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GENE EXPRESSION OF MIRNA-138 AND CYCLIN D1 IN ORAL LICHEN PLANUS

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ABSTRACT

Objectives: To evaluate microRNA-138 (miR-138) gene expression and its target cyclin D1 (CCND1) gene and protein expression in oral lichen planus (OLP) mucosa in an attempt to investigate their possible roles in OLP immunopathogenesis.

Methods: Sixty oral biopsy specimens were harvested from 30 healthy subjects and 30 OLP patients; subdivided into reticular, atrophic and erosive groups (n=10 each). Samples were subjected to quantitative real-time polymerase chain reaction analysis for quantification of miR-138 and CCND1 relative gene expression and immuohistochemical analysis to determine CCND1 protein expression.

Results: Samples from OLP patients had a significant underexpression of miR-138 gene and overexpression of CCND1 at both gene and protein levels compared to normal mucosa samples. The lowest levels of miR-138 expression were observed in atrophic and erosive OLP compared to reticular OLP and the highest levels of CCND1 gene and protein expression was in atrophic OLP. An inverse correlation was demonstrated between the miR-138 expression and both CCND1 gene and protein expression in OLP patients. A significant positive correlation between CCND1 gene and protein expression was also observed.

Conclusion: Down regulation of miR-138 increases the gene and protein expression of its potential target CCND1 in OLP mucosa which might have a pivotal role in the disease pathogenesis.

Clinical relevance: This research implied that miR-138 may have a role in identification of symptomatic OLP lesions. MiR-138 might be considered as a potential tool in future OLP molecular therapy.

INTRODUCTION

Oral lichen planus (OLP) is a relatively common chronic inflammatory mucocutaneous disease. It affects 1-2% of the adult population, women more than men, mainly middle aged and elderly [1,2]. Clinical presentation of OLP ranges from asymptomatic reticular white straie to symptomatic

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atrophic-erosive red lesions with symptoms of burning, irritation and pain. To date, OLP is regarded as a T-cell-mediated disease, however, the precise pathogenesis remain obscure [3, 4]. OLP is classified as a potentially malignant disorder by the World Health Organization (WHO) with a highly variable risk of developing into head and neck squamous cell carcinoma (HNSCC) (0.4%-12.5%) [5-7]. Currently, there are few prognostic markers to identify which chronic OLP lesions are at a higher risk for progression. Therefore, it is extremely urgent to unravel the underlying molecular mechanisms of malignant transformation of OLP so that accurate diagnosis can be made and new therapeutic approaches can be developed [8].

Recent studies revealed an important role of microRNAs (miRNAs) in the pathogenesis of OLP [8,9]. MiRNAs are endogenous small non-coding 22-nucleotide-long RNA molecules that control the target gene expression at the post-transcriptional level [10, 11]. Each miRNA can control a large number of target mRNAs and each mRNA can be controlled by many miRNAs [12, 13]. MiRNAs participate in the regulation of diverse cellular processes including proliferation, differentiation, development and apoptosis [11, 14]. Recently, alterations in miRNAs expression were reported to be involved in many physiological and pathological processes, including chronic inflammatory, autoimmune diseases [15, 16] and HNSCC [17-21]. Moreover, studies suggested that there is a particular miRNA signature associated with the malignant progression of oral premalignant lesions [22-24].

MiRNAs are functionally integrated into many crucial cell cycle control pathways [25]. MicroRNA-138 (miR-138) is a multi-functional molecule regulator that regulates a variety of biological processes [26]. Studies reported that the downregulation of miR-138 was involved in the pathogenesis of different diseases [27, 28]. While deregulation of miR-138 has been frequently observed in HNSCC [29-32], the precise role of miR-138 in tumorigenesis is still elusive. Moreover, Liu et al [33] identified the cyclin D1 (CCND1) gene as a novel direct target of miR-138 using bioinformatics estimation. CCND1 is a proto-oncogenic positive regulator of the cell cycle, driving cells from G1 into S phase checkpoint [34]. It is a 45 kD protein encoded by CCND1 gene located on chromosome 11q13 [35]. Previous reports suggested that CCND1 might have a role in the pathogenesis of OLP [36-38]. In addition, other studies reported alterations in the expression of cell cycle regulatory proteins in oral premalignant and malignant lesions[35,39-41]. Nonetheless, our recognizing of the regulatory networks underlying the altered expression of these genes in the development of OLP is far from complete. Given that miRNAs appear to constitute one of the largest classes of gene regulatory molecules, understanding their mode of action and their pathological roles is essential [42].

