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ORIGINAL ARTICLE

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Evaluating the role of tissue microRNA-27b as a diagnostic marker for oral lichen planus and possible correlation with CD8

Sana Maher Aghbari[1] | Shaimaa Omar Zayed[2] | Olfat Gamil Shaker[3] | Abdelrahman Ibrahim Abushouk[4]

[1]Oral Medicine, Faculty of Dentistry, Aden University, Aden, Yemen

[2]Oral Pathology, Faculty of Dentistry, Cairo University, Cairo, Egypt

[3]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine Cairo University, Cairo, Egypt

[4]Faculty of Medicine, Ain Shams University, Cairo, Egypt

Correspondence
Sana Maher Aghbari, Faculty of Dentistry, Aden University, Aden, Yemen.
Email: Sana_aghbary@hotmail.com

Background: MicroRNA-27b (miR27b) is a small, non-coding RNA that is involved in physiological keratinocyte differentiation and regulating inflammatory processes. We performed this study to investigate the value of miR27b as a diagnostic marker for oral lichen planus (OLP) and the correlation between CD8 (cytotoxic T-cell marker) and miR27b tissue expression in OLP patients.

Methods: Forty participants (including 20 OLP patients and 20 controls) underwent oral biopsy. The obtained specimens were examined by immunostaining and quantitative RT-PCR for CD8 and miR27b tissue expression, respectively. We used the Spearman rank correlation test to evaluate the correlation between both variables.

Results: Our analysis showed that in comparison with healthy tissues, OLP tissue samples exhibited significantly higher CD8 levels ($P < 0.01$), as well as a significant downregulation of miR27b expression ($P < 0.0001$). Upon comparing different OLP subgroups, no significant difference was detected in terms of miR27b expression; however, the tissue levels of CD8 varied significantly (highest in the erosive subgroup and lowest in the papular/plaque/reticular subgroup). The Spearman rank analysis showed a negative correlation between tissue expression of miR27b and CD8; however, this was not statistically significant ($P > 0.05$). Further, the receiver operating characteristic curve of tissue miR27b as an OLP biomarker revealed 100% sensitivity and 65% specificity at cutoff value of 4.4.

Conclusion: This study demonstrated increased CD8 levels and downregulation of miR27b in OLP tissues, compared to healthy tissues. Moreover, it revealed the potential of miR27b as an OLP disease biomarker. The possible negative correlation between CD8 and miR27b tissue expression requires further investigation in larger studies.

KEYWORDS
CD8, cytotoxic T cells, miR27b, oral lichen planus

1 | INTRODUCTION

Oral lichen planus (OLP) is an inflammatory mucocutaneous disease that follows a chronic clinical course and affects about 2% of the worldwide population.[1] It is recognized by the World Health Organization (WHO) as a premalignant condition.[2,3] Pathologically, it is characterized by (a) degeneration of the basal epithelial cells; (b) rupture of the basement membrane; and (c) sub-epithelial infiltration by T-helper and T-cytotoxic lymphocytes.[4,5] However, to date, the exact etiology, pathogenesis, and malignant transformation mechanisms of OLP are not yet clear.

Within the past decade, researchers have focused on the role of microRNAs in different physiological and pathological processes. These small, non-coding RNA molecules are involved in the control of the cell cycle, and their alterations are associated with inflammatory, autoimmune, and carcinogenic processes.[6-8] Building on their role in immunity control, several miRNAs (including miR125, miR137, miR147a, miR155, miR181, and miR223) were investigated and found involved in the pathogenesis of OLP and its malignant transformation via alteration of their tissue expression and functions.[9-11]

MicroRNA-27b (miR27b) is a member of the miRNAs family that has been shown involved in the pathogenesis of atherosclerosis,[12,13] infections,[14,15] and cancer.[16] Hildebrand and colleagues demonstrated the involvement of miR27b in keratinocyte differentiation, both in vitro and in vivo.[17] Moreover, Zhang and colleagues reported significant downregulation of miR27b expression in OLP patients.[18] Based on these findings, we hypothesized that miR27b could be used as a diagnostic biomarker for OLP. Therefore, we performed this study to investigate this hypothesis, as well as the correlation between miR27b and CD8 (cytotoxic T-cell marker) tissue expression in OLP patients.

