Expression levels of microRNA-21 and microRNA-146a in patients with Oral Lichen Planus

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Abstract: Increasing evidence indicates that microRNAs (miRNAs) play a critical role in the pathogenesis of inflammatory diseases. Oral lichen planus (OLP) is a chronic inflammatory disease involving the oral mucosal tissues. The role of miRNAs in the pathogenesis of OLP has not been investigated. Therefore, the aim of this study was to investigate the expression levels of miRNA-21 and miRNA-146a in oral tissue samples from patients with OLP and matched healthy controls using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Our results showed a significant over-expression of miRNA-21(3.2-fold) and miRNA-146a (5.6-fold) in OLP patients compared to healthy controls. These results indicate that miRNAs may be the novel candidate biomarkers for the implication of miRNAs in the pathogenesis of OLP.
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Introduction

Oral lichen planus (OLP) is a chronic, T-cell mediated inflammatory oral mucosal disease of unknown etiology [1, 2]. The OLP lesions may coexist with cutaneous and genital lesions, or may be the only disease manifestations [3]. The epidemiology of OLP is not easy to calculate with reported incidence ranging between 1-2% of the general population. Recent meta-analysis calculated a 1.27% incidence in the general population [4]. The World Health Organization (WHO) has classified OLP as a "potentially malignant disorder" with a 0.4-5.6% transformation rate into oral squamous cell carcinoma [5, 6]. Recently, the etiology and pathogenesis of OLP has been the focus of much research, and several antigen-specific and non-specific inflammatory mechanisms have been put forward to explain the disease pathogenesis.

Micro-RNAs (miRNAs) are small, single stranded non-coding RNAs that regulate messenger RNAs (mRNAs) expression at the post-transcriptional level, thus targeting them for degradation or translational repression [7, 8]. To date, thousands of miRNAs have been identified in mammalian genomes, and up to 30% of human genes are regulated by these miRNAs [9]. More than 100 different miRNAs are expressed by cells of the immune system; they have the potential to broadly influence the molecular pathways that control the development and function of innate and adaptive immune responses [8]. Recently, miRNAs have been recognized as a novel player in normal immune function and inflammation [10]. Further-more, it has been suggested that over- or under-expression of miRNAs may induce deregulation of specific mRNAs. These factors may affect human immune response and then result in many pathogenic disorders [11]. In particular, T cell-mediated immune responses are associated with changes in the expression of specific miRNAs. CD4+ T-cells have also been found to express different miRNA subsets that are linked to cell differentiation, maturation, activation and function [12-15]. In the immune system, miRNA-21 is one of the most abundant miRNAs in T-cells indicating that its function is critical for T-cell homeostasis [16]. The expression of miRNA-21 dynamically changes during antigen-induced T-cell differentiation, with high expression in effector T cells in comparison to naive T-cells and memory T-cells [16, 17]. The involvement of miRNA-21 in inflammatory responses is particularly interesting [18, 19]. Over-expression of miRNA-21 was seen in atopic eczema and in psoriasis, the two most common types of chronic skin inflammation [18].

Similarly, miRNA-146a was one of the first miRNAs identified to be involved in the regulation of immune functions and inflammatory responses [17, 20]. Recently, altered miRNA-146a expression has been shown in several inflammatory diseases, including psoriasis [18], rheumatoid arthritis [21, 22], osteoarthritis [23], as well as systemic lupus erythematosus (SLE) [24]. As miRNAs have been shown to play important roles in inflammatory and autoimmune diseases, together with the fact that the pathogenesis of OLP is not fully understood, the aim of the present study was to investigate the expression levels of miRNA-21 and miRNA-146a in OLP lesions and matched healthy controls.

2. Patients and Methods

The present study was conducted on 20 patients, thirteen females and seven males, with a mean age of 48 years (age range: 43-59 years) suffering from OLP. Among the OLP patients, four patients had reticular lesions only, while the remaining sixteen had also erosive and/or atrophic types of OLP. They were diagnosed clinically and confirmed by histopathological examination according to the WHO's clinico-pathological diagnostic criteria for OLP [25]. The control group comprised15 healthy controls, twelve females and three males, with a mean age of 47 years (age range: 42-58 years). The controls were healthy volunteers without any systemic disease or inflammatory oral lesions. They were treated for third molar extraction, and they agreed to donate a sample of healthy oral mucosa.

All patients and healthy controls were selected from the Outpatient Clinic, Department of Oral Medicine and Periodontology, Faculty of Oral and Dental Medicine, Cairo University. A full medical history of each subject was obtained according to the detailed questionnaire of the modified Cornell Medical Index [26]. All subjects were free from any systemic disease and did not receive any topical or systemic medication during the last 3 months before the sample collection. Moreover, patients with suspected restoration-related reaction or gingival inflammation were excluded from the study. All patients and volunteers enrolled in this study gave their informed consent.

