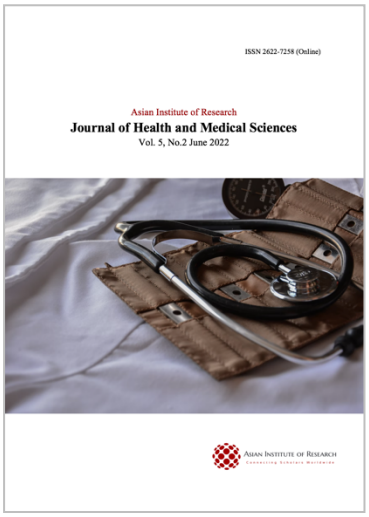

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Immunohistochemical Expression of Cyclin D1 in Oral Lichen Planus

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Abstract
Background: Many studies have reported that a condition initially classified as oral lichen planus (OLP) has a variable chance of developing cancer progression over time, although these results are still questionable. Cyclin D1 controls the mitotic cell cycle's transition from G1 to S. Cyclin D1 has a significant function in carcinogenesis due to its critical involvement in cell cycle control. Cyclin D1 deregulation or overexpression may result in shortened G1 phase, enhanced cell replication, and decreased reliance on growth regulators, causing disruption in conventional cell cycle progression and the progression of cancer. The aim of the current study was to evaluate the expression of Cyclin D1 in histopathologically confirmed OLP samples and to predict the rate of malignant transformation in these lesions by comparing the level of expression with some clinical parameters. Materials and Methods: The immunohistochemical procedure was used to examine the expression of cyclin D1 on paraffin-embedded sections of (40) OLP lesions and (10) specimens of normal oral mucosa. We used the SPSS software version (18.0) to do the statistical analysis in this work. Results: There is a very significant difference between the people investigated (P = 0.01), which is statistically significant. CyclinD1 was found in all of the oral lichen planus cases in the research (100%), but it was also found (80%) in the normal oral mucosa. There was also no statistically significant difference between the clinical types of OLP and the Cyclin D1 scoring (P = 0.942), which revealed that the reticular type was the most frequent (7) in mild scoring, whereas the erosive type was the most frequent in severe scoring, which accounted for the majority of the cases. Conclusion: This research found that higher cyclinD1 expression increases the risk of cancer and many of the markers should be tested. Furthermore, larger sample numbers, improved designs, and gene-reduction studies should be performed. OLP patients should be continuously monitored for OSCC progression.

Keywords: Immunohistochemistry, CyclinD1, Malignancy, Oral Lichen Planus

1. Introduction

Oral lichen planus (OLP) is a mucocutaneous chronic autoimmune condition that impacts the oral mucosal surface and is widely encountered. This condition affects approximately 0.5–2.2 percent of the population (Kurago, 2016; Georgescu et al., 2017; Giannetti et al., 2018). Various antigen-specific and nonspecific processes are considered

to be involved in the development of OLP, which is categorized as a premalignant condition (Roopashree et al., 2010; Sagari et al., 2016). Despite the fact that the normal age of patients is 50 to 60 years, it is commonly seen in middle-aged women and younger-aged men, and it affects women more often than men. OLP is relatively uncommon in children and frequently occurs in combination with cutaneous disorders (Bardellini et al., 2013; Kurago, 2016; Giannetti et al., 2018). OLP appears clinically in three types: reticular, atrophic, and erosive. They are classified as either erosive (erosive abnormalities) or non-erosive (reticular and atrophic abnormalities) (Zhou et al., 2012; Lorenzini et al., 2013; Giannetti et al., 2018). It may manifest as asymptomatic reticular white streaks or as symptomatic ulcers with burning, irritability, and discomfort. OLP is now thought to be a T-cell-mediated disorder, although the specific pathophysiology remains unknown (Sugerman et al., 2002; Van der Meij et al., 2003; Lavanya et al., 2011). It's been shown in the past that immunologic pathways can play a big part in the development or spread of oral lichen planus (Van Der Meij and colleagues 2007; Agha-Hosseini et al., 2009; Lavanya et al., 2011). OLP is classified as a precancerous disease by the World Health Organization (WHO), and its most serious consequence is the development of cancer (Shen et al., 2011; Peng et al., 2017). In contrast to carcinoma, which may be anticipated by precancerous lesions that do not always lead to cancer, malignancy is a complex mechanism in which carcinoma can develop (Siar et al., 2011; Dragomir et al., 2012). The ability to multiply more cells is one of the processes required for malignancy to progress and grow (De Sousa et al., 2009). The limited range of apoptotic events in OLP epithelial cells indicates that it may provide a suitable substrate for cancer development (Bascones et al., 2005). For several years, the potential of OLP malignancy has been considered and described (Fitzpatrick et al., 2014; Giuliani et al., 2019). A meta-analysis was carried out to look at the risk variables for OLP carcinogenic transition to squamous cell carcinoma (Aghbari et al., 2017). Information sources such as PubMed and Scopus were systematically analyzed to establish the cancerous transformation rate of OLP and associated risk factors (Giuliani et al., 2019). The link between OLP and squamous cell carcinoma has aroused people's importance, and researches on this issue have been conducted (Fitzpatrick et al., 2014; Giuliani et al., 2019). While OLP is already recognized as a condition with malignant potential, its precancerous potential and how it progresses to malignancy are not entirely understood (Fitzpatrick et al., 2014). The primary impediment to examining the cancer progression of OLP is indeed the lack of broadly agreed diagnostic criteria for OLP (Gandolfo et al., 2004). Mortazavi et al. found that if OPMDs are found and treated early, they have a much lower chance of becoming cancerous (Mortazavi et al., 2014). Several studies have been conducted in an effort to establish OLP's malignant alteration risk. These investigations demonstrate that a disease initially identified as OLP may undergo malignant change over time. Nevertheless, these conclusions continue to be challenged (Mignogna et al., 2001; Tizeira et al., 2003). The evidence suggests that the most likely proportion of malignant transformation of OLP is between 0.1-3 percent (Irani et al., 2016; Hadzi-Mihailovic et al., 2017).

