

ASSESSMENT OF miR-155 IN SALIVA OF ORAL LICHEN PLANUS PATIENTS AS A POTENTIAL BIOMARKER OF MALIGNANT TRANSFORMATION BEFORE AND AFTER TOPICAL CORTICOSTEROID THERAPY

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ABSTRACT

Background: Immune dysregulation is undoubtedly implicated in the pathogenesis of oral lichen planus (OLP) and considering the importance of miR-155 in the regulation of the immune response the present study is conducted to assess salivary levels of miR-155 in patients with oral lichen planus lesions and compare these levels before and after corticosteroid therapy to explore its possible implication in the pathogenesis OLP.

Methods: Thirty patients were enrolled in this study, 15 healthy individuals with normal mucosa as controls and 15 patients diagnosed with atrophic/erosive OLP lesions. Patients were treated with topical corticosteroid. Salivary levels of miR-155 were assessed before and after 4 weeks of topical steroids therapy. Clinical improvement was assessed via VAS score and Thongprasom scale.

Results: A significant reduction of miR-155 levels four weeks post treatment was recorded with a value of 55.67%. There was a highly significant direct correlation between miR-155 levels and VAS score.

Conclusion: The overexpression of miR-155 in OLP patients before treatment with its obvious reduction four weeks after therapy suggests it could be directly involved in OLP pathogenesis and shedding a light on the potential of miR-155 to act as an innovative therapeutic target for OLP.

KEY WORDS: miR-155, oral lichen planus, pathogenesis.

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INTRODUCTION

Oral lichen planus (OLP) is an immune mediated disorder that involves the oral mucosal tissue with a diversity of clinical appearances, such as reticular, atrophic, erosive and ulcerative forms¹, affecting 1–2% of the population mainly females². It has the potential for malignant transformation; where 1–2% of OLP lesions have been reported to turn into oral squamous cell carcinoma³. Though there is no precise understanding of the pathogenesis or etiology of OLP up till now, it has been hypothesized that OLP is a T cell-induced autoimmune disorder, characterized by an exacerbated Th1 and cytotoxic T- lymphocytes (CTLs) responses¹. Several cytokines work as mediators between inflammatory cells and keratinocytes playing a vital role in the immune mediated destruction of keratinocytes occurring in OLP⁴.

Several researches have proposed that microRNAs (miRNAs) could also be implicated in the pathogenesis of OLP¹. miRNAs are short (20- to 22-nucleotide), single-stranded noncoding RNAs that regulate gene expression. The effects of miRNAs in different biologic processes, as proliferation, apoptosis, differentiation, and oncogenesis are under thorough investigation⁵. miRNAs have been connected to immune mediated and inflammatory disorders via deregulating the expression of target mRNA⁶.

There is a positive correlation between the release of inflammatory cytokines and miR-155 expression. Moreover, its expression is augmented in several types of cancer and it primarily functions as a tumor-promoting factor⁷. Furthermore, miR-155 is upregulated in activated immune cells indicating that it has a significant impact on the immune response. Thus, the disturbed miR-155 expression adversely affects the immune responses leading to development of various diseases⁸.

In a microarray analysis of miRNAs in mucosal tissues of OLP patients, significant dysregulation was demonstrated in about 70 miRNAs¹. Where an increase in miR-155, miR-21, miR-146a, and miR-31 levels was recorded⁹. The association of

miRNAs with OLP has been previously reviewed, and a close association was revealed between miR-155 and cytokines linked with OLP, making it a perfect research nominee in OLP¹⁰. A positive relation was recognized between IFN- γ and miR-155, resulting in a Th1-mediated immune response in OLP³. Moreover, it has been proposed that miR-155 is involved in T cells differentiation toward Th1 via IFN- γ signaling. Nevertheless, the specific role of miR-155 in OLP pathogenesis is up till now largely obscure³.

Considering the importance of miR-155 in the regulation of the immune response and the strong implication of immune dysregulation in the pathogenesis of OLP, the present study is conducted to assess salivary levels of miR-155 in patients with OLP lesions and compare these levels before and after corticosteroid therapy to explore its possible implication in the pathogenesis OLP.

SUBJECTS AND METHODS

Thirty patients were recruited from the outpatient clinic of Oral Medicine Department and the Diagnostic center, Faculty of Dentistry - Cairo University. Each patient was educated about the study details and a signed consent form was obtained from each patient.

Eligibility Criteria

Inclusion Criteria

Patients clinically and histopathologically (if required) diagnosed as suffering from OLP. Patients free from any visible oral lesions other than OLP. Patients who agreed to take supplied medications. Patients who agreed for the biopsy (when required).

