Evaluation of the miRNA-146a and miRNA-155 Expression Levels in Patients with Oral Lichen Planus

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ABSTRACT

Background: Oral Lichen Planus (OLP) is a chronic autoimmune disease that could be considered as a potential premalignant status. **Objective:** To evaluate the miRNA-146a and miRNA-155 expression levels in patients with oral Lichen planus lesions compared to healthy subjects with normal oral mucosa. **Methods:** Forty patients with oral lichen planus and 18 healthy age and gender-matched controls were recruited in this case-control study. Oral lichen planus was diagnosed clinically and pathologically. The expression levels of two miRNAs in peripheral blood samples were determined using commercial TaqMan MicroRNA Assays. Relative quantification of gene expression was calculated by the $2^{-\Delta\Delta ct}$ method. **Results:** The expression levels of miRNA-146a and miRNA-155 in patients with oral Lichen planus were significantly higher than the of the healthy control group. Also, a direct but insignificant correlation was found between miRNA-155 and miRNA-146a expression levels among the patient group. **Conclusion:** Our findings indicate that miRNA-146a and miRNA-155 could be potential biomarkers for the immunopathogenesis of oral lichen planus.

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Keywords: miRNA-146a, miRNA-155, Oral Lichen Planus

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INTRODUCTION

Lichen planus is a chronic inflammatory disease mediated by T lymphocytes that involves stratified squamous epithelial tissue (1-3). Although this autoimmune dermatosis normally affects the oral mucosa, it may involve the skin, nails, and genital mucosa (4,5). Oral Lichen Planus (OLP) is characterized by multiple clinical features including white striations, white plaques or papules, erythema, blisters, and erosions (2,6). Diagnosis of OLP may need histopathological examination. It is widely accepted that patients with OLP are predisposed to develop oral carcinoma; though the risk is low (6). The prevalence of OLP, which is more common in middle-aged women and is rarely seen in children (6,8,9), has been reported in the literature between 0.5 to 4% (1,2,4,7). Although the exact etiology of OLP is not completely understood, the important role of inflammatory factors (e.g. cytokines) has been highlighted besides some other causal factors including anxiety, diabetes, autoimmune diseases, drugs, stress, infections and genetic predisposition (8,10). Abnormal expression of cytokines as potential key players in the immunopathogenesis of OLP is characterized by the imbalance between Th1 and Th2 cytokines (11). Considering the microRNAs (miRNAs) engagement in the regulation of cytokines gene expression, unraveling the exact role of miRNA-mRNA network in the pathogenesis of OLP may have clinical importance (11). MicroRNAs are small conserved non-coding RNA molecules that regulate gene expression post-transcriptionally by targeting 3′-untranslated region (UTR) of specific messenger RNAs (mRNAs) for degradation or translational repression. Regulation of microRNA-mediated gene is critical for normal cellular functions such as cell cycle, differentiation, apoptosis, immunological functions, and autoimmune disease prevention (12-16). It has been suggested that both over- and under-expression of miRNAs may induce dysregulation of specific mRNAs, which may affect immune responses and lead to the pathologic conditions (17). To the best of our knowledge, only one study has demonstrated a relationship between miRNA-146a and miRNA-155 expression levels and OLP (4). To further explore and confirm this correlation in Iranian patients, as a different ethnic population compared to above study, we designed the present study to evaluate the expression levels of the miRNA-146a and miRNA-155 in a blood samples of OLP patients in comparison with healthy subjects with normal oral mucosa.

MATERIALS AND METHODS

Patients. This case-control study was conducted in School of Dentistry, Hamadan University of Medical Sciences between May 2013 and September 2014. Forty patients with confirmed disease according to diagnostic hallmarks of lichen planus including clinical symptoms and histopathological characteristics of OLP irrespective of sex and age were recruited. All types of OLP such as reticular, plaque-like, and erosive forms were examined in the study. Next, 9 out of 40 patients with therapeutic interventions were classified as a treated group and the remaining 31 ones were classified as new cases without medication (untreated group). Eighteen age- and gender-matched healthy subjects without any specific disease or medications were selected among persons who referred to the Department of Oral Medicine for routine dental examination as the controls.