Furthermore, there are certain prognostic indicators that may be used to determine which chronic OLP lesions are most likely to progress. Hence, it is critical to understand the molecular pathways leading to OLP's oncogenesis in order to facilitate accurate diagnosis and the development of novel treatment options (Lavanya et al., 2011; Zuo et al., 2015). Molecular biomarkers may target individuals with possibly malignant tumors before malignant cells are detected histologically at the main location (Partridge et al., 2005). Several molecular methods have been reported to identify the transformation from healthy epithelium to precancerous lesions to cancer (Pitiyage et al., 2009). The cell cycle is controlled by cyclin-dependent kinases (CDKs) and their primary blockers, p16, p21, and p27, all of which are tumor suppressors (Poomsawat et al., 2011).

Cyclin D1 is a proto-oncogene that is encoded by the CCND1 gene and is located on chromosome 11q13. It is a part of the molecular system that observes and controls the cell cycle during the transition from the G1 to the S phase of the cell cycle (Basnaker et al., 2014; Batool et al., 2019). Cyclin D1 is the first cyclin to accumulate throughout the cell cycle; it rises during the G1 phase but is not detected during the S phase. It stimulates CDK4 and the cyclin D–CDK4 complex in the G1 phase, phosphorylates the Rb protein, and stimulates cell proliferation upon E2F liberation. Cyclin D1 overexpression is associated with a shortened G phase and aberrant cell growth. From healthy oral tissues to dysplastic abnormalities and OSCCs, cyclin D1 levels increase sequentially (Pitiyage et al., 2009; Ramakrishna et al., 2013). Previous research has suggested that CCND1 may play a significant role in the etiology of OLP (Zhang et al., 2010; Abid and Merza, 2014). Additionally, further studies have shown changes in the expression of some cell regulatory proteins in premalignant and malignant lesions of the oral cavity (Poomsawat et al., 2010). The goal of this research was to determine whether or not OLP had malignant potential

based on Cyclin D1 expression. In addition, the relationship between Cyclin D1 and clinical and histological characteristics in OLP was investigated.

2. Materials and methods

Forty specimens of oral lichen planus (OLP) and ten cases of normal oral mucosa were obtained from the documents of the oral pathology laboratory/oral diagnosis department/Baghdad University. Formalin-fixed, paraffin-embedded tissue blocks from each case were collected, as well as clinical information on the patient's health (age, gender, and type) as described in the oral and maxillofacial reports. Two professional pathologists independently verified the diagnosis by examining hematoxylin and eosin (H&E) stained tissue segments from each patient. Tonsil tissue was selected and used as a positive tissue control in each immunohistochemistry run in accordance with the Cyclin D1 manufacturer's data sheets. Pathnsitu/USA produced and employed primary monoclonal antibodies throughout the experiment. The PolyExcel detection system was developed for use with primary antibodies generated against mouse and rabbit to qualitatively identify proteins in paraffin-embedded normal and pathological tissues, cryostat tissues, or cellular preparations using light microscopy. PathnSitu is unequaled in terms of specificity and sensitivity. PolyExcel's two-step detection method is a non-biotin-based micropolymer-based recognition system that significantly decreases or removes background avidin or biotin levels. It is based on a polymer that is HRP-labeled and conjugated to secondary antibodies.

