group's tissue biopsies. The biopsy site was determined by its clinical appearance with the worst clinical picture being that of non-homogenous, corrugated, indurated, or nodular areas (Poh et al., 2008).

A third party pathologist who was not a participant in the study did the histopathological assessment to avoid information bias. The histopathologic grade was recorded for all patients with oral cancer according to the WHO grading system (Broders, 1920; El-Naggar et al., 2017).

The third group of participants of this study were healthy individuals, the general information, past medical history, dental history, and family history were taken. In this group, no biopsies were taken, clinical examination was enough to exclude any abnormalities.

2.3 Index Test:

All the subjects gave a salivary sample for Gamma synuclein level analysis. Subjects were asked not to eat or drink before the collection of the sample for one hour, approximately 20 mm of unstimulated salivary samples were then collected by asking the patients to spit in a sterile falcon tube.

The collected salivary samples were stored in a -20 ^{o}C freezer at the Biochemistry Department-Faculty of Medicine - Cairo University until the time of analysis. The collected samples were discarded according to safety protocols placed by the Faculty of Medicine -Cairo University.

Saliva samples were centrifuged at 4000Xg. Then supernatant was collected for detection of Gamma synuclein by Elisa kit Catalogue Number: SL2683Hu (SunLong Biotech Co.,LTD, China). This ELISA kit uses Sandwich-ELISA as the method.

3. **Results:**

Demographic data of the included subjects is shown in table (1). OLP patients were divided equally into 6 cases with papular type, and 6 with ulcerative type. Seven patients were found to harbor signs of dysplasia. Mild dysplasia was found in 5 cases (41.7%), while severe dysplasia was found in 2 cases in this group (16.7%). Eight cases from the oral cancer group had grade II severity (66.7%) and four cases had grade III severity (33.3%). Figure 1 shows oral lesions from both groups and their gamma synuclein results.

Regarding the level of gamma synuclein, intergroup comparisons showed a statistically significant difference between all the study groups ($p<0.001$). The highest gamma synuclein value was found in group II which represents oral cancer (2.58±0.45), followed by group I (1.68±0.40), while the lowest value was found in the control group III (0.80±0.04).

Analysis of ROC curve for the diagnostic accuracy of gamma synuclein with group (I) as the negative status and group (II) as the positive status is shown in table (2) with the cut off value chosen based on a value of >2.01ng/ml according to Youden's index. Youden's index to differentiate between normal and patients with OLP and cancer was set at a value of >0.86 ng/ml.

Gamma synuclein level was not related to the type of OLP ($p=0.081$). However, the ulcerative OLP seems to have a higher value (1.89±0.49) than that found in the papular type (1.48±0.12). There was, however, a significant association between gamma synuclein and the degree of dysplasia observed in OLP with cases showing signs of dysplasia having a significantly higher value (1.88±0.40) than cases with no dysplasia (1.40±0.19) ($p=0.032$). Lastly, analysis of association between gamma synuclein levels and cancer differentiation revealed no significant association between different cancer grades ($p=0.220$).

Subgroup analysis of the smoking status in each group showed no significant association between gamma-synuclein levels and smoking ($p=0.687$) in OLP patients. While, in the oral cancer group, the association was statistically significant with

non-smokers having significantly higher value (p=0.042)

Table (1): Demographic data and clinical characteristics of the study groups

Parameter	Value		Group (I) (OLP)	Group (II) (OSCC)	Group (III) (Controls)	p-value
Sex	**Male**	**n**	2	7	3	**0.072ns**
		%	16.7%	58.3%	25.0%	
	Female	**n**	10	5	9	
		%	83.3%	41.7%	75.0%	
Age (years)	**Mean±SD**		47.25±8.54	53.75±8.32	46.75±13.61	**0.162ns**
Smoking	**No**	**n**	9	6	0	**0.206ns**
		%	75.0%	50.0%	0.0%	
	Yes	**n**	3	6	0	
		%	25.0%	50.0%	0.0%	
No of cigarettes	**Mean±SD**		4.58±11.57	14.17±16.21	NA	**0.153ns**
Lesion site	**Buccal mucosa**		9 (75.0%)	4 (33.3%)		
	Tongue		3 (25.0%)	2 (16.7%)		
	Lip		0 (0%)	2 (16.7%)		
	Palate		0 (0%)	2 (16.7%)		
	Retromolar area		0 (0%)	2 (16.7%)		

Table (2): Diagnostic accuracy for gamma synuclein (ng/ml) (groups I and II)

Sensitivity	Specificity	PPV	NPV	+LR	-LR	AUC	95% CI
100%	83.33%	85.70%	100.0%	6.00	0.00	0.955	0.784 to 0.999

