Salivary and serum expression of TNF-α and Ki-67 in oral potentially malignant lesions

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Abstract

Background: Oral potentially malignant lesions (OPML) are suspicious lesions that may turn into malignancy according to the degree of dysplasia. Early diagnosis of these lesions allows their treatment before malignant transformation. Saliva is a liquid biopsy that can be obtained easily from the patient and assessed for biomarkers expression. Our study aimed to investigate the possibility of using salivary and serum TNF-α and Ki-67 in early detection of OPML. Patients with OPML were selected according to histopathological evaluation of the suspected lesions. The lesions were stained immunohistochemically by tumor necrosis factor alpha (TNF-α) and Ki-67. Saliva and blood samples were collected from the patients for detecting the expression of the markers by ELISA.

Results: The level of TNF-α and Ki-67 was higher in patients with OPML than healthy individuals regarding both saliva and serum. TNF-α was significantly higher in saliva than in serum unlike Ki-67 which was insignificantly higher in saliva than in serum. The OPML show negative expression of TNF-α in OPML immunohistochemically unlike Ki-67 which show intense positive expression in OPML immunohistochemically.

Conclusions: Saliva can be used as an early noninvasive biomarker detector depending on selecting the appropriate salivary biomarker. Salivary TNF-α can be used in early diagnosis of OPML.

Keywords: Oral potentially malignant lesions, Salivary biomarkers, Serum biomarkers, TNF-α, Ki-67

Background

Oral potentially malignant lesions (OPML) are group of doubtful lesions with different appearance, the most common OPML include erythroplakia, leukoplakia, lichen planus, actinic keratosis (Waal 2009), submucous fibrosis, dyskeratosis congenital, epidermolysis bullosa and discoid lupus erythematosus (Lingen et al. 2011; Warnakulasuriya et al. 2007). The World Health Organization in 2005 termed them oral potentially malignant lesions/disorders instead of oral premalignant lesions/disorders (Liu et al. 2016). Oral cancer usually starts by OPML though most of OPML do not advance to cancer (Yang et al. 2018), and the risk for malignant transformation is 12.1% (Leemans et al. 2011). OPML have a degree of epithelial dysplasia which may be mild, moderate, severe or carcinoma in situ. Dysplasia is the existence of epithelial histological changes as the degree of dysplasia increases the risk of malignant transformation increase (Warnakulasuriya and Ariyawardana 2016).

The diagnosis of these lesions warrants clinicians to treat cancer at intraepithelial level to improve survival rate and reduce morbidity of the patients as some studies found that clinically normal mucosa may show dysplasia or initial invasion (Liu et al. 2016; Messadi 2013).

The diagnosis of OPML by biopsy and histological examination is the gold standard technique for diagnosis, but because it is invasive, it is not accepted by some patients especially those with normally appearing mucosa. So, noninvasive diagnostic techniques are needed (Mercadante et al. 2012).
There are molecular biomarkers which can predict the risk for malignant transformation by detecting epigenetic and genetic modulation observed in OPML (Liu and Duan 2012; Mehrotra and Gupta 2011; Langer 2012). TNF-α is a cytokine, it has different effects on individual cells, and among its effects are the activation of monocytes/ macrophages, neutrophils, lymphocytes and the advancement of coagulation. It has a binary action in natural killer cells (Krishnan et al. 2014). In inflammation, TNF-α is an early cytokine in the inflammatory cascade, and also, it promotes carcinogenesis (Cheng et al. 2013). Salivary and serum TNF-α show diagnostic and prognostic value in detection of oral cancer. A meta-analysis showed that TNF-α may participate in development and spread of oral cancer (Chen et al. 2013; Juretić et al. 2013).