Exclusion Criteria

Patients suffering from any systemic disease. Treatment with any systemic drugs such as systemic corticosteroids, other immunosuppressive drugs or NSAID at least eight weeks. Treatment with any oral topical medicines for at least four weeks prior to the study. Pregnant and lactating women.

Interventions

Intervention group: composed of 15 patients diagnosed with atrophic/erosive OLP lesions. Patients were treated with topical corticosteroid (Kenacort A Orabase: triamcinolone acetonide 0.1% 5 grams adhesive paste – Dermapharm) four times daily, and miR-155 was assessed in saliva before and after 4 weeks of topical steroids

Application

Patients were instructed to apply the topical corticosteroid regularly four times a day after food and before sleep for 4 weeks. They were instructed to apply the drug by finger rubbing on dried lesions and to refrain from food and liquid for 30 minutes after the application.

Control group: composed of 15 healthy individuals with normal mucosa, salivary levels of miR-155 were assessed.

Outcomes

Primary outcome: Salivary level of miR-155 using qRT-PCR.

Salivary miR-155 assay

Total RNA with conserved miRNAs was extracted from 200 μ L saliva by miRNA easy extraction kit (Qiagen, Valencia, CA, USA). Reverse transcription was done on 5ng of total RNA in 20 μ L RT reactions (incubated for 60 min at 37°C, 5min at 95°C, and then kept at 4°C) utilizing the miRNA easy Reverse Transcription Kit (Qiagen, Valencia, CA, USA) following the instructed protocol. Expression of miR-155 was appraised by qRT-PCR analysis following the guidelines. The housekeeping miRNA SNORD 68 was utilized as the endogenous control. For real time PCR, 5 μ L of diluted RT products (cDNA template) was mixed with SYBER Green Master Mix (Qiagen, Valencia, CA, USA) in a final volume of 25 μ L and added to a custom 96 well plate miScript miRNA PCR arrays which is enhanced with miRNA forward and reverse- miRNA-specific primer. The plate was sealed with optical thin wall 8-cap strips. Real-time

PCR reactions were achieved utilizing an Applied Biosystems 7500 Real Time PCR System (Foster city, CA, USA) with these circumstances: 95°C for 15 min, then 40 cycles at 94°C for 15s, and 55°C for 30s and 70°C for 34s. The cycle threshold (CT) is the number of cycles needed for the fluorescent signal to cross the threshold in real-time PCR. miRNAs expression was described as Δ Ct value which is reached by subtracting the CT values of miRNA SNORD68 from the CT values of the target miRNA. As there is an inverse correlation between miRNA expression level and Δ Ct, increased miRNA were associated with lower Δ Ct values. The resulting normalized Δ Ct values were utilizing in calculating relative expression values by using $2^{-\Delta(\text{Ct})}$, which are directly correlated to the miRNA expression levels. The $2^{-\Delta\Delta(\text{Ct})}$ technique was utilized to assess relative-quantitative levels of individual miRNAs.

Secondary outcome

Pain; measured by VAS

Visual Analog Scale (VAS) was utilized for pain appraisal at baseline and week 4 after treatment, it is denoted by a 10cm horizontal line, where the left endpoint indicating no symptoms and the right endpoint indicating the worst thinkable symptoms. Intensity of symptoms is indicated by the distance from the left endpoint to the mark the patient made measured in millimeters¹¹.

Clinical improvement; measured by Thongprasom scale

Clinical examination and assessment of the atrophic and erosive lesions were carried out and the severity and the number of sites involved were quantified at base line and four weeks after treatment based on the scale used by Thongprasom et al¹². Where 0 indicates normal mucosa; 1 indicates mild white striae, no erythematous area; 2 indicates white striae with atrophic area < 1 cm²; 3 indicates atrophic area > 1 cm²; 4 indicates White striae with erosive area < 1 cm²; 5 indicates erosive area > 1 cm².

Statistical analysis:

All Data were collected, tabulated and exposed to statistical analysis. Statistical analysis is performed by SPSS in general (version 16), while Microsoft office Excel is utilized for data handling and graphical presentation.

Measured variables are described by the Mean, Standard Deviation (SD), the Range (Minimum – Maximum), Standard Error (SE) and 95% confidence interval of the mean.

Shapiro-Wilk test of normality is used to test normality hypothesis of all quantitative variables for further choice of appropriate parametric and non-parametric tests. Almost all the variables are found not normally distributed leading to the use of nonparametric tests. Kruskal Wallis test followed by Mann and Whitney U test was used for comparison with control while Wilcoxon signed rank test is used for comparing pre and post measurements for the same group. Correlation is performed using Pearson correlation coefficient.

Significance level is set at $P < 0.05$ (S); and $P < 0.01$ is highly significant (HS). Two Tailed tests were performed for all statistical tests.