Oral mucosa samples from OLP patients were obtained after administration of ring block anesthesia and a surgical double wedge incisional biopsy [27]. Each biopsy specimen was divided into two parts; one part was formalin fixed and paraffin embedded for histopathological examination to confirm the diagnosis of OLP and the other part was kept frozen and stored at -80°Cuntil assayed for RNA extraction procedures.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of miRNA-21 and miRNA-146a

Total RNA was extracted from tissue samples using TRIzol® Reagent (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA), according to the manufacturer's protocol. TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used for determination of the expression levels of miRNA-21 and miRNA-146a. Briefly, 10 ng of total RNA was reverse-transcribed using gene-specific stem-loop primers to each miRNA [28] (TaqMan MicroRNA Reverse Transcription kit, Applied Biosystems), according to the manufacturer's instructions. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miRNA-21 and miRNA-146a specific TaqMan primers and TaqMan Universal PCR Master Mix in a 7500 Sequence Detection System real-time PCR machine (Applied Biosystems). PCR cycles were started at 95°C for 10 min, followed by40cycles of 95°C for 10 sec and 60°C for 1min.miRNA-21 and miRNA-146a expression was normalized to endogenous RNU6B (TaqMan Micro RNAassay, Applied Biosystems) as internal control.

Dissociation curve analysis was performed at the end of 40 cycles to verify the identity of the PCR product. The $2^{-\Delta\Delta Ct}$ method [29] was used to calculate relative changes in the gene expression determined from real-time quantitative PCR experiments. The value of each control sample was set at 1 and was used to calculate the fold change in the target miRNAs.

Statistical analysis

Data were presented as mean and standard deviation (SD) values. The differential expression levels of miRNA-21 and miRNA-146a studied in OLP lesions and control biopsy samples were analyzed by independent two-tailed Student's t-test. A p value <0.05 was considered statistically significant. All tests were performed using the GraphPad statistical software (GraphPad Software Inc, La Jolla, CA).

Results

Twenty OLP patients and fifteen healthy controls were included in this study. A total of thirty five oral tissue biopsy samples were analyzed for the expression profiles of miRNA-21 and miRNA-146a by qRT-PCR. Relative quantification of miRNA-21 and miRNA-146a in OLP lesions was higher than in normal oral mucosa in all the studied samples ($P < 0.0001$). Our results revealed that there was a statistically significant 3.2-fold increase in the expression level of miRNA-21 in OLP compared to healthy controls ($P < 0.0001$). Moreover, there was a statistically significant increase in the expression level of miRNA-146a in OLP at 5.6-fold ($P < 0.0001$) above healthy controls (table 1 and figure 1).

Table 1: Changes in miRNA-21 and miRNA-146a expression levels in OLP compared to healthy controls.

miRNAs	Reference sequences	Fold change*(mean± SD)	P value**
miRNA-21	NR_029493	3.2±1.09	P < 0.0001**
miRNA-146a	NR_029701	5.6±1.12	P < 0.0001**

*The values represent the mean fold changes of miRNA-21 and miRNA-146a in OLP compared to healthy controls. **Data were analyzed by independent two-tailed Student's t-test, P < 0.01 was considered highly statistically significant.

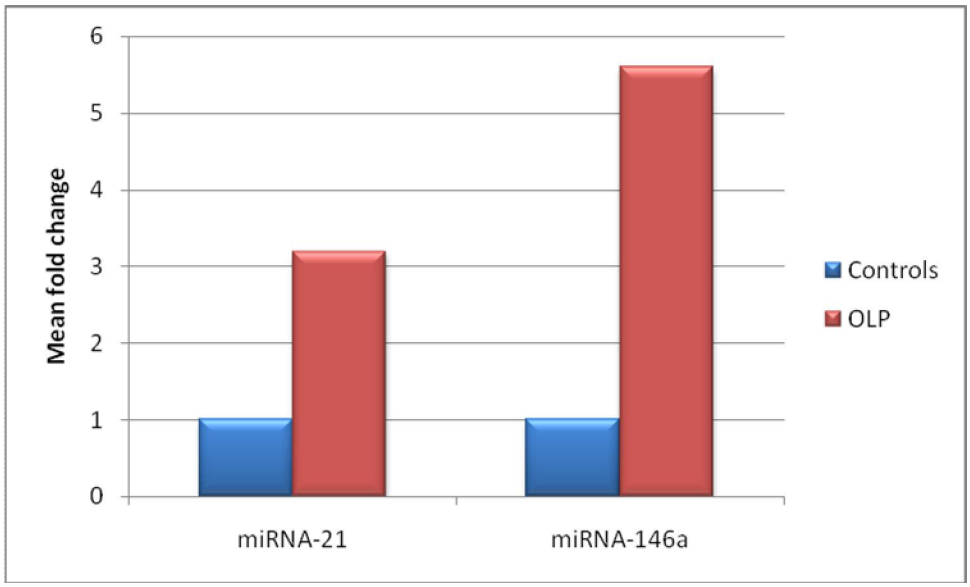

Figure 1: Bar chart representing the mean fold changes in miRNA-21 and miRNA-146a in OLP and healthy controls.