Although OLP is currently discussed as a status with malignant potential, the premalignant potential of OLP and how it develops into oral cancer is not fully elucidated [43, 44]. In light of the regulatory properties of miRNAs and its impact on tumor development, there is an urgent and great need to explore the molecular mechanisms underlying the pathogenesis of OLP, indicating new therapeutic targets. Based on the authors' knowledge, no previous studies in the literature have investigated the target relationship between miRNA-138 and CCND1expression in OLP mucosal tissue. Accordingly, the aim of the present investigation was to evaluate the expression of miR-138 and its target CCND1 protein and gene expression in OLP lesions compared to healthy controls utilizing quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry. This study was conducted in an attempt to investigate the possible role of miR-138 on the regulation of CCND1 in OLP immunopathogenesis.

MATERIALS AND METHODS

This clinical trial has been registered at ClinicalTrials.gov (identifier NCT02834520)

Study population

The entire study sample comprised 60 subjects. Thirty patients suffering from OLP (19 female, 11 male; their age ranging from 46 to 69 years) and 30 age-sex matched control subjects (19 female, 11 male; their age ranging from 45 to 65 years) who were healthy normal individuals free form any inflammatory oral lesions.

Inclusion and exclusion criteria

All individuals enrolled in this study were selected from the Outpatient clinic, Department of Oral Medicine, Periodontology and Diagnosis, Faculty of Oral and Dental Medicine, Cairo University during the period from May 2015 to December 2015. A detailed medical history of each subject was obtained according to the detailed questionnaire of the modified Cornell Medical Index [45]. To qualify for the study, patients were diagnosed with OLP according to the World Health Organization's (WHO's) clinicopathological diagnostic criteria for OLP [46]. According to the clinical features, the OLP patients were divided into three subgroups, including 10 reticular OLP, 10 erosive OLP and 10 atrophic OLP cases. The presence of Wickham's striae confirmed the clinical diagnosis. These striae got accentuated by stretching of the surface mucosa and were not eliminated by rubbing. The oral lesions were bilateral and extended to involve the buccal mucosa, labial mucosa and tongue which varied from one patient to the other. Duration of the disease ranged from 5 to 6 months with periods of remission and exacerbation. Exclusion criteria included; patients with suspected restoration-related reaction or under any medication that could cause lichenoid reaction during the 3 months before the study, gingival inflammation, any systemic disease, inflammatory oral lesions, immunodeficiency, autoimmune disorders, hepatitis, or human immunodeficiency virus infection, pregnancy or lactation, and former or current smokers. Patients did not use any topical or systemic medications for treatment of OLP in the last 3 months.

Following an explanation of the study as well as the information about the sampling procedures, each subject signed a written informed consent form approved by the Faculty Research Ethics committee (September 2014). After obtaining patients' written consent, 60 tissue biopsy specimens were harvested from representative lesions clinically diagnosed as OLP (n=30) and from healthy normal oral mucosa as control (n=30) under local anesthesia with 2% xylocaine adrenaline. All tissue specimens were processed by the Department of Oral Pathology, Faculty of Oral and Dental Medicine, Cairo University. Analysis were performed on coded samples by one of the authors (KR), who was masked with regard to the subjects' diagnoses until all analysis were finished. The histopathologic diagnosis and examination of all specimens was confirmed by two oral pathologists (FS, KR).

Tissue specimens

A total of fresh 60 specimens were transformed into paraffin impregnated tissue blocks including normal oral mucosa as a control group (n=30) and OLP (n=30; 10 reticular OLP, 10 atrophic OLP and 10 erosive OLP). Thin (5 μm) paraffin sections of each tissue specimen were stained with H&E to reconfirm the diagnosis other 4 μm paraffin sections of each specimen were mounted on positively charged glass slides (Optiplus;Biogenex, Milmont Drive, CA, USA) for immunostaining with anti-cyclin D1 antibody. Ten sections of 5 μm thickness were cut from each sample and placed in plastic eppendorf to be subjected equally to a qRT-PCR analysis for miR-138 and cyclin D1 gene expression.

