ORIGINAL RESEARCH

Sensitivity and Specificity of Serum and Salivary CYFRA21-1 in Detecting Malignant Changes in Oral Potentially Malignant Lesions (Diagnostic Accuracy Study)

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Abstract

Aim and objective: To identify the specificity and sensitivity of CYFRA21-1 in differentiating between oral malignancy and oral potentially malignant lesions (PML) and to be able to early diagnose malignant changes in oral lesions.

Materials and methods: It was a prospective pilot study. Twenty-eight participants were collected in a convenience series and divided into three groups. Group I: 12 patients suffering from PML. Group II: eight patients with oral squamous cell carcinoma (OSCC). Group III: eight participants with no oral lesions. Serum and salivary CYFRA21-1 levels were measured using enzyme-linked immunosorbent assay and correlated with the histopathological examination to confirm the diagnosis.

Results: The OSCC group showed the highest levels of both salivary and serum CYFRA21-1 followed by the group of PML than the control group. The differences in means were statistically significant. At a cutoff value of 0.4 ng/mL, salivary CYFRA21-1 showed 87.5% sensitivity and 100% specificity in differentiating PML from OSCC, with 95% accuracy. Serum CYFRA21-1, at a cutoff value of 1.03 ng/mL showed 100% sensitivity, specificity, and accuracy.

Conclusion: Serum and salivary CYFRA21-1 could be considered promising biomarkers for the diagnosis of oral malignancy and could help detect early malignant changes in PML, especially oral lichen planus and leukoplakia.

Keywords: CYFRA21-1, Diagnostic accuracy study, Oral cancer, Potentially malignant lesions, Saliva.

World Journal of Dentistry (2021): 10.5005/jp-journals-10015-1825

Introduction

It is well recognized that most of the oral cancers are preceded by potentially malignant lesions (PML) of which leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen planus (OLP), and actinic cheilitis are recognized as the most prevalent. These lesions are mostly asymptomatic, except for erosive OLP, and could be missed during routine dental care, leading to late diagnosis of malignant changes that might take place within such lesions.[1] The International Agency for Research on Cancer (IARC) had reported that cancer is considered the second leading reason of death in underdeveloped countries and the major cause of death in developed countries.[2]

Late discovery and diagnosis of oral cancer is primarily accused of its high mortality rate, where its 5-year survival rate is around 50% and remained unchanged over >20 years.[2,3] Also, a high recurrence rate after treatment of oral cancer complicates the situation, especially in oral squamous cell carcinoma (OSCC) which comprises 90% of oral cancers.[4] These issues underline the importance of discovering and developing new diagnostic methods. Therefore, it is necessary to identify biomarkers with high sensitivity and specificity which could be collected easily with no patient discomfort for early detection of malignant transformation.[5] Fortunately, the intimacy between saliva and oral lesions gives the detection of salivary biomarkers high importance as a noninvasive method.[6]

It is well recognized that cytokeratin 19 (CK19) and/or its fragment CYFRA21-1 correlate with tumor diagnosis and progression in several types of cancer, as lung cancer,[7–9] ovarian cancer,[10] and cervical cancer.[11] In a meta-analysis, urinary and serum CYFRA21-1 levels were found to be valuable diagnostic biomarkers for bladder cancer.[12] Similarly, a meta-analysis utilizing serum CYFRA21-1 to diagnose head and neck cancer (HNC) showed promising results and concluded that serum CYFRA21-1 level could be a good marker for diagnosis and prognosis of HNC patients.[13]

For years, salivary biomarkers were researched vs serum and researchers have developed advanced technologies in a trial to validate a lot of salivary biomarkers looking forward to turn the use of saliva into a clinical reality. Among salivary biomarkers, CYFRA21-1 holds great promise as a biomarker for early detection of oral malignancy as well as PML. Consequently, the present study

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How to cite this article: Afifi S, Zahran F, Shaker O, *et al.* Sensitivity and Specificity of Serum and Salivary CYFRA21-1 in Detecting Malignant Changes in Oral Potentially Malignant Lesions (Diagnostic Accuracy Study). World J Dent 2021;12(3):200–207.