The inflammation-mediated carcinogenesis in OPML and oral cancer was detected by using biomarkers, and it was found that the most common inflammatory biomarkers are TNF-α, IL-6, IL-10, VEGF, IL-8 and IL-4. TNF-α has been used as a diagnostic marker in oral cancer and was highly detected in saliva than serum (Deepthi et al. 2019). TNF-α has dual action in the process of carcinogenesis, it may be cytotoxic to cancer cells by restraining the progression of the tumor, or it may stimulate proliferation, migration, angiogenesis and survival of cancer cells (Kaur and Jacobs 2015).

Molecular biomarkers are expressed in saliva and can be used for early detection, surveillance and treatment of different diseases. Saliva can be considered as a promising noninvasive, highly available and financially suitable diagnostic tool (Polz-Dacewicz et al. 2016; Korostoff et al. 2011).

Ki-67 is a proliferation marker. It is exclusive to cell proliferation and aggressiveness of malignancy. Ki-67 is found in prophase and metaphase during cell cycle but not found in resting cells, and this makes it a helpful marker in detecting the prognosis of different types of cancer (Dash et al. 2020; Dadfarnia et al. 2012). Using proliferation markers as Ki-67 in biomolecular examination can detect the progress of malignant tumors. Elevated salivary Ki-67 has been associated with severe dysplasia (Triani et al. 2021).

Routine examination of the oral cavity cannot easily detect malignancy, so researchers aimed to find an easy and inexpensive method to help in early detection of malignancy (Rezazadeh et al. 2017).

The aim of this study is to detect the potential of salivary Ki-67 and TNF-α in diagnosing OPML versus serum.

Subjects and methods
Patients
Patients with OPML were selected from the oral medicine clinic, Faculty of Dentistry, Cairo University. Patients included in the study having oral lesion which was diagnosed according to the standard clinical criteria and confirmed by incisional biopsy to be an OPML. Healthy individuals were selected without any oral lesion. Subjects neither had gingival and/or periodontal inflammation. Patients exclusion criteria were patients with pregnancy, lactation, hepatitis, diabetes mellitus, autoimmune disease or systemic infection, tumors and patients who are smoking.

For all patients, full history was taken.

Ethical approval
The present study was conducted with the Code of Ethics of the World Medical Association, according to the principles expressed in the Declaration of Helsinki. This study has been approved by the local Ethics Committee of National Research Centre, Cairo, Egypt, with approval number 13132032021, and a written informed consent was provided by each participant prior to their inclusion in the study.

Sample size calculation regarding Ki-67
Sample size was calculated depending on a previous study (Triani et al. 2021) as reference. According to this study, the minimally accepted sample size was 13 per group, when the response within each subject group was normally distributed with standard deviation 20.9, and the estimated mean difference was 23.9, when the power was 80% and type I error probability was 0.05.

Sample size calculation regarding TNF-α
Sample size was calculated depending on a previous study (Polz-Dacewicz et al. 2016) as reference. According to this study, the minimally accepted sample size was 13 per group, when the response within each subject group was normally distributed with standard deviation 17.44, and the estimated mean difference was 20.3, when the power was 80% and type I error probability was 0.05.

Study design
The study included two groups: The first group (OPML group) include 25 patients with OPML. The OPML patients were selected from the Oral Medicine Clinic, Faculty of Dentistry, Cairo University. The second group (control group) include 13 healthy individuals. Subject’s age ranged from 18 to 58 years.
Methods

Histopathological evaluation
Tissue specimen of suspicious lesions was taken from the suspected lesions to confirm the definitive diagnosis. The specimen was formalin fixed, paraffin embedded and stained with hematoxylin–eosin stain.

Immunohistochemical evaluation
Consecutive slides from paraffin-embedded tissue blocks were cut by microtome into sections of 4 μm thickness and were mounted on positively charged glass slides (Opti-Plus, BioGenex Laboratory, USA). Then, the sections were dewaxed and labeled for the following commercially available markers (Anti-Ki-67 antibody produced in rabbit with catalog number SAB4501880 and Anti-Tumor Necrosis Factor-α Antibody, clone 195 with catalog number MBA1096) purchased from Sigma-Aldrich, all of which were ready to use using automated stainer (Thermo Scientific, Lab Vision Corporation TM, USA). The immunoreactions were visualized using the ultraView Universal DAB (diaminobenzidine) Quanto Detection Kit in an automated autostainer (Thermo Scientific, Lab Vision Corporation TM, USA).