Exclusion Criteria. The following patients excluded from the study: those with (a) systemic metabolic disease particularly diabetes mellitus and hepatitis C, (b) those with probably unilateral lesions that occurred due to drugs or contact reactions, (c) those with periodontal diseases, (d) cigarette smokers, (e) those with history of acute oral trauma, (f) history of bone marrow transplantation and (g) history of medications during the last months (at least 3 months), which may induce lichenoid reactions such as oral hypoglycemic agents, anti-hypertensive, and ACEs inhibitors. This case-control study was approved by our institutional ethics committee. Moreover, all patients who fulfilled the clinical characteristics and histopathological criteria and healthy control subjects gave written informed consents approved by the ethics committee of Hamadan University of Medical Sciences in accordance with the World Medical Association Declaration of Helsinki.

Blood Sample Collection and Total RNA Extraction. Two milliliters of peripheral blood was collected in EDTA containing tubes. Afterward, total RNA was extracted from whole blood using TRIZOL Reagent (Invitrogen, CA, USA), according to the manufacturer's protocol. The concentration of total RNA was determined by spectrophotometer and agarose gel electrophoresis was applied to assess the integrity of extracted RNA.

cDNA Synthesis and Quantification of miRNA Expression. Synthesis of cDNA was performed from 10 ng of total RNA using Taq-Man MicroRNA Reverse Transcription kit (Invitrogen, CA, USA) according to manufacturer's instructions. After that, quantification of miRNAs expression levels was carried out by applying individual TaqMan MicroRNA Assays (TaqMan MicroRNA Assay, Applied Biosystems, USA) for miRNA-146a and miRNA-155 in an Exicycler real-time PCR 96-well optical plate (Bioneer, Korea). Each assay was performed in technical duplicates. The expression levels of miRNA-155 and miRNA-146a were normalized to RNU48 as an internal control. The average threshold cycle (Ct) for two replicates per sample was used to calculate ΔCt. Relative quantification of gene expression was calculated by the $2^{-\Delta\Delta ct}$ method.

Statistical Analysis. The mean values of quantitative variables between groups were compared using an unpaired t-test for data distributed normally and Mann-Whitney test for non-normal data. Also, correlation analyses were carried out to determine the relationship between pairs of variables using Pearson's and Spearman's rank correlations. The P values ≤ 0.05 were considered as significant difference. The ANOVA or Kruskal-Wallis test was used to compare means among two or more groups. Data analyses were performed using the SPSS Version 16.0 for windows and GraphPad Prism 5 (GraphPad Software, San Diego, CA) software packages.

RESULTS

The expression levels of miRNA-146a and miRNA-155 in patients with lichen planus were significantly higher than that of healthy individuals (Fig. 1). Also, treated OLP patients demonstrated significantly up-regulated miRNA-146a and miRNA-155 expressions compared to the control group (Fig. 2). The expression levels of miRNA-155 and miRNA-146a did not differ significantly between treated and untreated patients (Fig. 3). Pearson's correlation analysis depicted a direct correlation between miRNA-

146a and miRNA-155 expression levels among patients compared to healthy controls but, it was not statistically significant (P=0.32).