Source of support: Nil

Conflict of interest: None

was carried out in an attempt to measure the salivary and serum levels of CYFRA21-1 in patients suffering from oral PML as well as patients with OSCC to evaluate the validity of this biomarker in diagnosing oral malignancy and, hopefully, in early detecting malignant transformation of PML.

Materials and Methods

Study Design

It is a pilot diagnostic accuracy study.

Participants

Participants included in the present study were recruited consecutively (convenience sample) from the outpatient clinics of Oral Medicine and Periodontology Departments at Faculty of Dentistry, Cairo University and Faculty of Dentistry, Fayoum University as well as from National Cancer Institute (NCI), Cairo, Egypt. The start of recruitment was in October 2018 and ended in January 2020.

Eligibility Criteria

Inclusion criteria: Participants were divided into three groups:

Group I: patients suffering from PML as defined by World Health Organization.[1] Group II: patients suffering from OSCC. Group III: healthy participants who are systemically free, non-smokers, and not suffering from any oral mucosal lesions.

Exclusion Criteria

- Patients taking any drugs inducing any changes that could affect the salivary flow.
- Pregnant females.
- Patients having any allergies, infectious diseases, or active dental abscesses within 1 month before saliva sampling.
- Patients receiving any drugs related to the oral lesions during the 6 months before sample collection.

Ethical Considerations

- This research involved human participants and has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' Institutional Review Board (The Ethical Committee of Faculty of Dentistry, Cairo University) with serial number: 18948.
- Informed consent was obtained from all participants included in the study.
- The full study protocol can be accessed on clinicaltrials.gov; NCT03686020.

Index Test

Preanalytical Phase

Clinical examination: All included participants underwent thorough oral examination along with documentation of their identification data, medical history, and description of their oral lesions.[1]

Salivary sample collection:[14] Whole unstimulated saliva samples were collected in the morning between 9 am and 12 pm by requesting participants to swallow first, tilt their heads forward, and expectorate 10 mL saliva into a sterile centrifuge tube. Participants were asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least half an hour before saliva collection. The saliva was immediately centrifuged at 9880*g* and the clarifying supernatant was filtered and stored at –20°C until used.

Serum sample collection: Peripheral venous blood samples (5 mL) were taken by standard venipuncture from patients and controls using plain tubes. The procedure for serum collection was done under aseptic conditions. Samples were immediately centrifuged and the clarifying supernatant was filtered and stored at –20°C till assayed.

Analytical Phase (Assay Procedure)

The concentration of CYFRA21-1 in saliva and serum was quantitated using an enzyme-linked immunosorbent assay (ELISA) kit (Catalogue Number: SL0589Hu) that was supplied by SunLong Biotech Co., LTD (Hangzhou, China). The main principle applied in the used ELISA kit is Sandwich enzyme immunoassay.[15]

Reagents Preparation and Assay Procedure

- Dilution of standards.

Ten wells were set for standards in a Microelisa strip plate. In well 1 and well 2, 100 µL standard solution and 50 µL standard dilution buffer were added and mixed well. In well 3 and well 4, 100 µL solution from well 1 and well 2 were added, respectively. Then, 50 µL standard dilution buffers were added and mixed well. Then, 50 µL solution was discarded from well 3 and well 4. In well 5 and well 6, 50 µL solutions from well 3 and well 4 were added, respectively. Then, 50 µL standard dilution buffers were added and mixed well. In well 7 and well 8, 50 µL solutions from well 5 and well 6 were added, respectively. Then, 50 µL standard dilution buffers were added and mixed well. In well 9 and well 10, 50 µL solutions from well 7 and well 8 were added, respectively. Then, 50 µL standard dilution buffers were added and mixed well. Then, 50 µL solutions were discarded from well 9 and well 10. After dilution, the total volume in all the wells was 50 µL and the concentrations were 3.6, 2.4, 1.2, 0.6, and 0.3 ng/mL, respectively.