Immunohistochemistry

The 60 paraffin embedded tissue sections on positively charged slides were immunostained with anti-cyclin D1 antibody with super sensitive

biotin–streptavidin staining technique. Tissue sections were deparafinized, rehydrated, and treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer, pH 6.0 for 10–20 min followed by cooling at room temperature for 20 minutes. The positive test slides were incubated with the primary antibody rabbit polyclonal antibody anti-cyclin D1 antibody (Cat #RB-9041-R7) Thermo Scientific, Labvision, Kalamazoo, MI, USA) for 30 min at room temperature in a humified chamber. On the other hand, the negative control slides were not exposed to the primary antibody. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin-labeled link antibody for 30 minutes, then the streptavidin conjugated horseradish peroxidase was used. The diaminobenzedine chromogen was applied to visualize the antigen antibody reaction. All these reagents belong to the universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code no. K0673 DakoCytomation, Glostrup, Denmark) All the slides were immersed in Mayer's hematoxylin for counterstaining. Finally, the sections were covered by cover slips using aqueous mounting medium.

The ordinary light microscope was used to detect and localize the immunostaining of anti-cyclin D1 antibody. Cells with nuclear staining were considered positive. The number of cyclin D1 positive cells in the basal and parabasal layers of control and OLP cases were counted. The parabasal layers were defined as the second and third row above basement membrane of stratified squamous epithelium. At least 500 basal and parabasal cells were counted. The cyclin D1 labeling index was expressed as the number of positive cells per total number of cells in the basal and parabasal layers [47].

MicroRNA Extraction

MicroRNAs was extracted from paraffin blocks sections by miRNeasy extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted microRNA was stored at –80°C until use.

Quantitative real-time PCR (qRT-PCR) analysis for miR-138 expression

Reverse transcription was carried out on extracted microRNA in a final volume of 20 uL RT reactions using the miScript II RT kit (Qiagen, Valencia,CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using a MiScript SYBR Green PCR kit (Qiagen, Valencia,CA, USA) and miScript primer assay miR-138 (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions using Rotor-gene Q Real-time PCR system (Qiagen, USA). After the PCR cycles, melting curve analyses were performed to validate the specific generation of the expected PCR product. SNORD 68 was used as an endogenous control. The expression level of miR-138 was evaluated using the ΔCt method. The cycle threshold (Ct) value is the number of quantitative PCR cycles required for the fluorescent signal to cross a specified threshold. ΔCt was calculated by subtracting the Ct values of SNORD 68 from those of target microRNA. ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the disease samples. The relative gene expression of miR-138 was calculated by the equation $2^{-\Delta\Delta Ct}$ [48].

Quantitative real-time PCR (qRT-PCR) analysis for cyclin D1 expression

The commercial QIAamp RNA Mini Kit (Qiagen, USA) was used for RNA extraction. The RNA integrity and concentration was determined by nanodrop measurement at 260 nm. One μg of extracted RNA was reverse transcribed to cDNA with High cDNA Reverse Transcriptase Kit. cDNA was amplified for the expression of Cyclin D1 and β-actin with SYBR green fluorophore following the manufacturer's recommended amplification procedure. The sequence of primers used for Real-time PCR analysis were: cyclin D1 forward

5'-CGGAGGACAACAAACAGATC-3' and reverse 5'-GGGTGTGCAAGCCAGGTCCA- 3' and β actin forward primer 5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' and reverse 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3'. The relative quantification of cyclin D1 gene was determined using the comparative CT method. The ΔCt was calculated as the difference between the average Ct values of the β-actin from the average Ct value of cyclin D1 gene. The ΔΔCt was determined by subtracting the ΔCt of the control from the ΔCt of the OLP. Samples were run in triplicate. Relative expression of the target gene was calculated by the equation $2^{-\Delta\Delta Ct}$ [48] which was the amount of cyclin D1 product, normalized to the endogenous control (β-actin) and relative to the control sample.

Statistical and power analysis

Using G-power analysis program [49] sample size was determined by comparing cyclin D1 index in the control and OLP groups according to Hirota et al. [47]. A total sample size of 44 patients was calculated to be sufficient to detect effect size (f = 1.15) by considering level of significance $\alpha = 0.05$, with 95 % power. This number was increased to 60 patients (30 in each group) to increase the validity of the results. Data were presented as mean ± standard deviation (SD) or median (min–max). Mann-Whitney U test was used for pair wise comparisons when Kruskal-Wallis test was significant and for correlation analyses, Spearman's rank correlation coefficient was performed. Results were displayed by the use of box plots, with the rectangle representing 50% of the cases and the whiskers going out to the smallest and largest value. The median value is displayed by the line inside the rectangle. All tests were two sided, and P values of ≤ 0.05 were accepted for statistical significance. All data were processed with a computerized statistical package (SPSS 15.0 for Windows, SPSS Inc., Chicago, IL).