2 | MATERIALS AND METHODS

2.1 | Patients

Forty participants (20 OLP patients and 20 controls) were recruited from the Oral Medicine and Periodontology Department Outpatient Clinic at Cairo University during the period between January 2013 and December 2016. To be eligible for inclusion, patients must have been (a) diagnosed with OLP according to the modified WHO criteria[2] (Hematoxylin and Eosin staining was performed later to confirm the diagnosis); (b) nonsmokers, nonalcoholics, and free of oral and systemic diseases; and (c) did not receive any treatments during the 90 days, preceding the oral biopsy. These patients were further divided into erosive, atrophic, and popular/reticular/plaque OLP subgroups. Prior to participant recruitment, our protocol was approved by the Research Ethics Committee at Cairo University and all patients gave a fully-informed consent as per the declaration of Helsinki (1978, as revised in 2008).

2.2 | Biopsy and histopathological examination

Oral mucosal biopsy was obtained from the non-inflamed gingiva in participating controls and from the lesion mucosa in OLP patients. A part from each specimen was fixed in formalin and paraffin, and then cut using a microtome into 4 μm thick sections. These sections underwent immunostaining by CD8 antibody, and binding was detected using a BIOGENEX detection kit (Fremont, CA, USA). A computerized method was later used to assess and compare CD8 tissue staining between OLP and control groups.

2.3 | MicroRNA extraction and quantification

We used miRNeasy extraction kits (Qiagen, CA, USA) to extract miRNAs from the biopsy specimens. After storage at −80°C, the extracted miRNAs underwent reverse transcription and real-time quantitative PCR (RT-PCR) using miScript II RT kit and Green (MiScript SYBER) PCR kits (Qiagen, CA, USA), respectively, according to the manufacturers' protocol. Using SNORD as an endogenous control, we performed melting curve analyses to confirm the generation of miR27b. We used the ΔΔCt method to compare miR27b tissue expression between OLP and control groups.

2.4 | Statistical analysis

To achieve a statistical power of 80% (using MedCalc Software (version 15.2.2), Mariakerke, Belgium), 20 OLP patients and a similar control sample size were selected for inclusion. The obtained data were summarized as means ± standard deviations (for continuous data) or frequencies and percentages (for categorical data). All statistical analyses were performed using the SPSS software Inc. (version 15 for Windows), Chicago, IL, USA. The following tests were performed: (a) Student's *t* and Wilcoxon's signed-rank tests for between-group comparisons of continuous data; (b) the chi-square and the Fisher's exact tests for between-group comparisons of categorical data; and (c) the Spearman rank coefficient to evaluate the correlation between different variables. The level of statistical significance was assigned at P value ≤ 0.05.

3 | RESULTS

3.1 | Baseline characteristics

The OLP and control groups included 6 (30%) and 13 (65%) males, respectively. The mean age of OLP patients (48.8 ± 8.9 years) was significantly higher than controls (32 ± 10); $P < 0.05$. The enrolled OLP patients had the following clinical subtypes: atrophic (n = 8), erosive (n = 7), papular (n = 2), plaque (n = 2), and reticular (n = 1). Patients were followed for variable durations from 1 to 48 months (Table 1).

3.2 | CD8 tissue expression

Figures 1,2,3 show the immunohistochemical staining of CD8 T cells in the sub-epithelium of atrophic, erosive, and papular OLP tissues, respectively. We found a statistically significant ($P < 0.01$) increase in CD8 tissue expression in the OLP group (4.7 ± 2.4), compared to the control group (0.7 ± 0.2). In addition, our analysis revealed a significant difference ($P = 0.001$) in the same parameter between various OLP subgroups (erosive OLP: 7.2 ± 1.1 > atrophic OLP: 4.1 ± 1.7 > papular/plaque/reticular OLP: 2.1 ± 0.9).

3.3 | MicroRNA27b tissue expression

A statistically significant ($P < 0.0001$) downregulation of miR27b tissue expression was noted in the OLP group (1.6 ± 1.1), compared to

TABLE 1 Shows the patient and lesion characteristics of enrolled oral lichen planus patients