- In the Microelisa strip plate, a well was left empty as blank control. Each set of standards, samples, and controls were duplicated.
- In sample wells, 40 µL sample dilution buffer and 10 µL sample were added (dilution factor is 5). Samples were loaded onto the bottom without touching the well wall. Mix well with gentle shaking was done.
- Incubation for 30 minutes at 37°C was done after sealed with a closure plate membrane.
- Dilution: the concentrated washing buffer was diluted with distilled water (30 times).
- Washing: carefully aspiration and refill with the wash solution were done. The wash solution was discarded after resting for 30 seconds. The washing procedure was repeated 5 times.
- 50 µL HRP-conjugate reagent was added to each well except the blank control well.
- Incubation was done for 30 minutes at 37°C after sealed with a closure plate membrane.
- Washing was done as described in step 5.
- Coloring: 50 µL chromogen solution A and 50 µL chromogen solution B were added to each well, mixed with gently shaking, and incubated at 37°C for 15 minutes with avoidance of light.

- Termination: 50 µL stop solution was added to each well to terminate the reaction. The color in the well was changed from blue to yellow.
- Absorbance OD was read at 450 ± 10 nm using a microtiter plate reader. The OD value of the blank control well is set as zero. The assay was carried out within 15 minutes after adding the stop solution.

Postanalytical Phase (Calculation of Results)

The average absorbance values were calculated for each set of duplicate standards, samples, and controls. Duplicates were within 20% of the mean. A linear standard curve was created by plotting the known concentrations of Human CYFRA21-1 Standard and its corresponding optical density value on the log scale (*x*-axis) and the log scale (*y*-axis), respectively. The Professor who performed the index test was not aware of the clinical information and reference standard results.

Reference Standard

The reference standard was tissue biopsy followed by histopathological examination because it is considered the gold standard for diagnosing malignancy and PML.[16,17] Histopathological examination was performed by third party pathologists who were neither aware of the study nor the index test results.

Statistical Analysis

The statistical package used for this study was R statistical package version 3.5.2 (Copyright © 2018. The R Foundation for Statistical Computing, Vienna, Austria). There were no indeterminate values in the index test because all values were used to form a cutoff. Regarding reference standards, participants with indeterminate results were not included in the study. There were no missing data as the reference standard was a requirement for the inclusion of a patient into the study.

Sample Size Calculation

The test of choice was a one-way analysis of variance (ANOVA) power calculation for more than two groups. Means and standard deviations (SDs) needed for calculations were determined according to Rajkumar et al.[18] based on the difference in serum CYFRA21-1 levels in different study groups. The results showed that at a power of 85% and a two-sided significance level of 5%; a total sample size of 24 participants would be adequate to reject the null hypothesis that the group means are equal.

Descriptive and Comparative Analyzes

The Shapiro–Wilk test for normality was used. Numerical parameters were described in terms of mean, SD, and range. Gender was described in terms of frequencies and percentages. Normally distributed data required using the parametric one-way ANOVA to assess the differences between groups regarding numerical data and Pearson's Chi-squared test was applied for gender. To assess the pairwise between-group differences regarding numerical data, Tukey's *post hoc* test was applied.

Testing Diagnostic Capabilities of Predictors

An optimal cutoff value for each predictor was assessed using the Youden Index method. The receiver operating characteristics (ROC) curve and area under the curve (AUC) were plotted. Area under the curves were compared using DeLong's test. The significance level was verified at $p \leq 0.05$.

Results

The present study was carried out on 28 participants (12 males and 16 females) divided into three groups. The first group included 12 patients suffering from oral PML (8 OLP and 4 leukoplakia). Group II included eight patients who suffered from OSCC in various locations. The third group involved eight healthy controls who were selected to match the age and gender of both groups I and II.

The Shapiro–Wilk test for normality results showed that all numerical data (including age, serum, and salivary CYFRA21-1 levels) were normally distributed. Descriptive analysis of numerical data in each group and between-group comparisons are shown in Table 1. Concerning CYFRA21-1 levels in serum and saliva, the malignancy group had the highest mean value followed by that of the PML group than that of the control group. The differences in means were statistically significant (p value < 0.0001).