RESULTS

Table 1 shows demographic data for all subjects included in this study.

Immunohistochemical detection of cyclin D1 protein in OLP

Evaluation of CCND1 protein expression occurred by the use of immunohistochemistry performed by one of the investigators (KR).

TABLE (1) Demographic and clinical data of subjects.

Clinical characteristics of subjects	Mean (SD) of age (year)	Range of age (year)	Gender (male/female)	Tobacco habits (n)	n (%)
Control group (n=30)	58.77 (5.47)	45-65	11/19	0	-
Oral lichen planus group (n=30)	60.73(6.0)	46-69	11/19	0	
Reticular OLP (n=10)	59.33 (5.02)	50-65	4/6		
Erosive OLP (n=10)	60.5 (2.55)	46-64	3/7		
Atrophic OLP (n=10)	63.7 (7.15)	47-69	4/6		
Localization of OLP lesion					
Buccal mucosa					27 (90)
Labial mucosa					15 (50)
Tongue					21 (70)
Gingiva					12 (40)
Palate					6 (20)

In normal oral epithelium (n=30) no expression of CCND1 protein was seen, (immunonegative). In all specimens (n=30) of OLP (100%) CCND1 protein was expressed prominently compared with those in the control mucosa. The positivity of CCND1 was detected as dense nuclear stain which was concentrated at the basal and the parabasal cell layers. The subepithelial connective tissue zone which is just below the epithelium contains dense band of lymphocytes (Fig. 1). Statistical analysis of the CCND1 labeling index revealed that CCND1 protein was significantly highly expressed in atrophic OLP cases compared to reticular OLP group ($P < 0.05$). Although erosive OLP cases also showed high CCND1 protein expression, yet it was not significant when compared to both reticular OLP ($P=0.96$) and atrophic OLP groups ($P=0.242$) (Table 2).

Relative gene expression of miRNA-138 in OLP by qRT-PCR

MiRNA-138 relative gene expression was successfully detected in all 60 samples by qRT-PCR. Results showed significantly lower expression of miR-138 ($P<0.001$) in OLP samples compared to normal oral mucosa (Fig. 2a). No differences in miRNA-138 expression in OLP epithelium based on sex or age could be detected. The least miRNA-138 expression was observed in atrophic OLP group followed by erosive OLP and reticular OLP groups. Moreover, subgroup analysis revealed that miRNA-138 expression was significantly