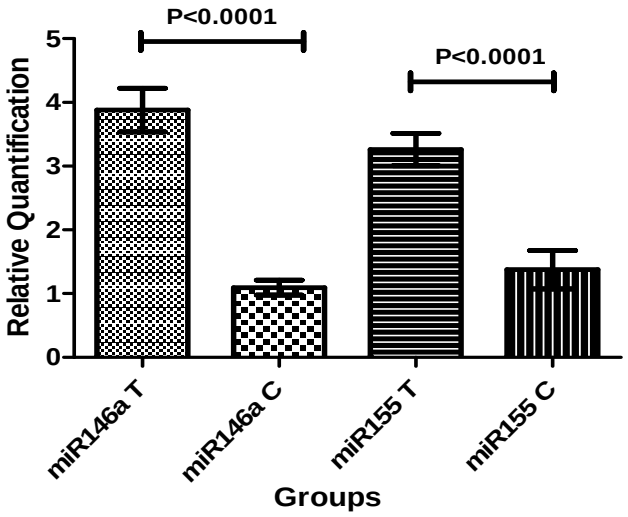

Figure 1. Comparison of the expression level of miRNA-146a and miRNA-155 between patients (n=40) and healthy controls (n=18). T: Patients, C: Controls.

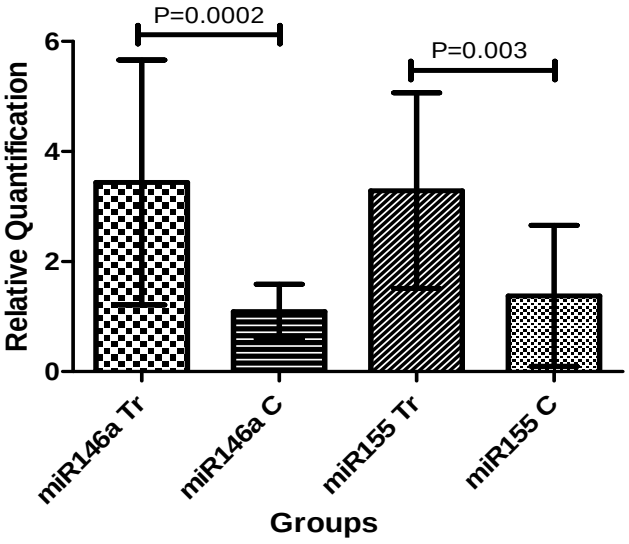

Figure 2. Comparison of the expression level of miRNA-146a and miRNA-155 between patients with a history of previous treatment (n=9) and healthy controls (n=18). Tr: Treated patients, C: controls.

Analysis of demographic data for the study subjects showed that differences in the expression levels of two miRNAs according to demographics including gender, age, body mass index, treated or untreated, types of lichen planus (reticular, erosive, popular, plaque type, erosive and reticular, and bullous), family history, and smoking were not

statistically significant. The only significant difference was found in increased levels of miRNA-146a expression in patients with cutaneous involvement compared to those without skin pathology (P=0.05, Table 1).

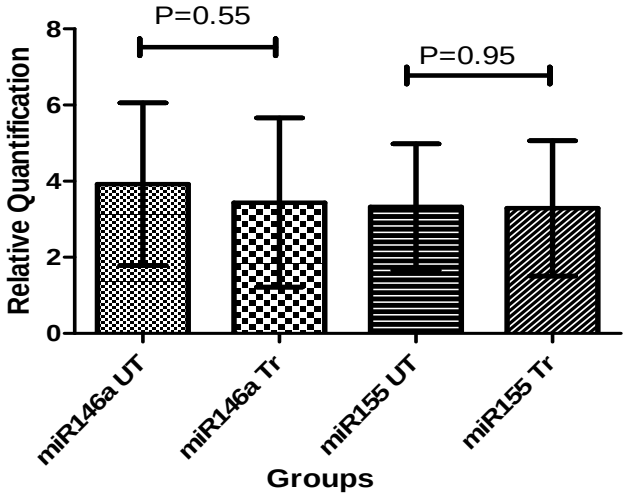

Figure 3. Expression state of miRNA-146a and miRNA-155 in patients with a history of treatment (n=9) in comparison with untreated patients or new cases (n=31). UT: Untreated patients, Tr: Treated patients.