Pt. No	Sex	Age (year)	Follow-up duration (months)	Clinical type	Site (oral and extra-oral lesion)	Pain score[a]	Ulcer N and size
1	F	40	-	Papular	Buccal mucosa bilateral	0	-
2	F	60	6	Atrophic	Left buccal mucosa, bilateral buccal vestibules, and upper alveolar edge	5	-
3	M	48	3	Erosive	Tongue, left buccal mucosa, and lower right alveolar edge	7	N = 1 (3 mm)
4	M	60	4	Atrophic	Bilateral buccal mucosa and tongue	7	-
5	M	47	>12	Plaque	Bilateral buccal mucosa	0	-
6	F	58	>3	Atrophic	Bilateral buccal mucosa, labial mucosa, and tongue	2	-
7	F	59	9	Atrophic	Bilateral buccal mucosa	5	-
8	F	26	1	Atrophic	Bilateral buccal mucosa	5	-
9	M	45	48	Erosive	Bilateral buccal mucosa	8	N = 4 (5-11 mm)
10	F	43	2	Erosive	Bilateral buccal mucosa and tongue	9	N = 2 (5-6 mm)
11	F	55	5	Bullous Erosive	Bilateral buccal mucosa and tongue	8	N = 1 (10 mm)
12	F	50	6	Atrophic	Bilateral buccal mucosa plus genitalia lesion	5	-
13	F	34	1	Erosive	Bilateral buccal mucosa and tongue	4	N = 3 (3-9 mm)
14	F	49	-	Plaque	Right buccal mucosa and lateral border of the tongue	0	-
15	M	47	-	Atrophic	Bilateral buccal mucosa plus skin lesion	2	-
16	F	49	5	Erosive	Bilateral buccal mucosa and left lateral border of the tongue	9	N = 2 (0.5-5 mm)
17	F	42	2	Papular	Bilateral buccal mucosa and alveolar mucosa	0	-
18	F	53	1	Erosive	Bilateral buccal mucosa and tongue	7	N = 2 (3-5 mm)
19	M	55	-	Reticular	Right buccal mucosa and alveolar edge	0	-
20	F	57	4	Atrophic	Left buccal mucosa, palate, and tongue	5	-

F, female; M, male; OLP, oral lichen planus.
[a]Pain score measured on a 10-point visual analog scale (the greater score means more perceived pain by the patient).

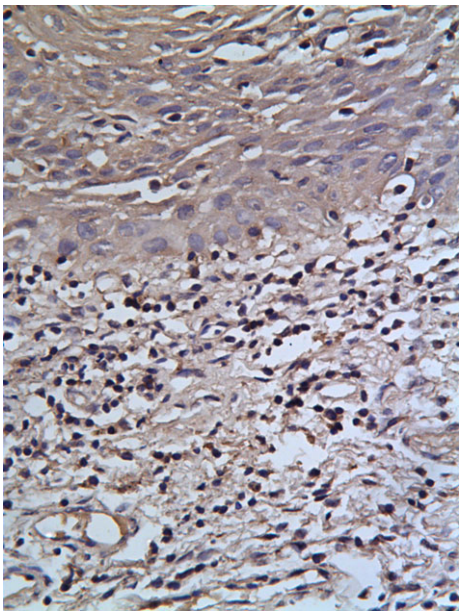

FIGURE 1 Immunostaining of CD8 protein (400×) shows the infiltration of CD8 cells in the sub-epithelium of atrophic oral lichen planus tissues. Note the CD8 cells between the basal and supra-basal epithelial cells

the control group (4.7 ± 1). However, upon comparing different OLP subgroups in this parameter, no statistically significant difference was identified ($P = 0.54$). The results of tissue expression parameters are illustrated in Table 2.

Our study data revealed a cutoff value of 4.4 for miR27b tissue expression. To assess the diagnostic accuracy of miR27b as a tissue biomarker for OLP diagnosis, the receiver operating characteristic curve analysis (Figure 4) revealed an area under the curve of 0.98 with 100% sensitivity and 65% specificity at the aforementioned cutoff value (positive predictive value = 0.74, negative predictive value = 1, positive likelihood ratio [LR] = 2.86, negative LR = 1).

3.4 | Correlation between miR27b and CD8 tissue levels

The Spearman correlation analysis showed a negative correlation between tissue expression of miR27b and CD8; however, this association was not statistically significant ($P > 0.05$). The correlation coefficient varied in different OLP subgroups ($r = -0.28$, -0.36 and -0.43 in the erosive, atrophic, and papular/plaque/reticular OLP subgroups, respectively).