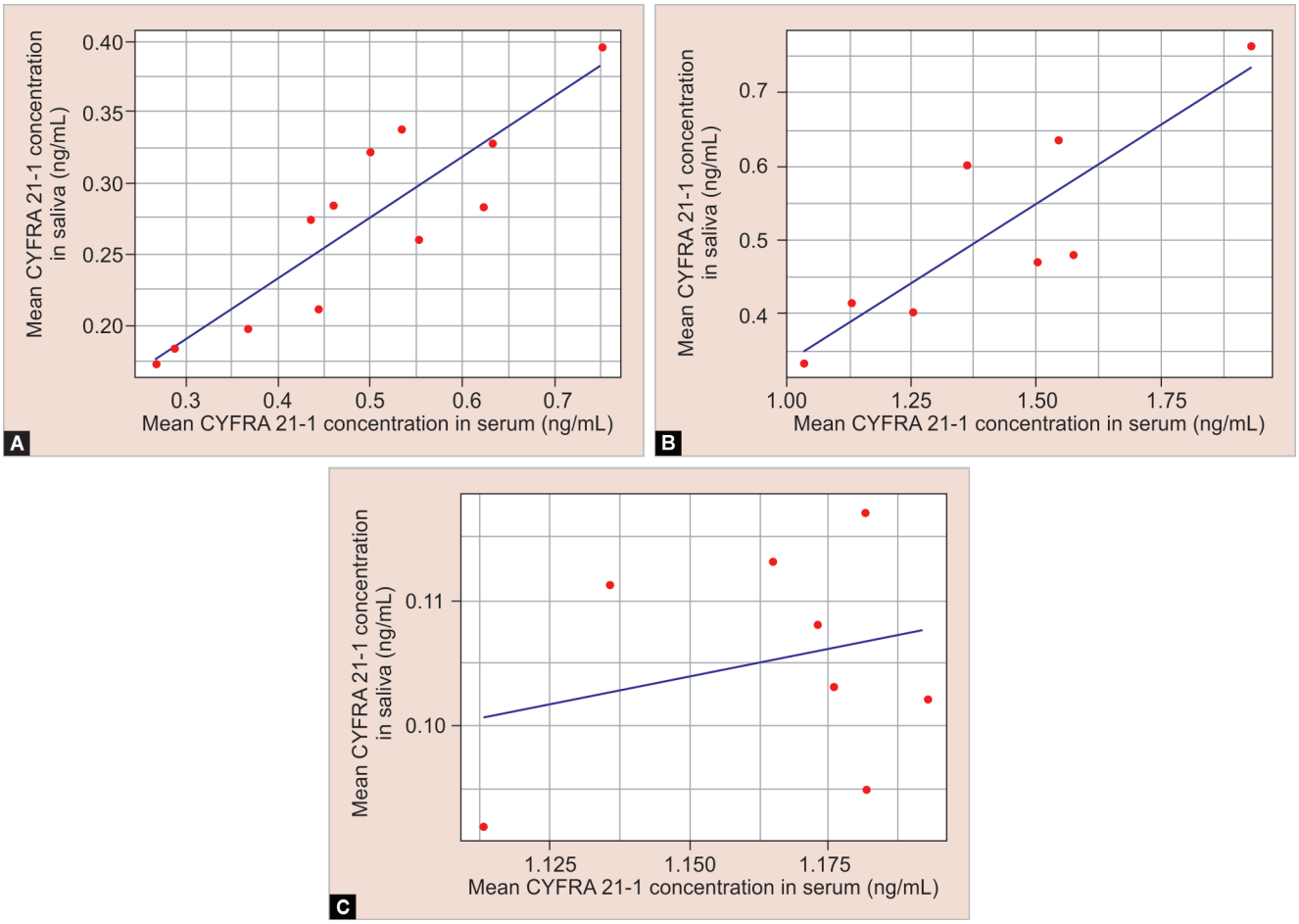
The results of Pearson's Chi-squared test showed that the difference in gender distribution between groups is statistically insignificant (p value = 0.8035). Number of PML patients were 12; 6 males (50%) and 6 females (50%). Cancer patients were eight including three males (37.5%) and five females (62.5%). Control group included eight healthy participants; three males (37.5%) and five females (62.5%). Results of Pearson's correlation coefficient test for the correlation between serum and salivary CYFRA21-1 are demonstrated in Figure 1.