Blood and saliva samples
Samples were collected from all subjects prior to the administration of any adjunct therapy or surgery.

Blood sample
5 ml of venous blood will be taken from each individual under complete aseptic conditions and kept in the refrigerator as described in previous reports (Ragab et al. 2018). After clotting, the specimen will be centrifuged at 5000 rpm to separate serum. Sera will be stored at freeze (−80 °C) till use.

Saliva sample
The collection protocol has been described in previous reports (Bandhakavi et al. 2011; Qin et al. 2013). Briefly, unstimulated saliva was collected in the morning at least 2 h after the last intake of food. The mouth was rinsed three times with physiological saline immediately before the beginning of the collection, and then, the subjects were requested to swallow first, tilt their head forward and then expectorate all saliva into sterile centrifuge tube for 5 min without swallowing. The samples were centrifuged at 5000 rpm for 20 min, and the clarified supernatants were drawn off and immediately frozen at −80 °C till use in the ELISA assay.

ELISA (enzyme-linked immunosorbent assay)
The ELISA kits of TNF-α and Ki-67 were purchased from Sunlong Biotech Co., Ltd., with catalogue number (SL1761Hu and SL3186Hu, respectively). Determination of the level of the TNF-α and Ki-67 was carried out according to the manufacturers’ instructions. Briefly, after preparing all needed buffers and reagents, serum and saliva samples were added to microwells and then antibodies of the selected markers were added according to the manufacturer's instruction and then the reaction was ended by adding stop solution. Subsequently, absorbance was read on an ELISA reader.

Statistical analysis
All data were expressed as mean ± standard deviation. Data were analyzed by SPSS 16® (Statistical Package for Scientific Studies), Graph Pad prism and Windows Excel to correlate between the level of the markers in serum and saliva. Differences among means were considered statistically significant at P<0.05. Exploration of the given data was performed using Shapiro–Wilks test and Kolmogorov–Smirnov test for normality which revealed that the significant level (P value) was insignificant as P value>0.05 which indicated that alternative hypothesis was rejected, and the concluded data originated from normal distribution (parametric data) resembling normal Bell curve. Accordingly, comparison between different groups was performed by using independent t test. Also, all correlations were performed by using Pearson’s correlation coefficient.

Results
According to the clinical examination, patients with suspected oral lesions were subjected to incisional biopsy to confirm the clinical diagnosis.

Histopathological evaluation
Lesions with dysplastic features were only included in the study. There were different dysplastic features in the selected lesions. But generally the lesions show mild-to-moderate dysplasia in different lesions as presented in Fig. 1. Some lesions have hyperkeratosis, basilar hyperplasia, loss of polarity, loss of cohesiveness between the cells. Also, abnormal mitotic figures and nuclear hyperchromatism can also be seen.

Immunohistochemical analysis
Staining the tissues with TNF-α antibody shows negative expression in epithelium in all cases and weak expression as sporadic spots in stroma as shown in Fig. 2, while staining with Ki-67 antibody shows intense positive nuclear staining of the basal and suprabasal cells, and also, there was negative expression of some basal cells in some cases as shown in Figs. 3, 4 and 5.
Statistical evaluation

Expression of TNF-α

In OPML group, the expression of TNF-α in saliva was (247.92 ± 78.34), while its expression in serum was (177.3 ± 83.94). Comparison between saliva and serum was performed by using independent t test which revealed that saliva was significantly higher than serum as \( P = 0.03 \), as presented in Table 1 and Fig. 6. Also, there was strong positive significant correlation in the expression of TNF-α between saliva and serum of OPML group as presented in Fig. 7.