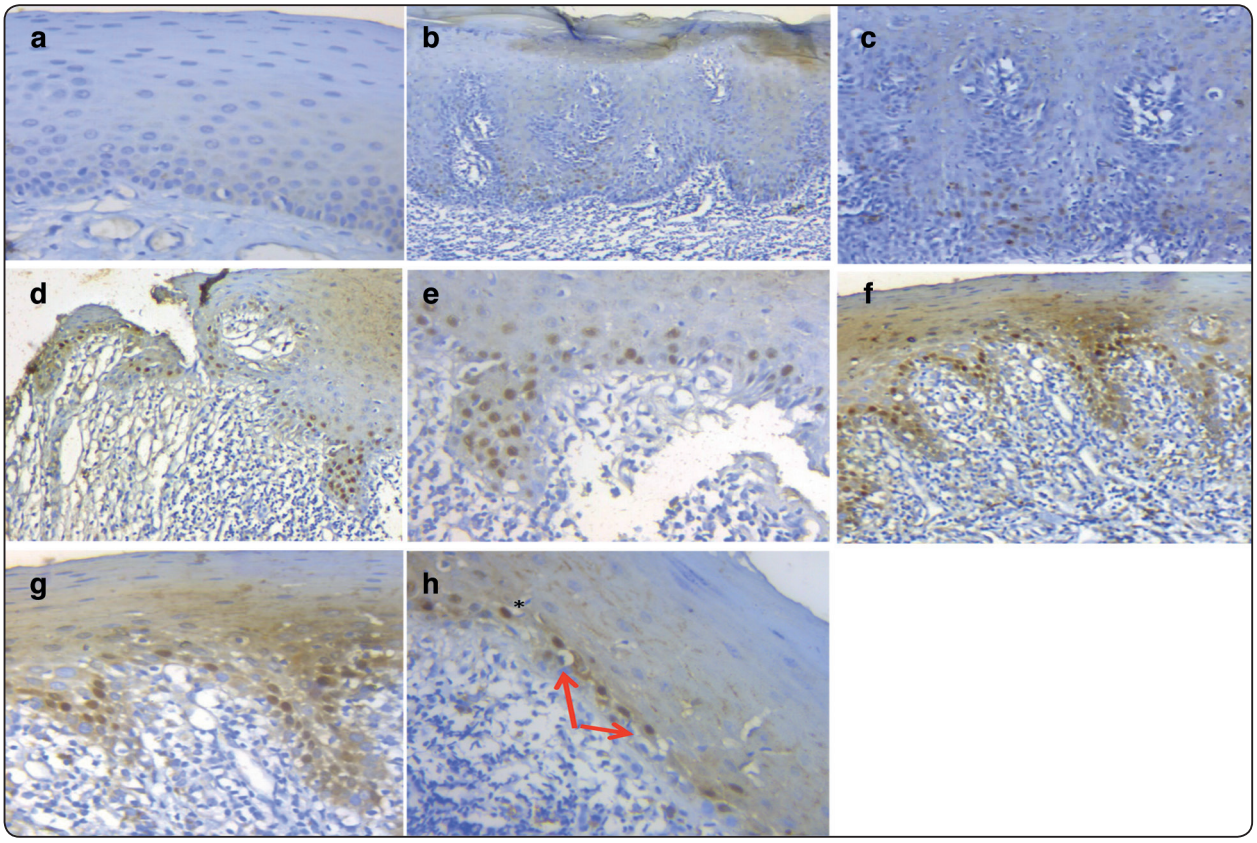

Fig. (1): (a) photomicrograph of normal oral mucosa showing negative CCND1 immunoexpression, anti-cyclin D1antibody X400. (b)&(c) photomicrographs of reticular OLP showing CCND1 positivity, Anti-cyclin D1 antibody x200&400. (d)&(e) erosive OLP showing CCND1 positivity, Anti-cyclin D1 antibody x200&400. (f)&(g) atrophic OLP showing CCND1 positivity, Anti-cyclin D1 antibody x200&400. (h) CCND1 positive basal cells (arrows), anti-cyclin D1 antibody X400.

higher (P<0.01) in reticular OLP compared to both atrophic and erosive OLP, however no significant difference (P=0.968) was detected between atrophic OLP and erosive OLP subgroups (Table 2). In addition, the fold change regarding miR-138 was approximately 0.4-fold in OLP lesions compared to normal tissue. In subgroup analysis, miR-138 fold change in reticular, atrophic and erosive OLP samples was approximately 0.8, 0.5 and 0.2 fold compared to normal controls, respectively.

Relative gene expression of cyclin D1 in OLP by qRT-PCR

CCND1 relative gene expression was successfully detected in all 60 samples by qRT-PCR. Similar to immunohistochemical analysis, results showed that CCND1 mRNA was significantly ($P < 0.001$) overexpressed in OLP samples compared to normal oral mucosa (Fig. 2b). The highest CCND1 gene expression was observed in atrophic OLP group followed by erosive OLP and reticular OLP groups. Although, subgroup analysis revealed that CCND1 gene expression was lower in reticular OLP compared to both atrophic and erosive OLP, however no significant difference (P=0.105) was detected between them (Table 2).

Table 3 represents delta Ct (ΔCt) of miRNA-138 and cyclin D1 in all studied groups.

Spearman's rank correlation coefficient analysis

In OLP tissue, a statistically significant negative correlation was detected between the miR-138 relative gene expression levels and its potential target CCND1 at both the protein level ($q = -0.417$, $n = 30$, $P < 0.05$) and mRNA gene level ($q = -0.483$, $n = 30$, $P < 0.001$) (Fig. 3 and 4 respectively). Furthermore, statistical analysis showed a statistically significant strong positive correlation between CCND1 relative gene expression and CCND1 protein expression ($q = 0.754$, $n = 30$, $P < 0.001$) in OLP tissue.

TABLE (2) Relative gene expression of miRNA-138 and cyclin D1 and cyclin D1 index in all studied groups.

	miRNA-138 (Relative gene expression)		**Cyclin D1 (Relative gene expression)**		**Cyclin D1 index (protein expression)**	
	Median	**min-max**	**Median**	**min-max**	**Median**	**min-max**
Control group (n=30)	4.6	3.1-6.88	1.24	0.34-2.55	-	-
Oral lichen planus group (n=30)	2.05*	0.11-4.32	7.74*	0.69-12.4	30	22-40
Reticular OLP group (n=10)	3.38	1.03-4.3	4.74	0.69-12.31	26	22-35
Erosive OLP group (n=10)	1.82#	0.11-2.5	9.88	0.74-12.3	30	28-40
Atrophic OLP group (n=10)	1.05#	0.5-4.32	11.13	0.88-12.40	33#	27-37

Statistically significant when compared to control (P < 0.001).