Discussion

In the last decade, many studies of the literature have focused on the role of miRNAs in the immune system. As a matter of fact, miRNAs are involved in T-cell selection, T-cell receptor sensitivity and regulatory T-cell development, suggesting that variants in miRNA gene expressions and their targets may play crucial role in the development of inflammatory and autoimmune diseases [18, 30, 31]. OLP is a T-cell mediated chronic inflammatory oral mucosal disease of unknown etiology [1]. Understanding the etiology and pathogenesis of OLP is one of the major challenges in our field. Therefore, the aim of this study was to investigate the possible role of miRNA-21 and miRNA-146a in OLP immuno-pathogenesis. Results of the present study showed a statistically significant increase in the expression levels of miRNA-21 (~3-fold, $P < 0.01$) and miRNA-146a (~5.5-fold, $P < 0.01$) in OLP compared to normal controls. This increased miRNA expression levels in OLP lesions suggest a participation of these miRNAs in the pathogenesis of the disease.

Results of this study are partially in agreement with those of ***Sonkoly et al.*** [18] who showed increased expression of both, miRNA-21 and miRNA-146a, in the chronic inflammatory skin disease, psoriasis compared to healthy skin. However, they reported increased expression level of only miRNA-21, not miRNA-146a, in atopic eczyma.

Moreover, expression of miRNA-21 [32] and miRNA-146a [24] were also increased in patients with SLE. Furthermore, ***Nakasa et al.*** [33] **and** ***Stanczyk et al.*** [34] reported increased miRNA-146a levels in rheumatoid arthritis. Their results further support our results.

In addition, results of the present study are in accordance with those of ***Wu et al.*** [35] who demonstrated increased miRNA-21 expression in active ulcerative colitis. Similarly, miRNA-21 was also found to be increased in the lungs of mice exposed to aerosolized lipopolysaccharide [36].

Results of the present study can be explained on the basis that both, miRNA-21 [37] and miRNA-146a [38] have been reported to play an important role in regulation of T-helper-1 (Th1) immune responses, which is known to be increased in OLP. Moreover, evidence has shown that miRNA-146a can impair nuclear factor kappa beta (NF-κB) activity [39] and suppress the expression of NF-κB target genes such

as interleukin(IL)-6, IL-8, IL-1β and tumor necrosis factor- alpha (TNF-α) [39,40]. Additionally, ***Li et al.*** [20] reported that miRNA-146a play an important role in the regulation of inflammatory response through a negative feedback pathway. Interestingly, increased miRNA-146a expression was only seen at high IL-1 β concentrations, which indicated that negative feedback is only activated during severe inflammation and that this might be crucial in preventing potentially dangerous inflammation from spiraling out of control [41].

On the other hand, ***Rhodus el al.*** [42] reported significantly higher levels of NF-κB-dependent cytokines; TNF-α, IL-1α, IL-6, and IL-8 in different oral fluids from OLP patients compared to normal controls. They concluded that NF-κB-dependent inflammatory cytokines may have diagnostic and prognostic potential for monitoring disease activity and making therapeutic decisions in patients with OLP [42]. Accordingly, we can speculate that increased expression of miRNA-146a may be involved in OLP pathogenesis through NF-κB pathway that initiate and amplify the local immune reaction which may contribute to the development of the disease.

Previous studies provided explanation for how miRNAs could regulate the immune response in inflammatory diseases through the action of other regulatory mechanisms. ***Lu et al.*** [19] showed that miRNA-21 was the most highly induced miRNA in an IL-13-induced asthma model. They reported thatmiRNA-21 is involved in inflammation, at least in part, by modulating cytokine responses [19]. Moreover, ***Li et al.*** [22] reported a significant positive correlation between miRNA-146a and TNF-α in both peripheral blood and synovial fluid of patients with rheumatoid arthritis. They postulated that the inflammatory milieu may altermiRNA-146aexpression in infiltrated T-cells [22]. One of these previous mechanisms may be involved in OLP pathogenesis.

In contrast to our results, ***Tang et al.*** [24] showed under-expression of miRNA-146a in patients with SLE as compared with patients with Behçet's disease and normal control subjects. They stated that miRNA-146a under-expression contributes to abnormal activation of type I interferon (IFN) signaling pathway in SLE.

OLP has been classified by the WHO as a "potentially malignant disorder". Interestingly, miRNA-21, one of the miRNAs connected to oral epithelial cancer, oral squamous cell carcinoma [43], was up-regulated in our results, supporting a trend towards a malignant connection for OLP. Consequently, we can conclude that, in addition to its role in inflammation, miRNA-21may have a possible role in malignant transformation of OLP. However, further studies are needed to delineate the exact role of both miRNA-21 and miRNA-146a in OLP, and to determine whether modulation of these miRNAs activity in vivo may become a novel therapeutic approach to treat this disease.

Conclusion

The current study demonstrated that there is a significant increase in the expression levels of miRNA-21 and miRNA-146a in OLP lesions compared to healthy controls. These data indicate that miRNAs may be the novel candidate biomarkers for the implication of miRNAs in the pathogenesis of OLP.

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