Youden Index method was applied to calculate the optimal cutoff points for serum and salivary CYFRA21-1 discretely which

Table 1: Descriptive analysis of numerical data and results of one-way ANOVA and Tukey's *post hoc* test for between-group comparisons

				Range		*Between-group comparison one-way ANOVA*	
		Mean	*SD*	*Min*	*Max*	*p value**	*Interpretation*
Age	Controls	52.00	5.18	44.00	60.00	0.241	Statistically insignificant difference
	PML	56.00	10.16	32.00	70.00		
	Malignancy	48.50	11.71	29.00	62.00		
CYFRA in saliva	Controls	0.11[a]	0.01	0.09	0.12	<0.0001	Statistically significant difference
	PML	0.27[b]	0.07	0.17	0.40		
	Malignancy	0.51[c]	0.14	0.33	0.76		
CYFRA in serum	Controls	0.17[d]	0.03	0.11	0.19	<0.0001	Statistically significant difference
	PML	0.49[e]	0.14	0.27	0.75		
	Malignancy	1.42[g]	0.29	1.03	1.93		
	p value	*<0.0001					

*Significance level at p value ≤0.05. Different superscripts in the same column mean they are statistically significantly different



Figs 1A to C: Results of Pearson's correlation coefficient test for the correlation between serum and salivary CYFRA21-1 in (A) PML group; (B) Malignancy group; (C) Control group. All correlations are direct (positive) and statistically significant

corresponds to the values of the best sensitivity and specificity. In an attempt to evaluate the ability of serum and salivary CYFRA21-1 in discriminating between patients with PML vs healthy controls, malignancy vs healthy controls, and PML vs malignancy; three ROC curves were plotted as shown in Figure 2, and the accuracy measurements were abridged in Table 2. At a cutoff value 0.4 ng/mL, salivary CYFRA21-1 showed 87.5% sensitivity, 100% specificity, and 95% accuracy in differentiating PML from OSCC (p value < 0.0001), while serum CYFRA21-1 at a cutoff value 1.03 ng/mL showed 100% sensitivity, specificity, and accuracy (p value = 0.0001). By contrasting AUCs using DeLong's test, there was no statistically significant difference between salivary and serum CYFRA21-1 in the accuracy of distinguishing cancer and PML patients (p value = 0.407).