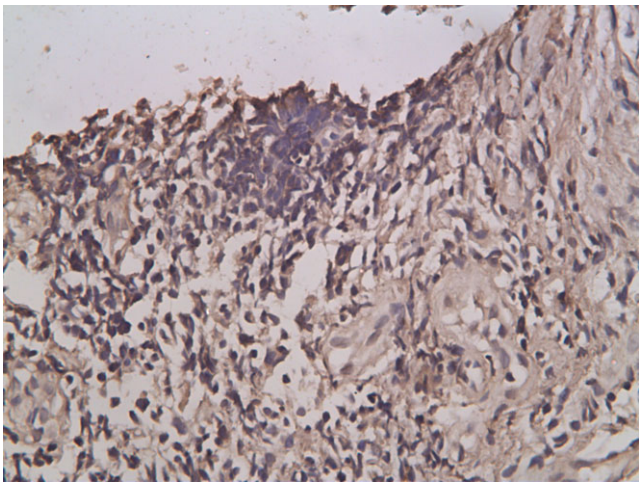

FIGURE 2 Immunostaining of CD8 protein (400×) shows the intense infiltration of CD8 cells in the sub-epithelium of erosive oral lichen planus tissues. Note the CD8 cells between the basal epithelial cells

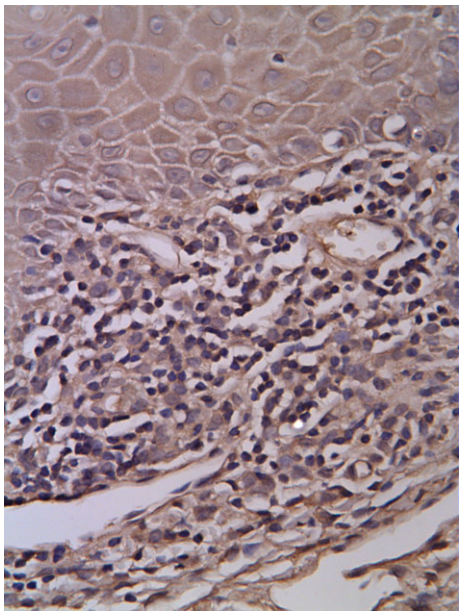

FIGURE 3 Immunostaining of CD8 protein (400×) shows the infiltration of CD8 cells in the sub-epithelium of papular oral lichen planus tissues. Note the few CD8 cells between the basal epithelial cells

3.5 | Tissue expression and patients' characteristics

Our analysis showed no significant association between the tissue expression of CD8 or miR27b and the patients' age (Student's *t* test: $P > 0.05$), gender, and duration of the disease (Fisher's exact test: $P > 0.05$).

4 | DISCUSSION

The main finding of the current study is that OLP patients have increased oral mucosal tissue levels of CD8, as well as down-regulated expression of miR27b in comparison with non-OLP controls. Although not significant, we found a negative correlation between tissue levels of CD8 and miR27b. Further, our study demonstrated that miR27b can be a sensitive biomarker for OLP diagnosis. These findings fit within the reported data in the literature of altered

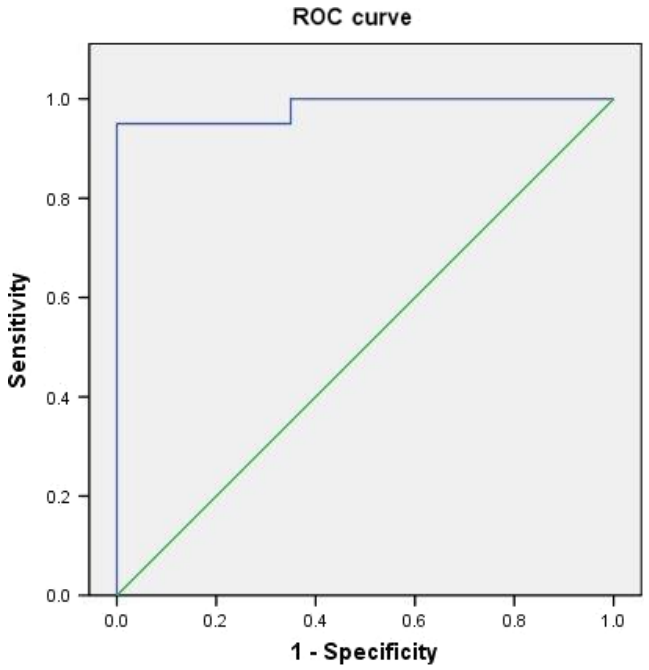

FIGURE 4 Receiver-operator curve of the diagnostic value of tissue miR-27b as a diagnostic marker of oral lichen planus

TABLE 2 Shows CD8 and MicroRNA-27b tissue expression values

	Oral lichen planus patients (N = 20)			Control group (N = 20)	*P* value
CD8 tissue expression	4.7 ± 2.4			0.7 ± 0.2	<0.01[a]
	Papular/reticular/plaque	Atrophic	Erosive		
	2.1 ± 0.9	4.1 ± 1.7	7.2 ± 1.1		0.001[b]
miRNA-27b tissue expression	1.6 ± 1.1			4.7 ± 1	<0.0001[a]
	Papular/reticular/plaque	Atrophic	Erosive		0.54[b]
	1.9 ± 1.3	1.8 ± 1.2	2.3 ± 1.1		

Data are means ± standard deviations (SD). The *P* value is significant if <0.05.
[a]Comparing OLP and control groups.
[b]Comparing different OLP groups.

miRNAs expression in OLP and the ability of such alterations to serve as diagnostic biomarkers for OLP.