 # Statistically significant when compared to reticular OLP (P < 0.05).

TABLE (3) Delta Ct (ΔCt) of miRNA-138 and cyclin D1 in all studied groups.

	ΔCt miRNA-138		ΔCt Cyclin D1	
	Median	**min-max**	**Median**	**min-max**
Control group (n=30)	-2.2	-1.63 – -2.78	-0.31	1.55 – -1.35
Oral lichen planus group (n=30)	-1.04*	3.184 – -2.11	-2.95*	0.53 – -3.63
Reticular OLP group (n=10)	-1.76	-0.04 – -2.1	-2.24	0.53 – -3.62
Erosive OLP group (n=10)	-0.86#	3.18 – -1.32	-3.3	0.43 – -3.62
Atrophic OLP group (n=10)	-0.07#	1– -2.11	-3.48	-0.18 – -3.63

** Statistically significant when compared to control (P < 0.001).*

 # Statistically significant when compared to reticular OLP (P < 0.05).

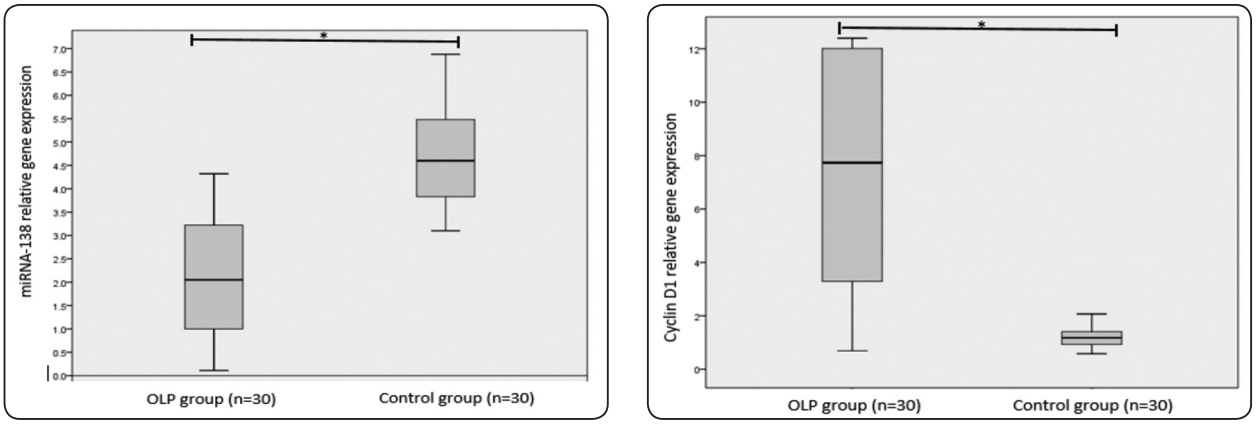

Fig. (2): Box plot showing relative gene expression of miR-138 (a) and CCND1 (b) in normal mucosa compared with OLP mucosa using qRT-PCR. The boxes stretch from the 25th to the 75th percentile; the lines across the boxes indicate the median values; the lines stretching from the boxes indicate extreme values. *Statistically significant when compared to control (P < 0.001).

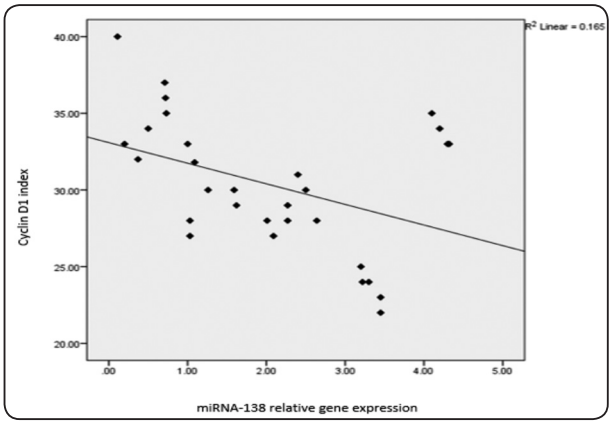

Fig. (3): miR-138 relative gene expression levels were inversely correlated with CCND1 index (protein expression) in OLP.