DISCUSSION

Oral Lichen Planus (OLP) is an autoimmune, resistant to medical therapy and sometimes severe and refractory premalignant disease with unknown exact etiology (2,3,18). Several studies have indicated that simultaneous evaluation of both mRNA and miRNA profiles could be a powerful tool to clarify the immunopathogenesis of autoimmune diseases and subsequently defining proper therapeutic interventions (19-21). In this context, various expression patterns of miRNAs including miR-146a, miR-155, miR-146b-5p, miR-27b, miR-21, miR-320a, etc. in blood or tissue and saliva samples from OLP patients have been documented (11,19,22).

Our results showed that the expression levels of miRNA-146a and miRNA-155 in OLP patients were significantly higher than those of healthy controls. Also, a higher level of miRNA-146a expression was observed in patients with cutaneous involvement compared to those without skin pathology. Additionally, there was a direct but insignificant correlation between miRNA-146a and miRNA-155 expression levels among patients group. In this regard, miRNAs have been shown to play an important role in development and progression of diseases such as cancer and certain autoimmune diseases (23,24). Given that miRNAs expression patterns have been evaluated locally (e.g., in biopsy specimens) and in sera, body fluids, and blood samples, the results from sera samples were comparable with tissue samples of the patients. Hence, applying less invasive and preferentially non-invasive samples for evaluation of clinically relevant molecular biomarkers could be very crucial (18).

Table 1. Comparison of miRNA expression levels according to demographic and clinical features.

	*miRNA 146a				* miRNA 155			
Variables	**Number**	**Mean**	**SD**	**P values**	**Number**	**Mean**	**SD**	**P values**
Gender								
Female	28	3.69	2.11		28	3.29	1.63	
Male	12	4.37	2.32	0.35	12	3.18	2.14	0.84
Side of involvement								
Unilateral	6	3.55	2.46		6	2.92	1.30	
Bilateral	34	3.94	2.15	0.69	34	3.32	1.67	0.57
Age								
<40 yrs	18	3.77	2.11		18	3.67	2.13	
>40 yrs	22	3.97	2.26	0.78	22	3.90	2.30	0.76
Body Mass Index								
Normal	15	4.39	2.30		15	3.29	1.60	
Over weight	18	3.91	2.20		18	3.21	1.28	
Obesity	7	2.69	1.52	0.23	7	3.32	2.51	0.98
Situation of treatment								
Untreated	31	3.96	2.16		32	3.22	1.58	
Treated	5	4.69	2.96		4	3.31	0.92	
Treating	4	2.40	0.82	0.30	4	3.53	2.68	0.75
Type of lichen planus								
Reticular	13	4.18	2.35		13	3.15	1.26	
Erosive	5	2.6	1.10		5	3.45	2.80	
Papullar	2	2.97	3.05		2	4.88	2.32	
Plaque type	2	4.93	3.36		2	4.11	0.94	
Erosive& reticular	18	4.00	2.15		18	3.00	1.46	
Bolluse	0	0	0	0.60	0	0	0	0.55
Site of involvement								
Buccal mucosa	23	3.81	2.26		23	3.29	1.35	
Gingiva	4	2.56	0.67		4	3.14	2.23	
Tongue	6	4.18	2.49		6	3.51	1.89	
Floor of the mouth	7	4.58	2.13	0.52	7	2.99	2.12	0.95
Cutaneous involvement								
Yes	5	5.64	1.69		5	2.90	1.43	
No	35	3.63	2.13	0.05	35	3.31	1.64	0.60
Other mucosal involvement								
Yes	2	2.45	0.64		2	2.72	2.60	
No	38	3.95	2.20	0.35	38	3.28	1.59	0.63
History of follow up								
1yr	7	4.80	2.54		7	2.80	0.84	
1.5yrs	0				0	0	0	
2yrs	7	3.47	2.38		7	3.85	1.50	
2.5yrs	3	2.70	2.07		3	4.54	2.69	
3yrs	2	5.33	4.17		2	3.21	058	
>3yrs	21	3.74	1.86	0.51	21	3.04	1.71	0.45
Family history								
Yes	2	3.67	2.98		2	4.50	3.61	
No	38	3.89	2.17	0.89	38	3.19	1.52	0.27
Smoking								
Yes	5	4.31	2.87		5	3.62	1.61	
No	35	3.82	2.10	0.64	35	3.21	1.52	0.60
Relation with food								
Yes	2	3.67	2.97		15	2.94	1.40	
No	38	3.89	2.17	0.89	25	3.44	1.73	0.35

* Fold changes of miRNAs expression levels in comparison with healthy controls.