Sample size calculation:

This power analysis used miR1155 fold change after treatment as the primary outcome. The effect

size (d_z) = 0.926 was calculated based upon a pilot study conducted on 3 LP patients. Using alpha (α) level of (5%) and Beta (β) level of (20%) i.e. power = 80%; the minimum estimated sample size was 12 subjects per group. Sample size was increased to 15 subjects per group to compensate for a drop-out rate of 25%. Sample size calculation was performed using G*Power Version 3.1.9.2.

RESULTS

The present study was conducted to assess salivary levels of miR-155 in patients with OLP lesions and to compare their levels at baseline and four weeks after corticosteroid therapy.

A total of 30 individuals participated in the study divided as follow: Intervention group: composed of 15 patients diagnosed with OLP lesions (6 males and 9 females) with mean age of 40.4 years, control group composed of 15 healthy individuals healthy (6 male and 9 females) with mean age of 38.47. Both gender and age ranges were closely matching in OLP and control groups. Descriptive data of miR-155 levels in the study groups are demonstrated in Table (1)

Kruskal Wallis test statistics revealed that the means of the three variables OLP group (baseline, post treatment) and control group are statistically highly significantly different for miR-155 ($P < 0.001$). All mean values are represented in fig (1).

TABLE (1) Descriptive Statistics of miR-155 in the study group and healthy controls.

Level of	Mean	SD	SEM	95% Confidence Interval for Mean		Min	Max	Median	IQ Interquartile range	
				Lower Bound	Upper Bound					
miR155 Fold change	Baseline	4.28	1.23	0.32	3.60	4.96	2.17	5.68	4.65	152.00
	After	1.90	0.42	0.11	1.66	2.13	1.03	2.36	1.99	0.54
	Healthy control	1.07	0.08	0.02	1.03	1.12	1.00	1.25	1.05	0.09

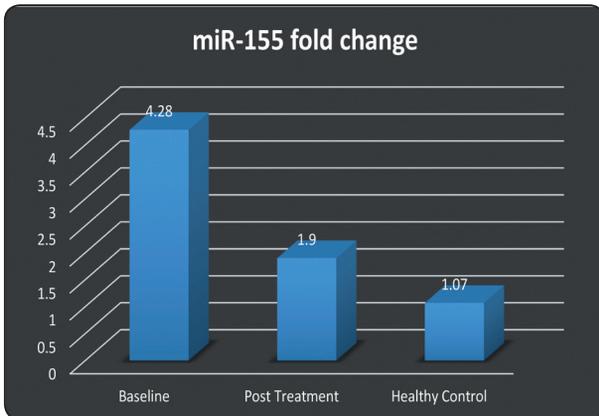


Fig. (1) Mean values of miR-155 in the study group and healthy controls.

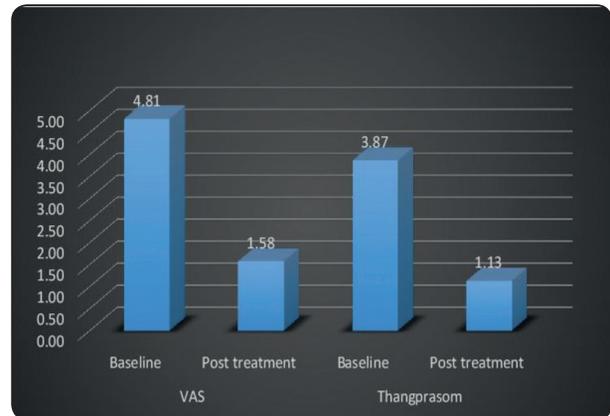


Fig. (2) Mean values of VAS score & Thongprasom at baseline and post treatment.

Comparisons with control using Mann and whitney U test revealed a highly statistically significant difference between mean levels of miR-155 at baseline and control and miR-155 post treatment and control ($P < 0.001$).

Wilcoxon Signed Ranks test was used for comparing mean levels of miR-155 at baseline with their mean levels four weeks post treatment and it revealed a highly statistically significant difference ($P < 0.001$) with a percent change of -55.67%.

Analysis of VAS & Thongprasom scores:

The mean values of VAS score & Thongprasom at baseline and post treatment are represented in fig (2). Wilcoxon Signed Ranks test was used for comparing VAS and Thongprasom scores at baseline and four weeks post treatment and the comparison showed a highly statistically significant difference ($P < 0.001$) for both with a percent change of -67.17% for VAS score and a percent change of -70.69% for Thongprasom score.

Correlations:

There was a highly significant direct correlation between miR-155 levels and VAS score (Table: 2).