In control group, the expression of TNF-α in saliva was (141.18 ± 54.05), while its expression in serum was (105.25 ± 29.24). Comparison between saliva and serum was performed by using independent t test which revealed that saliva was significantly higher than serum as \( P = 0.04 \), as presented in Table 2 and Fig. 8. Also, there was medium positive insignificant correlation in the expression of TNF-α between saliva and serum in control group, as presented in Fig. 9.
Fig. 2  Photomicrograph showing a negative expression of TNF-α in epithelium, b sporadic spots of weak expression of TNF-α in stroma (× 200)

Fig. 3  Photomicrograph showing positive immunexpression of Ki-67, a showing basal positive immune staining and some cells of the spinous cell layer, b showing basal and parabasal intense immunostaining (× 100)
Comparison between OPML and control groups

Comparison between both groups was performed by using independent t test, which revealed that the expression of TNF-α in OPML group was significantly higher than control group regarding both serum and saliva as $P<0.001$, as presented in Table 3 and Fig. 10.

Expression of Ki-67

In OPML group, the expression of Ki-67 in saliva was $(8.45\pm1.64)$, while its expression in serum was $(8.44\pm1.62)$. Comparison between saliva and serum was performed by using independent t test which revealed that saliva was insignificantly higher than serum as $P=0.98$, as presented in Table 4 and Fig. 11. Also, there was strong positive significant correlation in the expression of Ki-67 between saliva and serum of OPML group, as presented in Fig. 12.

In control group, the expression of Ki-67 in saliva was $(3.01\pm0.53)$, while its expression in serum was $(2.8\pm0.33)$. Comparison between saliva and serum was performed by using independent t test which revealed that saliva was insignificantly higher than serum as $P=0.09$, as presented in Table 5 and Fig. 13. Also, there was strong positive significant correlation in the expression of Ki-67 between saliva and serum of control group, as presented in Fig. 14.

Table 1  Mean and standard deviation of saliva and serum in OPML group

|          | Saliva | Serum |
|----------|--------|-------|
| $N$      | 25     | 25    |
| Mean     | 247.92 | 177.33|
| SD       | 78.34  | 83.94 |
| $P$ value| 0.03*  |       |
| $r(\bar{P})$ | 0.68 (0.001*) |
Comparison between OPML group and control group

Comparison between both groups was performed by using independent t test, which revealed that the expression of Ki-67 in OPML group was significantly higher than control group regarding both serum and saliva as $P<0.001$, as presented in Table 6 and Fig. 15.

| Table 2 | Mean and standard deviation of saliva and serum in control group |
|---------|------------------|
| **Control** | **Saliva** | **Serum** |
| $N$ | 13 | 13 |
| Mean | 141.18 | 105.25 |
| SD | 54.05 | 29.24 |
| $P$ value | 0.04* |  |
| $r(P)$ | 0.48 (0.15) |  |
Molecular biomarkers can be used for oral cancer detection, and they can be obtained from saliva which reflects the state of the body. Saliva as a diagnostic tool meets the need for a noninvasive, financially suitable and highly available diagnostic tool. Saliva can detect caries susceptibility, periodontal disease, salivary gland disorders, breast cancer, oral cancer and systemic disorders (Pesce).
and Spitalnik 2007). This diagnostic test can be done by unspecialized clinicians and can be used for screening to detect the tumors in early stage and monitor patients postoperatively (Deepthi et al. 2019).

Our study aimed to detect the ability to use saliva instead of serum for detecting biomarkers expressed by OPML. In carcinogenesis and inflammation process, cytokines have a significant role which may be proinflammatory or having anti-inflammatory effect. TNF-α

### Table 4 Mean and standard deviation of saliva and serum in OPML group

| OPML   | Saliva | Serum |
|--------|--------|-------|
| N      | 25     | 25    |
| Mean   | 8.45   | 8.44  |
| SD     | 1.65   | 1.62  |
| P value| 0.98   |       |
| r(P)   | 0.82 (0.000*) |       |

### Fig. 10 Bar chart showing mean and standard deviation of TNF in saliva and serum regarding control and OPML groups

### Fig. 11 Bar chart showing mean and standard deviation of Ki-67 in saliva and serum in OPML group
is a cytokine which has multiple biological activities as antiviral and antitumor activity (Prasad and McCullough 2013).