Understanding the etiopathogenesis of OLP is one of the major challenges in oral pathology. The complex network of cytokines in initiation and exacerbation of oral lesions in OLP results from the imbalance between Th1 and Th2 immune responses, which are the hallmark of autoimmunity (11,19). MicroRNAs are known as one of the important regulators of gene expression and this epigenetic mechanism is more remarkable for the genes involved in the induction and regulation of the immune responses. The expression pattern of miRNA-146a and miRNA-155 and their potential target transcripts can affect the differentiation of CD4+ T cells towards Th1 or Th2 responses (4,15). In this context, some studies have shown upregulation of miRNA-146a and miRNA-155 expressions in tissue samples of OLP patients compared to healthy controls (4). Moreover, the altered expression levels of miRNA-146a and miRNA-155 have been reported in other chronic inflammatory situations such as periodontal diseases, rheumatoid arthritis, and Sjogren's syndrome (12,23,25,26). In line with these findings, we observed an increased level of miRNA-146a and miRNA-155 expressions in peripheral blood samples of OLP patients.

Rodriguez *et al.* showed that the downregulation of miRNA-155 in mouse dendritic cells results in impaired T-cell response to the antigen (27). In addition, it has been suggested that miRNA-155 is involved in differentiation of CD4+ T cells toward Th1 through IFN-γ signaling (28). Alternatively, miRNA-146a mediates the downregulation of signal transducers and activators of transcription 1 (STAT1), a transcription factor needed for differentiation of Th1 cells (29). Based on these observations, our findings may indicate that overexpression of miRNA-146a and miRNA-155 in OLP may contribute to Th1 differentiation in response to an unknown autoantigen. It seems that miRNA-146a and miRNA-155 play a role in favor of increased Th1 response in OLP; however, the exact role of miRNA-146a and miRNA-155 in the immunopathogenesis of OLP has not been determined yet. Induction of these miRNAs expression through unknown mechanisms may lead to the imbalance of Th1/Th2 cytokines toward Th1 immunity (IFN-γ production) that, in turn, stimulates or reinforces the local immune responses against an antigen in favor of disease progression. Moreover, a higher expression of miRNA-155 is related to inflammatory cytokines in human retinal-pigmented epithelial cells through JAK/STAT signaling pathways, which may be involved in the pathogenesis of OLP (4). The lack of differences in miRNA expression levels between treated and untreated groups of the patients as well as the higher expression of both miRNAs in treated patients versus healthy controls could be an indicative for contribution of other genetic and non-genetic factors (e.g. environmental factors) involved in the regulation of gene expression that was not evaluated in the current study. Additionally, recruiting a low number of patients, performing a single but not serial assessment of both miRNAs and their target mRNAs could be the plausible explanations for these results and definitely needs to be investigated by further studies and preferentially by examining a panel of relevant miRNAs. In conclusion, up-regulation of miRNA-146a and miRNA-155 in OLP as an inflammatory disease may indicate their potential role in the development of the disease or even in the progression of OLP lesions to malignancy. However, our findings should be interpreted cautiously due to the lack of target transcripts analysis in the present study. Nonetheless, characteristics of miRNAs such as high stability, changes in the early stage of diseases, presence in body fluids, and their high specificity make these molecules as potential biomarkers in clinical applications particularly immunotherapeutic interventions for

autoimmune diseases (30,31). Finally, further studies are required to explore the exact role of miRNAs in the immunopathogenesis of OLP.

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