2.1. Immunohistochemical technique

The paraffin block technique was used to obtain the control and study tissue samples. Tissue samples were sliced into two pieces following formalin fixation. In order to increase tissue adhesion during immunohistochemical staining, one 5 mm segment was put on a positively charged microscope slide, while another 5 mm section was mounted on a pre-cleaned microscope slide. As positive controls, 5 mm thick tissue sections were placed on positively charged slides. The antibody was diluted (1:50) and applied to the tissue samples for 30–60 minutes at room temperature in humid environments. The segments were thoroughly cleaned with PBS, dried, and rinsed three times with PBS (5 min. for each). As a negative control, each slide received a second tissue section stained with PBS. A drop of soluble rabbit anti-mouse antibody was incubated at 37 °C for 15 minutes before being washed twice with PBS and dehydrated. A HRP-labeled polymer was incubated at 37oC for 15 minutes with secondary antibodies before being rinsed multiple times with PBS and left to dry. This mixture was applied to the tissue slice (15–20l) and incubated at 37 °C for 5–10 minutes before being cleaned with ordinary water and dried. The slides were gently rinsed with distilled water after being immersed in Mayer's Haematoxylin for 1-2 minutes. An optical microscope was used to examine the presentations after serial dipping in alcohol and xylene, then fixing with 1-2 drops of DPX fixing medium and immediately covering with cover slips.

2.2. Immunohistochemical evaluation

According to the product's datasheet, the appearance of a brown granular DAB pigment pattern within the cellular or tissue compartment targeted by primary antibodies on positive control tissue slides and the lack of darkening on negative control tissue samples indicated immunohistochemical signal specificity. The expression of CyclinD1 was assessed in a semiquantitative manner. All study presentations were evaluated independently, with no prior knowledge of any other features. Subsequently, the slides were reviewed by an expert pathologist.

2.3. Interpretation of immunohistochemical expression

The severity of nuclear cyclin D1 immunoreactivity expression was assessed through the intensity scale criterion given by Sharada et al. The level score criteria were as follows: 0 indicates no definite cells, 1 indicates a mild score, 2 indicate a moderate score, and 3 indicate a strong score. For quantitative immunohistochemical analysis, The H score was computed and tallied for each sample using the formula as given below (Sharada et al., 2018).

1× (% cells 1+) +2 × (% of cells 2+) +3× (% of cells 3+)

The micrographs collected were organized and saved on the computer in appropriately designated files. The Imagej® program, a freely available image processing tool built in Java that operates on a computer's operating system, has been used to assess photomicrographs.

2.4. Statistical Analysis

In order to evaluate and appraise the results, this research used the statistical program (SPSS) version (18.0) for descriptive analysis (mean and standard deviation) and graphs (bar and pie charts, histograms) to display the information. In order to examine and evaluate the results of the research, the data analysis approaches listed below were used. The Spearman rank correlation and the Pearson correlation coefficient are both employed in inferential data analysis, in addition to chi square tests and they are utilized in the same way. The presence of a p-value of less than 0.05 was considered statistically significant.

3. Results

As indicated in Table 1, the general clinical characteristics of the 40 patients who participated in the current investigation are summarized. It appears that there are no distinct changes in the age group distribution of the study subjects, denoting that the chance of confirmed disease doesn't really differ according the dispersion of age categories, and also the age shifted the focus at the sixth decade of life with mean and standard deviation (52.75±12.588), (Table 1).

In regards to gender, there are no statistically significant differences between the analyzed cases, and it can be inferred that the chance of the reported analyzed disorder does not alter depending on the sex of the patients (Fig.1).

According to the results of the study, there is a highly significant difference at P=0.01 among the participants examined, and in conformance with this finding, it is reasonable to state that the statistical likelihood of documented explored disturbed patients has represented important variations according to dissemination of clinical type, as it has been clearly indicated by the wide range of patients with "Reticular" type (Table 1).

Immunohistochemical analysis showed that brown nuclear positivity of CyclinD1 was expressed in all oral lichen planus cases of the study (100%) and 8 (80%) in normal oral mucosa (Fig.3).