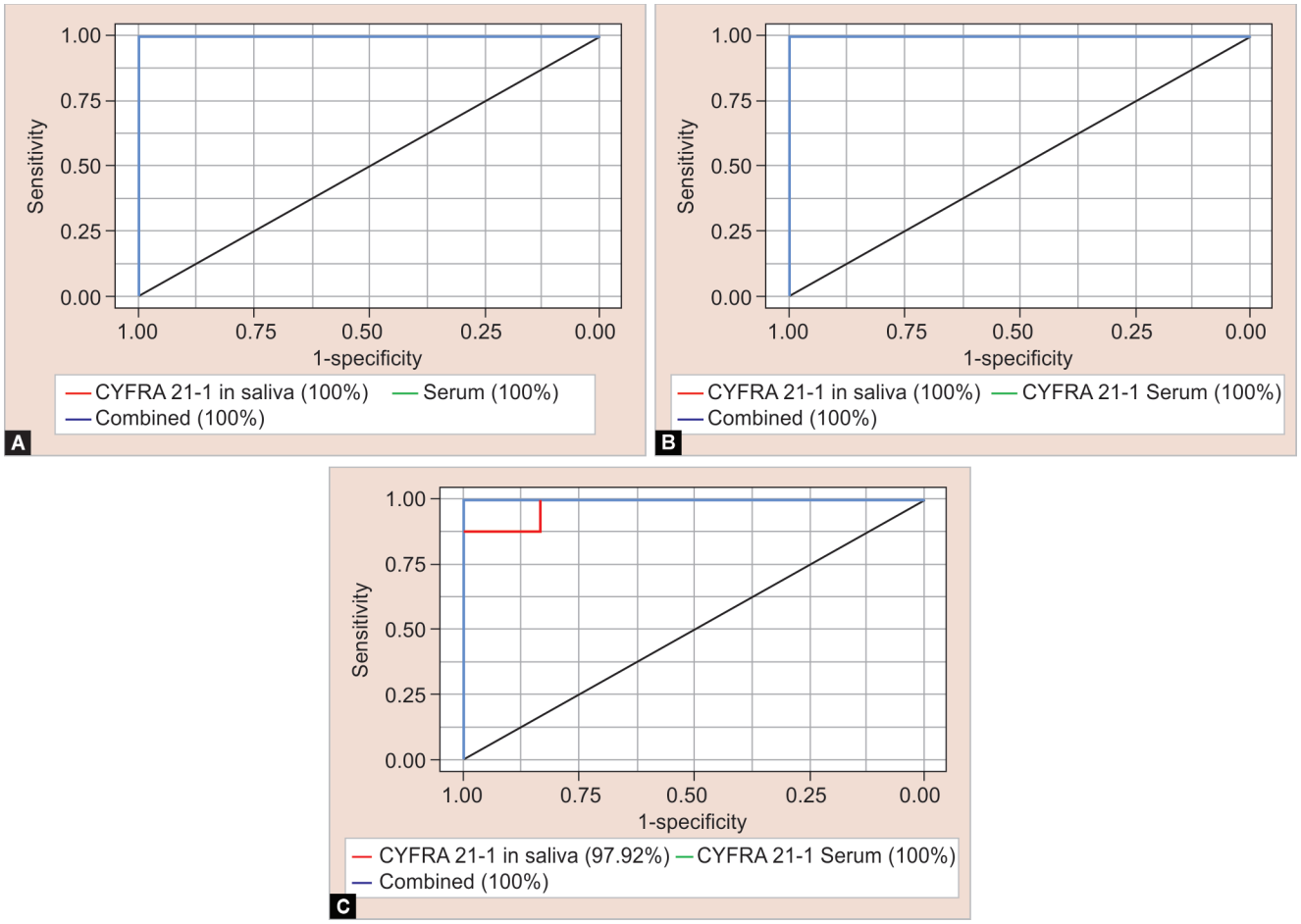
Discussion

Egypt, according to the report of GLOBOCAN 2018 had the uppermost incidence and mortality rates of oral and oropharyngeal cancer among the 22 countries of the Arab nations.[19] Unfortunately, the majority of cases registered among the Egyptian population were diagnosed at advanced stages.[20] These basic incidence and mortality ratios are expected to be doubled by 2030 especially in Egypt, among other countries as Sudan, Morocco, Iran, and Turkey. This rise is far beyond that of the estimated global rate.[21] Identification of high-risk PML and their close follow-up, with the possible early detection of malignant transformation, could constitute one of the keys to reduce the mortality, morbidity, and cost of treatment associated with oral cancer.[22]

In the normal cell's cytoskeleton, CKs demonstrate very low solubility. Though, during the malignant transformation of normal cells, posttranslational modifications impact the biological activity of the intermediate filaments resulting in elevated solubility and filament re-organization leading to a rise in CKs concentrations in patients with epithelial cell-associated carcinomas as OSCC. Thus, it could be concluded that CKs can reflect tumor cell activity, predict disease status and the response of cancer to different therapeutic modalities via critical information about tumor growth activity.[23]

Consequently, in the current study, we selected one of the CK19 fragments which is CYFRA21-1 to be evaluated in serum and saliva of patients suffering from PML as well as OSCC cases, compared to healthy participants, utilizing sandwich ELISA technique as index test. The ELISA technology was preferred because it is considered the most sensitive, well-established, widely available technology and is cost-effective for the detection of protein markers in different body fluids.[24] The reference standard selected was the histopathological examination of biopsy specimens (the gold standard) obtained from patients with PML and OSCC.[25]