TABLE (2) Correlation Coefficients between baseline and post treatment values for all variables

		r	P Value
miR155	VAS	0.86	0.00005**
miR155	Thongprasom	0.36	0.18406
VAS	Thongprasom	0.27	0.33577

*: Significant at $P < 0.05$, **Highly significant at $P < 0.001$

DISCUSSION

The immune system has several regulatory factors acting in harmony to achieve the vital balance between up-regulation and down-regulation, which facilitates effective self-defense against any challenge. Dysregulation of these factors predispose to many immune mediated diseases including OLP¹ where it is characterized by an exacerbated Th1 response that expresses TNF- α , IL-2, and IFN- γ , all of which are powerful mediators in the stimulation of CTLs and hence can induce local immune reactions².

miR-155 has an augmenting effect on many paths involved in cytokine production and immune activation, and it is important for T cells differentiation into Th1 cells¹³. Thus we conducted this research to explore the relation of miR-155 and

its possible implication in the pathogenesis of OLP through assessing its salivary levels in patients with OLP lesions and evaluating the effect of treatment of such lesions on its levels at baseline and four weeks after corticosteroid therapy which remains the most consistently and frequently used treatments for OLP.

Results of the current study discovered that mean levels of miR-155 levels were statistically significantly higher in OLP patients at baseline than post treatment levels that were also significantly higher than control group miR-155 levels. Hence, corticosteroid therapy caused a significant reduction of miR-155 levels four weeks post treatment with a 55.67% reduction in mean miR-155 levels.

The significantly elevated miR155 levels encountered in our study in OLP patients before starting corticosteroid therapy in comparison with controls were in line with former studies recording an upregulated expression of miR-155 in OLP patients¹. A different study reported an overexpression of miR-155 in peripheral blood monocytes in OLP lesions compared with controls and revealed a close association of its expression with the disease severity³. Another study surveyed the erosive type of OLP and identified a positive miR-155-IFN- γ interaction, further enhancing the Th1 mediated immune response in OLP¹⁴.

This upregulated miR-155 expression in OLP patients was further confirmed in another study that also reported that even treated OLP patients still demonstrated significantly up-regulated miR-155 expressions compared to the control group¹⁵ which was also in accordance with our results where miR-155 post treatment levels were still significantly higher than control group such findings may indicate that overexpression of miR-155 in OLP might enhance Th1 differentiation in response to an unknown auto antigen and could be directly involved in OLP pathogenesis¹⁵.

miR-155 is involved in controlling many immunologic pathways via targeting hundreds of genes in various cell types¹⁶. Overexpression of

miR-155 is known to increase Th1 differentiation which is vital for the initiation, preservation, and exacerbation of chronic inflammatory reactions possibly via producing IFN- γ . Elevated levels of IFN- γ attracts and stimulates diverse immune cells, thus enticing more Th1 cells to inflammatory site. Consequently, the Th1 response will be preserved and exaggerated, leading to chronic inflammation¹⁷.

Regarding the clinical results of the present study a highly significant improvement was recorded in VAS and Thongprasom scores after the therapy period reaching a 67.17% reduction of VAS score with which a highly significant direct correlation was found with miR-155 levels reflecting a direct correlation of the miR-155 levels with pain in OLP patients perceptibly because overexpression of miR-155 is related to excessive production of proinflammatory cytokines¹⁰. Additionally, a 70.69% reduction of Thongprasom score were evident four weeks after corticosteroid therapy.

Remarkably miR-155 was the first miRNA shown to be oncogenic as it has been reported to enhance cell proliferation, and impede apoptosis¹⁸. The miR-155 oncogenicity was certainly recognized in transgenic mouse models that revealed that the expression of miR-155 alone is enough to trigger the malignant transformation¹⁹. miR-155 levels are significantly upregulated in oral cancer tissue and often correlates with poor prognosis²⁰. The central oncogenic properties of elevated miR-155 levels appear to rise from its targeting of anti-inflammatory signal transduction or key oncogenic suppressors pathways²¹, for instance miR-155 acts as a negative regulator of the tumor suppressor p53 pathway which regulates differentiation and cell growth, a finding of potential significance to miR-155-associated malignancies²². Moreover, down regulation of miR-155 was found to restrain cell proliferation and cell cycle in oral cancer, potentially providing a novel molecular target for early oral cancer detection and management²³.

In light of that and considering the potentially malignant nature of OLP, we postulate that targeting miR-155 could provide a novel therapeutic strategy for OLP which not only improves clinical signs of the disease and reduces its severity and progression but more importantly could reduce the probability of malignant transformation of such lesions

CONCLUSION

There was an overexpression of miR-155 with its obvious reduction four weeks after therapy and this was associated with a significant improvement in clinical signs and symptoms proposing it could be directly involved in OLP pathogenesis. The present study offers a base for future research that is required for implication of miR-155 in the pathogenesis and management of OLP.

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Conflict of Interest: All authors affirm that they have no conflict of interest related to this study.

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