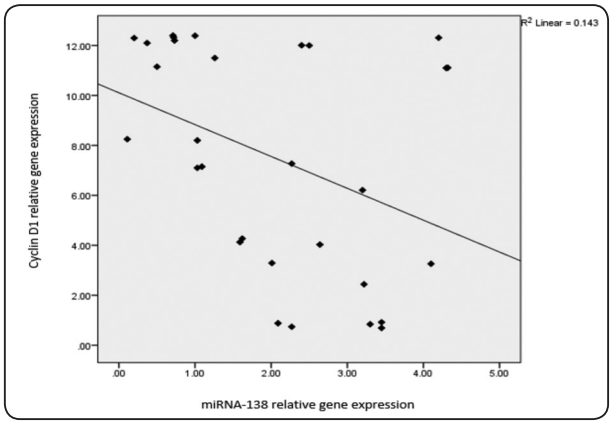

Fig. (4): miR-138 relative gene expression levels were inversely correlated with CCND1 relative gene expression in OLP.

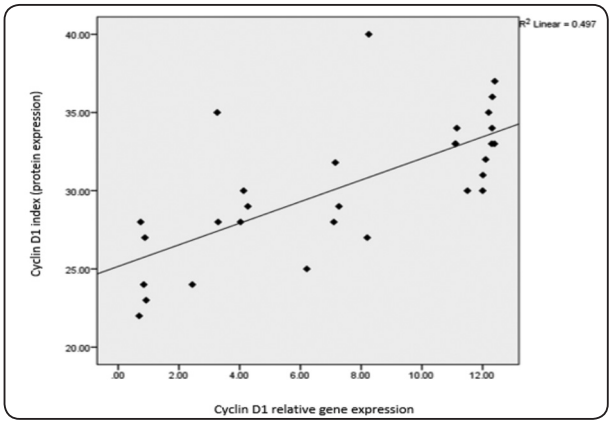

Fig. (5): CCND1 relative gene expression levels were positively correlated with CCND1 protein expression in OLP.

DISCUSSION

The comprehension of etiology and pathogenesis of OLP is one of the major challenges in oral pathology and oral medicine. MiRNAs are increasingly being recognized as critical regulators of tissue-specific patterns of gene expression [50]. Based on the authors' knowledge, this study revealed for the first time a significant lower miR-138 gene expression and higher CCND1 gene and protein expression in OLP patients compared to normal oral epithelium using qRT-PCR and immunohistochemical analysis.

The present immunohistochemical findings were similar to those reported by Hirota et al.[47], who suggested that the significant increase in CCND1 index could induce the proliferation status of OLP epithelium. As explained by the authors; in the basal and parabasal cell layers of OLP mucosa, most injured cells enter the cell cycle for proliferation and repair, while the remaining cells undergo apoptosis due to severe DNA damage. In this context, cell cycle arrest and up-regulation of proliferation activity are concomitantly seen, and these contradictory changes could contribute to the development of characteristic mucosal architecture and clinical manifestations of OLP [47] which supports the current observations. The present results were also consistent with other reports suggesting that CCND1 plays an important role in the occurrence and development of OLP [36-38]. This study showed positive CCND1 expression in 100 % of OLP cases, which is in line with Abid and Merza [38], Zhang et al [37] and Yao et al [36] who reported CCND1 positive expression in (84%), (71.67%) and (82%) of OLP cases respectively. In agreement with the above mentioned studies, these data suggests that the currently observed increase of CCND1 at both protein and mRNA levels in OLP may lead to increased cellular proliferation and might denote the hyperproliferative status of epithelial cells. Other studies reported the proliferation activity of epithelial cells in OLP showing alterations of the cell cycle regulatory mechanism [51, 52]. Although the mechanism of enhanced cell proliferation in OLP remains unsolved, Gonzalez-Moles et al.[53] proposed that this hyperproliferative status in OLP is a compensatory mechanism of epithelium to maintain its architecture in spite of aggressive lymphocyte attack. In addition, Rezaee et al [52] suggested that cyclin overexpression noted in OLP caused increased cell proliferation due to shortening in the cell cycle G1 phase.