The present investigation showed that the concentration of serum CYFRA21-1 in OSCC patients showed the highest value, with



Figs 2A to C: ROC curves of serum and salivary CYFRA21-1 in the diagnosis of (A) PML vs control; (B) Malignancy vs control; (C) Malignancy vs PML

a statistically significant difference when compared with either PML or control participants. These results were in agreement with previous studies that reported that serum CYFRA21-1 levels were significantly higher in HNC and OSCC than those of healthy individuals,[23,26–29] in addition to patients with laryngeal SCC.[30] Contradictorily, Alkotyfan et al.[31] evaluated oropharyngeal SCC by electro-chemiluminescence immunoassay (ECLIA) and the results showed that there was no significant correlation between the CYFRA21-1 serum level, at the time of initial diagnosis, with either the clinical or pathological parameters. However, they observed higher levels of CYFRA21-1 during follow-up in cases of local tumor recurrence or distant metastasis.[31]

In the present investigation, the serum CYFRA21-1 level of PML was statistically significantly higher than the control group; but still significantly lower than the malignancy group. These results were in accordance with Rajkumar et al.,[18] although they included cases with leukoplakia and oral submucous fibrosis, not OLP as the present study. However, they did not observe any significant difference in serum CYFRA21-1 in the PML group when compared with healthy controls, which is not the case in the present investigation. This could be attributed to the different sample sizes and different lesions included in the PML group.[18]

In the OSCC group, salivary CYFRA21-1 levels were significantly the highest among all groups. This finding was consistent with previous studies that reported higher salivary CYFRA21-1 values in oral cancer compared to healthy controls.[18,28,32–34] Likewise, other studies declared that salivary CYFRA21-1 was significantly higher in the PML group compared to healthy controls but still significantly lower than that of OSCC.[18,34]

Surprisingly, Awasthi[34] mentioned that the mean salivary level of CYFRA21-1 in the PML group was 5.9 (±2.4) and in the OSCC group was 17.5 (±15.5) which is much higher than that reported in the present study. Similarly, another study observed that salivary CYFRA21-1 in PML and OSCC group had a median of 7.39 and 16.56, respectively, compared to 0.28 and 0.48 recorded in the current study.[18] To explain this diversity, it should be noted that these levels depend on the expression and differentiation of the tumor tissue in case of malignancy, which may be heterogeneous as well as the localization of the lesion.[35] Another essential reason is the presence of confounding factors such as those in the study by Rajkumar et al.[18] where all the PML and OSCC patients had some of tobacco/pan chewing/smoking or alcohol intake habits taking into consideration that there is no clear evidence about the effect of these factors on CYFRA21-1 production. Moreover, Awasthi[34] did not mention the type of PML included in the study.

Receiver operating characteristic curve analysis revealed that at a cutoff value of 0.4 ng/mL, salivary CYFRA21-1 showed 87.5% sensitivity, 100% specificity, and 95% accuracy in differentiating PML from OSCC. For CYFRA21-1 in serum, the cutoff value was 1.03 ng/mL with 100% sensitivity, specificity, and accuracy. Similarly, a study analyzed the ROC curve at a cutoff value of >10.4 ng/mL; where salivary CYFRA21-1 had 75% specificity and sensitivity.[18] In

Table 2: Accuracy measurements of predictors that differentiate between healthy controls, PML, and malignancy cases at optimal cutoffs

							AUC[c]		
	Cutoff	*Sensitivity*	*Specificity*	*PPV*[d]	*NPV*[e]	*Accuracy*	*AUC*	*95% CI*[b]	*p value*[a]
PML vs control									
CYFRA in saliva	<0.17	100	100	100	100	100	100	100–100	0.0001
CYFRA in serum	<0.27	100	100	100	100	100	100	100–100	<0.0001
Combined	–	100	100	100	100	100	100	100–100	<0.0001
Malignancy vs control									
CYFRA in saliva	<0.33	100	100	100	100	100	100	100–100	0.0005
CYFRA in serum	<1.03	100	100	100	100	100	100	100–100	0.0003
Combined	–	100	100	100	100	100	100	100–100	0.0004
PML vs malignancy									
CYFRA in saliva	<0.4	87.5	100	100	92.31	95	97.92	92.99–100	<0.0001
CYFRA in serum	<1.03	100	100	100	100	100	100	100–100	0.0001
Combined	–	100	100	100	100	100	100	100–100	<0.0001