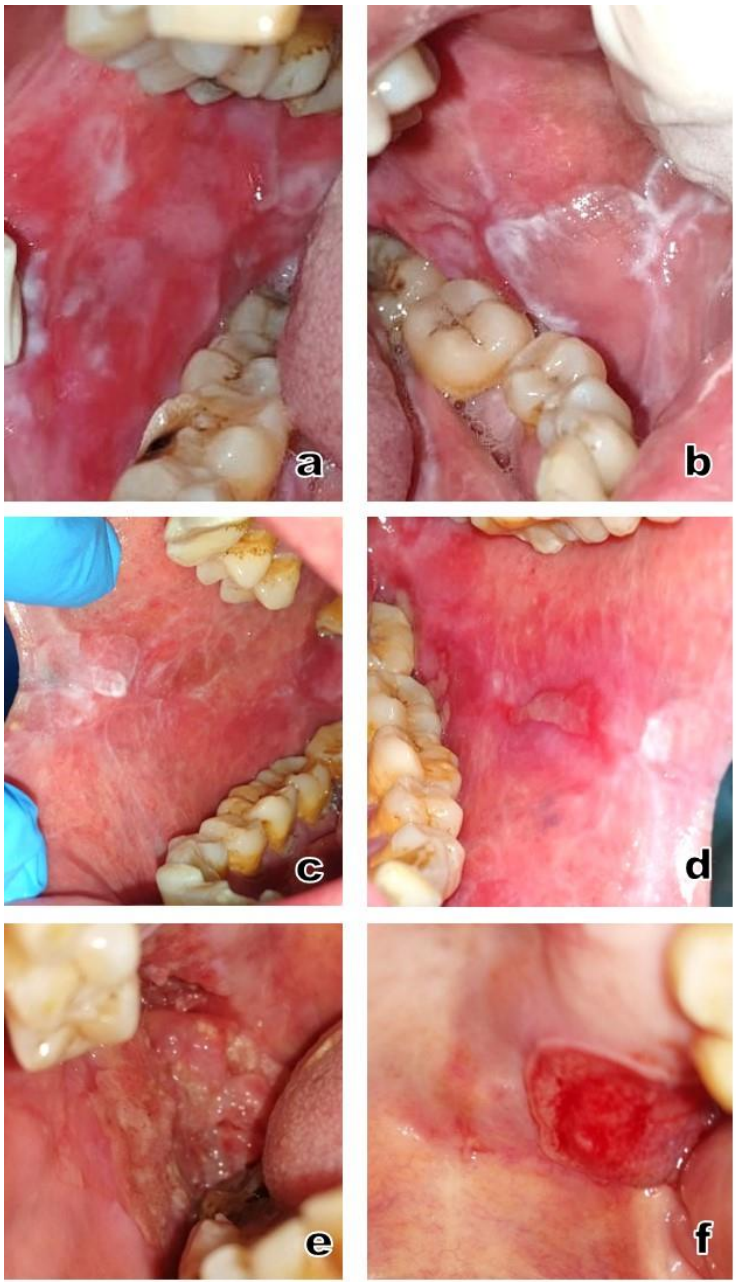

Figure (1): **a,b:** A 57-year-old female patient with ulcerative oral lichen planus in the buccal mucosa with mild dysplasia. Gamma synuclein level was 2.55 ng/ml. **c,d:** A 57-year-old female patient diagnosed with ulcerative oral lichen planus in the buccal mucosa bilaterally with mild dysplasia. Gamma synuclein level was 2.01 ng/ml. **e**: A 47-year-old male patient with squamous cell carcinoma of moderate differentiation in the retromolar area. Gamma synuclein level was 3.15 ng/ml. **f**: A 58-year-old male patient with squamous cell carcinoma of moderate differentiation in the palate. Gamma synuclein level was 2.95 ng/ml.

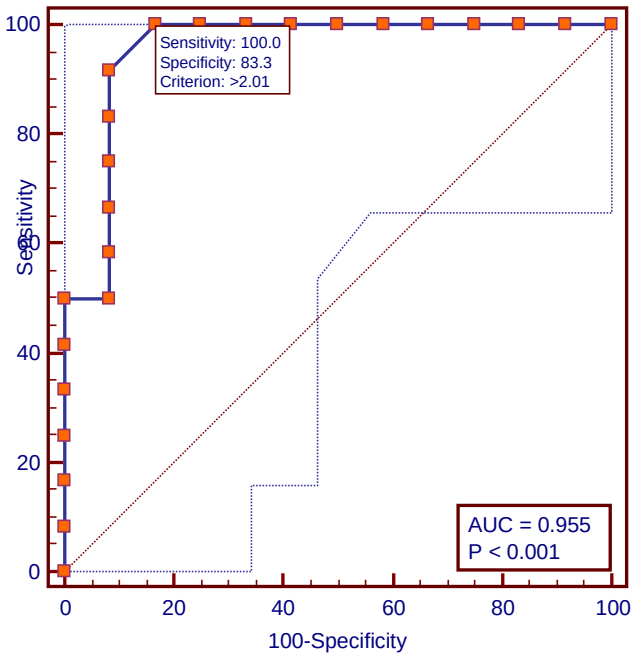

Figure (2): ROC curve (groups I and II)