The increased lesional tissue expression of CD8 in our study confirms the role of T cells in OLP pathogenesis.[19,20] Previous investigators reported T-cytotoxic cells as the predominant T-cell type in OLP lesions,[21] whereas others reported the same for T-helper cells.[22] These immunophenotypic differences may be related to disease stage that is, the increased T-helper cell infiltration in early disease prompts the T-cytotoxic cell influx at late stages.[19] Moreover, the T-helper/T-cytotoxic cell ratio has been found to vary in different OLP subtypes with increased ratio with the plaque form and decreased ratio with the atrophic form.[23] Our immunostaining findings confirm these results, as well as those of Vered et al[24] who reported variable CD8 tissue levels in different OLP clinical subtypes.

Further, we found a significant downregulation of miR27b tissue expression in OLP patients, compared to normal tissues, which is in agreement with the results by Zhang et al.[18] However, in comparison with their findings, we found no significant difference in miR27b expression between different OLP subgroups. This discrepancy may be related to the differences in sample size or disease stage, which should be accounted for in future studies. Wang and colleagues demonstrated that upregulation of miR27b expression improved keratinocyte migration, while miR27b downregulation induced keratinocyte apoptosis and generation of mitochondrial reactive oxygen species.[25] Taken together with the possible negative correlation between Cd8 and miR27b tissue expression (although not significant and should be further investigated), miR27b appears to have an anti-inflammatory or immunomodulatory function that is altered during OLP pathogenesis.

Similar to our findings on miR27b, former studies have investigated alterations in other miRNAs levels in OLP patients. In a recent review by Ma et al, the levels of more than 70 miRNAs were found to be altered in OLP patients. These miRNAs included blood miR155, miR146a (elevated), lesional miR21, miR203 (elevated), lesional miR121, miR26b, miR375 (reduced), salivary miR4484, and miR137 (elevated). The included studies in that review investigated the correlation between miRNAs and inflammatory cytokines levels, such as tumor necrosis factor-α, interferon-γ, interleukin (IL)-10, and IL-17.[26] Further, a recent study by Ghallab et al[27] showed an inverse correlation between oral mucosal levels of miR138 and cyclin D1 protein expression (a proto-oncogenic regulator of the cell cycle).

Further, recent data have shown correlations between miRNAs levels and lesional T-cell infiltration and activity. Our previous work showed a negative correlation between miR137 and CD8 tissue expression in OLP patients.[9] Moreover, Zheng and Li showed that miR214 suppresses CD44 expression, which prevents the apoptosis of activated CD8 T cells.[28] Another study by Hu et al[29] showed that elevated miR155 increases the proliferation of CD4 T cells. Collectively, the alteration in miRNAs levels may play a role in OLP pathogenesis by increasing mucosal T-cell infiltration and the release of pro-inflammatory cytokines.

In addition, we found a high sensitivity for tissue miR27b expression as a biomarker for OLP with a sensitivity as high as 100%. The cutoff value was 4.4 meaning that individuals with tissue values below 4.4 may have OLP, while those with higher values are probably free. These results open the possibility for considering miR27b as a surrogate biomarker for OLP activity, alone or in combination with other miRNAs.[30] Of note, our study failed to document a significant association between miR27b expression and patients' characteristics as age, gender, and disease duration, which may be due to our relatively small sample size and should be further investigated in future studies. Moreover, the potential of miR27b overexpression as a therapeutic strategy to reduce immune activity in OLP lesions should be considered.

In conclusion, our study demonstrated increased CD8 levels and downregulation of miR27b in OLP tissues, compared to healthy tissues. Therefore, miR27b expression may be a possible candidate for drug development. Moreover, it reveals the potential of miR27b as OLP disease biomarker. The possible negative correlation between CD8 and miR27b tissue expression requires further confirmation in larger studies.

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None.

CONFLICT OF INTERESTS

None to declare.

ETHICAL APPROVAL

Obtained from the Ethical Research Committee at Cairo University, Egypt.

INFORMED CONSENT

Obtained from all participants.

ORCID

Sana Maher Aghbari http://orcid.org/0000-0002-1600-9582

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