Our results detect the expression of TNF-α in saliva and serum of patients with OPML. And the expression of the markers was significantly higher in patients with OPML than in healthy individuals. Detection of TNF-α expression in saliva and serum of the selected subjects revealed high significant expression of TNF-α in saliva than in serum of both groups.

Our results are in accordance with Polz-Dacewicz et al. as they compare the expression of TNF-α in saliva and serum between oral squamous cell carcinoma (OSCC) patients and control, they found higher expression of TNF-α in saliva of OSCC patients than in serum, and they concluded that this is due to the proximity to the tumor (Polz-Dacewicz et al. 2016). Also, it was found that TNF-α was significantly higher in both serum and saliva of OSCC patients than control (Krishnan et al. 2014). Another study investigated the expression of salivary TNF-α in OPML and OSCC and found higher expression of the TNF-α in OPML patients and OSCC patients compared to normal individuals (Juretić et al. 2013).

Although salivary biomarkers cannot detect the origin of the tumor, but they can detect patients at risk and it is considered as a marker of OPML and OSCC (Deepthi et al. 2019). And it can be used as a prognostic marker in cancer (Rhodus et al. 2005a). The expression of salivary TNF-α was assessed in a study, and they found that TNF-α is highly expressed in OSCC than in leukoplakia than controls (Koontongkaew 2013) and this finding supports previous studies suggesting that TNF-α is a protumorigenic (Krishnan et al. 2014; Kaur and Jacobs 2015). And they concluded that TNF-α can be used as an indicator for transformation of OPML to neoplastic tumors (Koontongkaew 2013).

When studying the expression of TNF-α in serum and saliva of OSCC patients, higher expression of this marker was found in saliva than in serum, and the authors suggested that this may be due to local production of marker by tumor cells (SahebJamee et al. 2008).

Our results are in accordance with earlier study suggesting the diagnostic potential of TNF-α in screening OSCC as they found that TNF-α, IL-8, IL-6 and IL-1α were higher in saliva of OSCC patients compared with PML and controls (Rhodus et al. 2005b). Another study compared the expression IL-6, IL-8, IL-1 and TNF-α in

| Table 5 Mean and standard deviation of saliva and serum in control group |
|-----------------------------|-----------------------------|
| **Control** | **Saliva** | **Serum** |
| --- | --- | --- |
| **N** | 13 | 13 |
| **Mean** | 3.01 | 2.80 |
| **SD** | 0.53 | 0.33 |
| **P value** | 0.09 | 0.33 |
| **r(P)** | 0.81 (0.004*) | |
saliva of dysplastic oral lichen planus, OSCC and control and found significantly higher level of each marker in OSCC, sever and moderate dysplasia than in control (Juretić et al. 2013). It was also suggested that higher expression of TNF-α is correlated with speeding the disease progression (Brailo et al. 2012).

Table 6 Mean and standard deviation of saliva and serum regarding control and OPML and comparison between them

|         | OPML          | Control         | P value  |
|---------|---------------|-----------------|----------|
|         | M  | SD  | M  | SD  |          |          |
| Saliva  | 8.45 | 1.65 | 3.01 | 0.53 | <0.0001* |
| Serum   | 8.44 | 1.62 | 2.80 | 0.33 | <0.0001* |
Krishnan et al. found TNF-α to be a monitoring marker in transforming precancerous conditions into cancer. By detecting of the salivary level of the marker, they found significant increased expression of the marker in OPML but this increase was lower than OSCC group, and, contrast to our results there was insignificant difference between serum and saliva in OPML group, they suggested that this was due to the production of the marker by tumor cells and the rise in saliva was due to the expression of the marker in the local environment of the tumor (Krishnan et al. 2014).