Table (3): 2x2 table between the reference test and the index test (groups I and II)

Actual (Reference test)	**Observed (Index test)**	
	Negative	**Positive**
Negative	10	2
Positive	0	12

4. Discussion:

The aim of the present study was to determine the diagnostic accuracy of salivary gamma synuclein in patients with oral potentially malignant and malignant lesions. Oral potentially malignant lesions were represented by OLP, as OLP is one of the most common oral mucosal conditions seen at the outpatient clinic at the Faculty of Dentistry-Cairo University (Abd el-Aziz et al., 2020) , and the cases were unified to provide a homogenous group and avoid any variations in the marker's levels due to

differences in underlying pathogenic mechanisms.

Gamma synuclein levels differed significantly between all groups. The oral cancer group had the highest level with a mean of 2.58±0.45ng/ml, followed by the OLP group in which the value was almost halved (1.68±0.40 ng/ml) and further reduced by half in subjects with no oral lesions (0.80±0.04 ng/ml).

The increase in gamma synuclein levels throughout the three groups is justified on the cellular level as gamma synuclein is part of different pathways that orchestrate the process of carcinogenesis. These pathways mainly regulate cell function and apoptosis thus gamma synuclein has a crucial role in directing cells towards cancer formation. These pathways include the MAPK pathway that regulates cell differentiation and proliferation. Gamma synuclein can switch on the ERK1/2 and inhibit the JNK pathway, thus protecting the cell from apoptosis and promoting cell survival (Kim & Choi, 2010). Another pathway is the BubR1 that elongates the cell arrest phase until cell death occurs. Gamma synuclein was found to interact with BubR1 to inhibit apoptosis (Davies et al., 2010; Miao et al., 2014). Gamma synuclein was also found to increase the affinity of estrogen receptor ER-alpha36. This receptor regulates the estrogen-mediated activation of the MAPK/ERK1/2 pathways, which promotes cell growth and metastases (Jiang et al., 2004). Gamma synuclein can also bind to the AKT proteins causing the activation of pathways essential to cell survival and cancer progression (Liang et al., 2015; Ma et al., 2016). These pathways include the focal adhesion kinase, which regulates cell adhesion and metastases (Nagano et al., 2012). Furthermore, gamma synuclein can increase the levels of the phosphorylation of the Tyr397 thus causing cell motility, invasion and metastases (Liu et al., 2018).

However, in our study, gamma synuclein lost its value in differentiating between different stages of OSCC as there was no statistically significant difference between the two stages of OSCC (p =0.22). Similarly, Chen found that the levels of gamma synuclein in serum of OSCC patients did not correlate with the degree of differentiation, being non statistically significant with p value 0.580 (Chen et al., 2021).

The ROC curve between the OSCC and OPMLs in the current study revealed a perfect detection rate for the presence of malignant transformation (Sn=100%), and an 83.3% ability of ruling out the disease or a false positive rate of 16.67% (Sp=83.3%). In a clinical setting within a high prevalence population, such as a secondary or tertiary reference center, gamma synuclein can be used to rule out malignant transformation of OPMLs due to its perfect NPV (100%), while a positive result means that the patient has an 85.7% chance of the lesion having turned cancerous (PPV= 85.7%). Wang et al. evaluated gamma synuclein levels in the saliva of 79 patients with SCC. They reported a cut-off value of 0.8124 ng/ml, which gave sensitivity and specificity of 97.5% and 68.7% respectively (Wang et al., 2020). Meanwhile, Chen et al. found the sensitivity and specificity of the gamma synuclein levels in the serum between the OSCC and OPMLs (as a whole group of different potentially malignant diseases) to be 91.95%, 56.67% respectively with a cut-off value of 0.468 (Chen et al., 2021). The cutoff value used in this study to differentiate between malignant and premalignant is 2.01 ng/ml where a higher value indicated malignant transformation. Gamma synuclein values higher than 0.86 ng/ml were found to indicate the presence of a premalignant pathosis as opposed to normal tissues. The difference in the reported cut-off values is probably due to the different detection kits used.