[a]Significance level at p value ≤ 0.05
[b]95% Confidence interval
[c]Area under the curve (ROC curve)
[d]Positive predictive value
[e]Negative predictive valuec

the same study, a cutoff value of 2.5 ng/mL in serum CYFRA21-1 showed 90% specificity and 60% sensitivity in differentiating PML from OSCC.[18] These results are in agreement with Awasthi,[34] where salivary CYFRA21-1 showed a sensitivity of 90% and specificity of 97% at a cutoff value of 8.7 ng/mL. Furthermore, a study utilizing 3 ng/mL as a cutoff value for serum CYFRA21-1, revealed the sensitivity and specificity to be 88 and 78.2%, respectively.[28] In the same study, using a cutoff value of 8.5 ng/mL for salivary CYFRA21-1, the sensitivity was found to be 93.8% and specificity to be 84.3%.[28] Similarly, a previous study found that at cutoff value 2.17 ng/mL for serum CYFRA21-1 sensitivity was 60.36% and specificity was 81.03%.[29]

In a meta-analysis about the overall diagnostic accuracy of serum CYFRA21-1 for HNC, the pooled sensitivity and specificity were 51% and 97%, respectively. They suggested CYFRA21-1 as a valuable indicator in the diagnosis of HNC with high specificity. They also reported that an abrupt increase of CYFRA21-1 during follow-up would likely indicate disease progression and/or metastatic formation and provide early prognostic information, regardless of the cutoff value.[13]

We can explain the diversity of cutoff values by the expression of CYFRA21-1 even by healthy tissues, which leads to a wide range of CYFRA21-1 levels. Another reason might be a technical one since the measurements were done via two different technologies; ELISA and ECLIA. Another factor to be considered is the demographic differences since the age and gender distribution in those studies were heterogonous. Moreover, studies of the HNC involved participants with human papillomavirus (HPV) positive oropharyngeal cancer which expressed higher levels of CYFRA21-1 due to disturbance in the basal cell differentiation caused by viral infection leading to overproduction of CKs as CK19.[36,37]

There has been a great debate about the significance of serum CYFRA21-1 in the diagnosis and prognosis of patients with oral cancer, with highly variable cutoff values.[26,38,39] Yet, it is well recognized that elevated CYFRA21-1 in HNC patients are related to a worse overall prognosis and reduced survival.[26,27] Some studies revealed that CYFRA21-1 is inappropriate for the initial diagnosis of oral cancer but seems to be useful in the follow-up period for detection of a regional or distant tumor recurrence.[31,39] Others declared that CYFRA21-1 is a valuable biomarker in the diagnosis of OSCC and early detection of malignant transformation of PML,[13,18,29,34] thus perfectly fitting what the present investigation pointed out.

Concerning the limitations of this study, we may consider the small sample size as one despite it was calculated statistically with ANOVA test at a power of 85% as mentioned before. The results showed that a total sample size of 24 participants would be adequate to reject the null hypothesis. However, we intended to increase the number of PML participants to 12 patients (total participants = 28) without being overpowering. We selected leukoplakia and OLP as they are the most encountered PML in our daily practice in Egypt. Interestingly, there were no differences in CYFRA21-1 expression between them despite the inflammatory nature of OLP. This finding supported our conclusion on both as one group. However, we recommend further investigations considering re-classifying PML patients into different subgroups to establish a standardized cutoff point for CYFRA21-1 values indicative of a malignant transformation in different PML entities.

Conclusion

The present study suggests that salivary and serum CYFRA21-1 could be considered promising biomarkers for the diagnosis of oral malignancy and could be helpful in the detection of early malignant changes in PML, especially OLP and leukoplakia.

Author Contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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