The analyzed results showed no significant correlation between the expression Cyclin D1 and the age groups of the studied patients, P = 0.965, but these results revealed that the highest score was in seventh decade (Fig.2). Furthermore, there was no statistically significant difference between the clinical types of the OLP and the Cyclin D1 scoring, P = 0.942, which revealed that the reticular type was the most frequent (7) in mild scoring, whereas the erosive type was in severe scoring, which accounts for the majority of the cases (5) (Table 2).

Table 1: Frequencies of age groups, gender and clinical types for the patients with OLP lesions

Parameters	Age groups	Frequencies	(%)	P- Value
Age groups	(< 40)	8	20	P= 0.070 Non Sig.
	(40-50)	4	10	
	(50-60)	12	30	
	(60-70)	14	35	
	(> 70)	2	5	
	Total	40	100	
	Mean ± SD	52.75±12.588		
	Male	16	40	P = 0.268

Gender	Female	24	60	Non Sig.
Clinical types		Frequency	(%)	P = 0.000 Sig.
	Reticular	17	42.5	
	Erosive	15	37.5	
	Atrophic	3	7.5	
	Plaque	3	7.5	
	Bullous	2	5.0	
	Total	40	100.0	

NS: Non Sig. at P>0.05; S: Sig. at P<0.05; Testing are based on One Sample Chi Square Test.

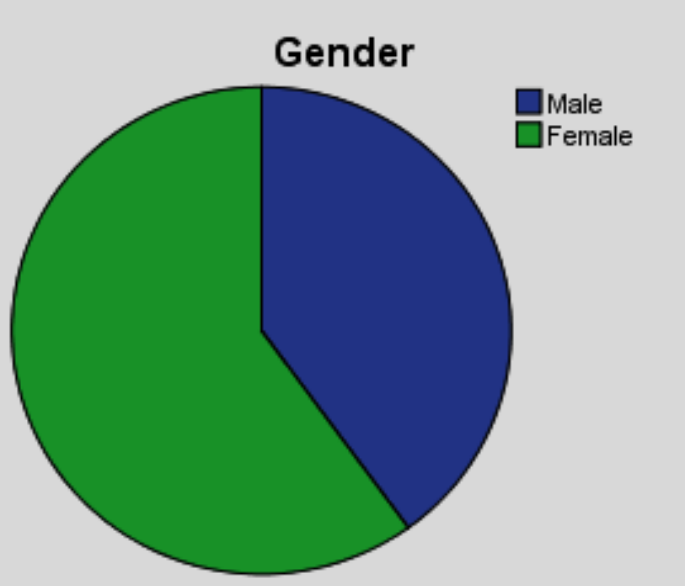

Figure 1: Pie chart shows the distribution of studied patients with OLP lesions according to gender factor.

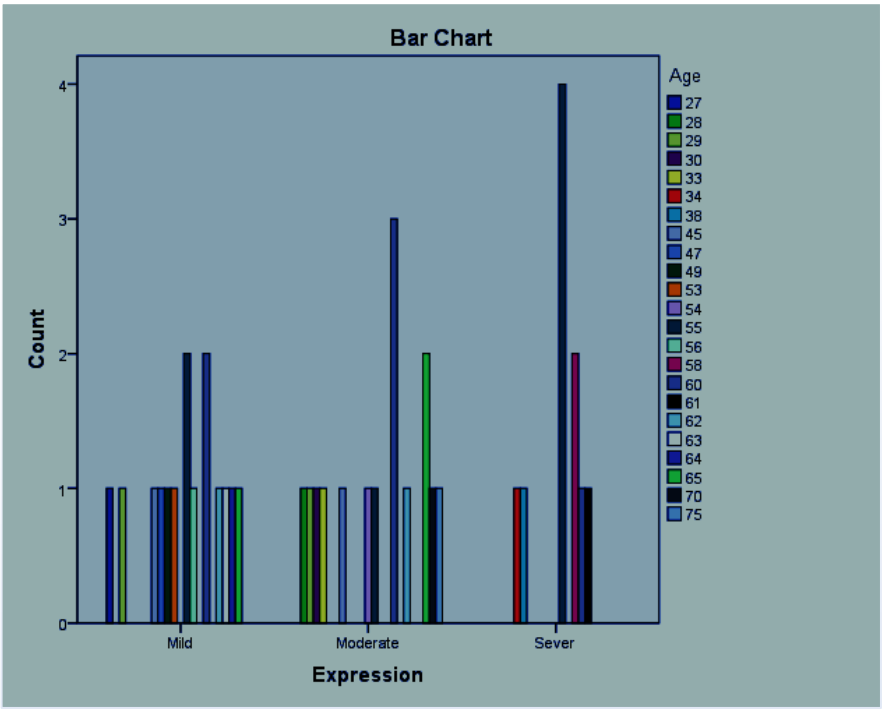

Figure 2: Bar chart shows the distribution of Cyclin D1 expression in regarding to the age groups of the patients with OLP lesions.