Likelihood ratios help in assessing the effect of a diagnostic test on the probability of disease. LRs can be used to project the change in

the pre-test probability of having a particular condition from what was initially assumed to the probability after the test results are interpreted (post-test probability). The positive likelihood ratio (+LR) gives the change in the probability of having a diagnosis in patients with a positive test result while the negative likelihood ratio (-LR) gives the effect of a negative test result on the probability of disease. Thus, tests with very high LR+ and very low LR− have greater discriminating ability and tests with LRs >10 or <0.1 are very useful in establishing or excluding a diagnosis (Ranganathan & Aggarwal, 2018).

In the present study, gamma synuclein was found to have a positive LR of six . A patient with OLP has a pretest probability of 1.4% malignant transformation rate of 1.4%. If the patient had a positive gamma synuclein test result, then the post-test probability of having undergone malignant transformation according to Bayes Theorem would be 7.8% (McGee, 2002). While a negative LR of zero means that, the probability of malignant transformation after obtaining a negative gamma synuclein result would go down to Zero.

Gamma synuclein is believed to be a nicotine-responsive protein; hence, an association between its salivary levels and the smoking status was sought in the present investigation in all studied groups. Previously, a study was performed on SCC cells that were treated with nicotine for 72 hours before analyzing the gamma synuclein levels. Nicotine was found to increase the expression of gamma synuclein in a dose-dependant manner, thus it could be concluded that gamma synuclein has an important role in nicotine-induced oral cancer (Hsu et al., 2020). However, in the present study, there was a statistically significant reduction in gamma synuclein level among smokers when compared to non-smokers within the oral cancer group. This contradiction could be due to the difference between in-vitro and in-vivo circumstances. In our case, the in-vivo environment of the oral cavity permitted the interplay of epigenetic mechanisms. Dawes et al. (2019) revealed that nascent smoking had an effect on salivary DNA methylation, leading to hindrance of expression of certain genes through epigenetic mechanisms, with the resultant decrease in the salivary level of the related proteins (Dawes et al., 2019). However, as the gamma synuclein level was massively increased in oral cancer cases, such an inhibitory effect did not affect the diagnostic accuracy of gamma synuclein as a marker for malignancy. As for the OLP group, there was another proof for the previously mentioned theory, where smokers registered lower gamma synuclein when compared to nonsmokers, but the difference did not reach the level of significance. This may be due to lower gamma synuclein levels in the group in general or due to differences in cellular kinetics and epigenetic factors in the absence of malignancy.

A more striking association was found between gamma synuclein levels and the presence of dysplasia, as patients with dysplastic changes had higher levels of gamma synuclein in their saliva ($p=0.032$). On the other hand, the clinical presentation of OLP did not yield any significant difference in synuclein level, where the erosive type did not differ from the papular one. This may suggest that gamma synuclein level is not related to the amount of accompanying inflammation usually found in OLP or the severity of the lesions. Conversely, these results suggest the specific relation of gamma synuclein levels to the presence of dysplastic changes. However, the small sample size did not permit to investigate the correlation between salivary gamma synuclein level and the degree of dysplasia.

Thus, the present results point out how helpful gamma synuclein level determination in unstimulated saliva could be as a diagnostic and

screening tool for the early detection of dysplastic changes and malignant transformation of oral mucosal lesions. The results also highlight that despite the obvious effect of age, gender and smoking status on salivary gamma synuclein, its diagnostic accuracy is not affected. However, it is of no help in discriminating between different grades of OSCC.

Further studies with larger sample sizes may produce better results regarding the ability of salivary gamma synuclein level to differentiate between oral cancer grades, and also can help investigate the ability of salivary gamma synuclein to detect the different degrees of dysplastic changes, and thus the severity of the disease and its prognosis.

In the light of our results, the relation between the gamma synuclein and age needs to be investigated, as well as the relation of gamma synuclein and smoking with its different levels.

Declarations:

Conflict of interests: The authors declare that they have no competing interests

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Ethical approval: The study was approved by the Research Ethics Committee of Faculty of Dentistry, Cairo University, approval number 4-3-21 and was conducted according to the declaration of Helsinki. Each patient was informed about the nature of the study and an informed consent was obtained from each patient.

Consent for publication: All patients who participated in the study were informed that photos of their oral lesions might be used in publication of the final manuscript and a statement was included in the Arabic and English consent forms.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: SM and NAA were responsible patient recruitment and biopsies of oral potentially malignant lesions and clinical examination, FMZ Conceptualized the study, contributed to clinical inclusion and did the data analysis and interpretation, while OS did the biochemical analysis and interpretation. All authors read and approved the final manuscript.

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