Regarding immunohistochemical expression of TNF-α, our results found negative expression of TNF-α immunohistochemically in epithelial cells but we found sporadic spots of weak expression of TNF-α in stroma and this was in accordance with Babiuach et al., who found notably altered expression of TNF-α in oral epithelial dysplasia specimens and suggested that TNF-α plays a proinflammatory role in process of malignant transformation within the oral lesions (Babiuch et al. 2020). In our study, the marker was highly expressed in saliva and serum and weakly expressed in the tissues and this may be due to the local production of the marker in the saliva and serum earlier than its expression in the tissue.

In studying tumorigenesis, it was found that there is an increase in cell proliferation as a precursor and indicator to neoplastic transformation. Ki-67 is a marker used to detect proliferation in cells. The level of dysplasia determines the speed of malignant transformation, and accordingly malignant transformation occurs 4.5 times more in lesion with high grade dysplasia (Liu et al. 2010).

Regarding expression of Ki-67 in OPML patients and control, there was significant high expression of Ki-67 in OPML patient than in control, but our results revealed insignificant higher expression of Ki-67 in saliva than in serum of both groups. Also, there was positive immune expression of Ki-67 in the basal, para-basal layer and some cells of the spinous layer in the epithelium and this was in the cases that was considered to be of moderate dysplasia. Also, we found some basal cells with negative immune expression and this was seen only in cases with mild dysplasia.

In accordance with our results, Dash et al. investigated the expression of Ki-67 in OSCC as well as different grades of epithelial dysplasia and found high expression of Ki-67 using immunohistochemistry (Dash et al. 2020), and this was in accordance with other studies (Raju et al. 2005; Dragomir et al. 2012; Ahmad et al. 2020) concluding that Ki-67 is a useful marker that can predict the severity of OPML as well as the aggressiveness of the tumor.

Triani et al. assessed the salivary expression of Ki-67 in betel nut chewers using ELISA and found that the marker expression increases fourfold when assessed in OPML than in normal oral mucosa, and they found also that marker expression based on grade of dysplasia and clinical symptoms of the patients (Triani et al. 2021). Other studies assessed the intense expression of Ki-67 immunohistochemically in a tissue biopsy of OPML as it is the gold standard tool for diagnosis (Beevi et al. 2019; Li et al. 2015).

To investigate the relation between cell proliferation, metastasis and progression of the tumor, Shpitzer et al. found increase in salivary Ki-67, LDH, MMP-9 and CysD1 in patients with OSCC. Therefore, these biomarkers are expected to be elevated in tumors (Shpitzer et al. 2009).
Conclusions

The present study concluded that saliva is a noninvasive diagnostic tool that can be used in diagnosis of OPML depending on selection of the appropriate salivary biomarker. Also, we confirmed the accuracy of TNF-α as a diagnostic salivary biomarker for detection of OPML and can differentiate OPML patients from healthy individuals.

Abbreviations

OPML: Oral potential malignant lesions; TNF-α: Tumor necrosis factor alpha; ELISA: Enzyme-linked immunosorbent assay; OSCC: Oral squamous cell carcinoma; IL-8: Interleukin 8; IL-6: Interleukin 6; IL-1α: Interleukin 1 alpha; LDH: Lactate dehydrogenase; MMP-9: Matrix metalloproteinase 9; CysD1: Cysteine synthase D1.

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Author contributions

HT planned the experiment design and was helped by ME in collecting samples, histopathological and immune-histochemical evaluation. BH was responsible for incisional biopsy. HE and HN performed the statistical analysis. HE analyzed the results helped by RM. RA and MH performed the ELISA. Both HT and ME analyzed the results. RA and MH performed the ELISA. All authors read and approved the final manuscript.

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

The present study was conducted with the Code of Ethics of the World Medical Association, according to the principles expressed in the Declaration of Helsinki. This study has been approved by the local Ethics Committee of National Research Centre, Cairo, Egypt, with approval number 13132032021, and a written informed consent was provided by each participant prior to their inclusion in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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