The lower expression of miR-138 in OLP tissue demonstrated in this study was consistent with numerous studies which proposed that alterations in miRNAs expression may play a critical role in the pathogenesis of OLP [50,54-58]. These studies suggested that the discovery of miRNAs is a new way to unveil the molecular mechanisms underlying OLP and might represent a novel candidate biomarker for diagnosis [8, 9] which supports the current data. Although it is not possible to identify the exact role of miR-138 in OLP, some hypothesis could be drawn. Recent reports provided explanation for how miR-138 could regulate the immune response in some chronic diseases through the action of others regulatory mechanisms [59,60]. Fu et al [59] showed that miR-138 expression was decreased significantly in psoriasis patients compared with healthy controls,

suggesting that that the downregulation of miR-138 induced an imbalance in Th1/Th2, involved in the pathogenesis of psoriasis. It is well established that OLP is characterized by the imbalance between the levels of Th1 and Th2 immune responses [3], consequently, this study hypothesized that miR-138 downregulation might contribute to the intense inflammation seen in OLP through its impact on Th1 and Th2. This might be one of the mechanisms that may be implicated in OLP pathogenesis. Furthermore, subgroup analysis revealed that miR-138 expression was significantly lower in cases of atrophic and erosive OLP than reticular OLP group. Similarly, Zhang et al [54] revealed that miR-27b was significantly lower in atrophic and erosive OLP compared with reticular OLP suggesting that downregulation of miR-27b was associated with the disease activity. Hu et al. [57] also showed that miR-125a has varied expression in different clinical forms of OLP and might be a novel candidate biomarker to estimate the severity of OLP. Along with the current literature which confirms that erosive and atrophic OLP show more severe clinical symptoms, pathological features and differ in treatment than reticular OLP [61,62], this study supports that different clinical forms of OLP may have a distinct immune modulatory background.

Despite current established evidence in the literature considering erosive and atrophic subtypes of OLP to have a higher risk of malignant transformations, still the mechanism is poorly understood [2,6]. Carcinogenesis can occur as a result of uncontrolled cell proliferation due to multiple genetic alterations associated with aberrant cell cycle regulation [35]. Consistent with this hypothesis, previous studies showed that overexpression of CCND1 were implicated in the pathogenesis of HNSCC [35, 63, 64]. Furthermore, Bascones et al [65] suggested that molecular alterations related to cell cycle control may produce an epithelial substrate that favors evolution to malignity in OLP. Since erosive and atrophic OLP epithelium is more susceptible to malignant transformation being more sensitive to carcinogenic exposures than normal oral mucosa [5, 52], hence, action on proliferating cells by oncogenic insults may lead to the development of a malignant cell phenotype in OLP patients [65]. Accordingly, this study agrees with other reports suggesting that overexpression of CCND1 at both protein and gene levels in OLP may disturb the normal cell cycle control providing tumor cells with a selective growth advantage [36, 38]. Moreover, Rezaee et al [52] reported that the increased cell proliferation observed in OLP favors the accumulation of cell cycle genetic alterations which might be considered as a strong indicator of malignant potential in OLP, which agrees with the current results.

Previous reports suggested that miR-138 might function as a tumor suppressor by negatively regulating cyclin genes in cancer [66]. Earlier studies showed that CCND1 and D3 levels were inversely correlated with miR-138 expression in nasophayngeal carcinoma [33] and hepatocelular carcinoma [27]. Numerous investigations also observed that dysregulation of miR-138 might contribute to the enhanced cell migration in HNSCC [26, 29-31]. Interestingly, this study demonstrated an inverse correlation between miR-138 and CCND1 expression in OLP patients. This is in agreement with Liu et al [33], who suggested that miR-138 negatively regulates cell cycle progression in OLP by targeting critical cell cycle regulators leading to overexpression of CCND1 and eventually cell proliferation. Recently, Yang et al [62] reported that miR-146a may be involved in the malignancy associated with erosive OLP and could be used as a potential biological marker to evaluate the severity of OLP. Based on the above mentioned data, it might be speculated that miRNA-138 might be considered as a tumor suppressor in premalignant lesions like atrophic and erosive OLP. However, further studies involving HNSCC are warranted to confirm this speculation.

In conclusion, this investigation showed that the downregulation of miR-138 increases the expression of their potential targets CCND1 in OLP mucosa which might indicate a pivotal role in the disease pathogenesis. The present data highlights the potential role of miRNA expression analysis in OLP, which will ultimately improve diagnosis and patient outcome. Moreover, this study suggests that miR-138 might be considered as a potential novel therapeutic target for atrophic and erosive OLP patients. Further characterization of miRNA and their target genes will advance our understanding of the pathogenesis of OLP and a large-scale patient based study will be needed to fully explore the mechanism that regulates the expression of this noncoding gene.

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