Table 2: Shows the frequencies of the CyclinD1 expression according to the clinical types of OLP and comparison's significant.

Clinical types * Expression Crosstabulation					
		Expression of Cyclin D1			Total
		Mild	Moderate	Sever	
Clinical types	Reticular	7	6	4	17
	Erosive	5	5	5	15
	Atrophic	1	2	0	3
	Plaque	1	1	1	3
	Bullous	1	1	0	2
Total		15	15	10	40

Pearson Chi-Square Test (Asymp. Sig. (2-sided), P = 0.942, Likelihood Ratio = 0.863

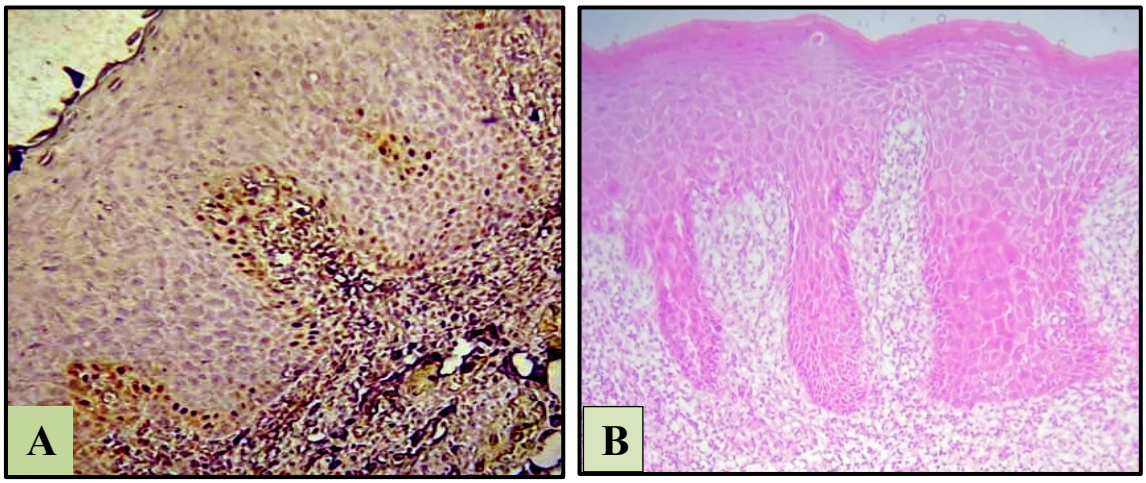

Figure 3: A- Immunohistochemical expression of Cyclin D1 in oral lichen planus, (magnification 20X), B- High power field of basal layer and subepithelial tissue of oral lichen planus (OLP) (Hematoxylin and Eosin staining, x20).

4. Discussion

Understanding the processes that lead to cancer development is critical in explaining how cancer develops. Oncologists may use this information to assess the likelihood of precancerous lesions progressing to malignancy and can use it to apply preventative interventions. So, in this study, we wanted to find out if Cyclin D1 was found in OLP, which is thought to be a condition that can lead to oral cancer.

Carcinogenesis, on the other hand, may arise as a consequence of unregulated cellular proliferation caused by numerous genetic changes coupled with abnormal cell signaling (Carlos et al., 2002; Martin-Ezquerra et al., 2010). Cellular proliferation is regulated by CDKs that interact with cyclin components. CDK4 and CDK6 interact with cyclin D and contribute to G1 phase advancement. By phosphorylating retinoblastoma proteins (pRb), Cyclin D, CDK4, and CDK6 create a complex that promotes cell progression from S to G1 (Poomsawat et al., 2011). Moreover, Bascones et al. proposed that translocates linked to cell cycle regulation may result in an epithelial substrate that promotes the progression of malignancy in OLP (Bascones et al., 2005).

For decades, many studies that estimated the rate of malignant transformation in the OLP lesions were performed by using different techniques (Fitzpatrick et al., 2014; Laniosz et al., 2019; Hwang et al., 2020). Because different types of OLP have the same chance of becoming cancerous, unique clinical criteria can't be used to describe the malignant transformation of OLP conditions (Mattsson et al., 2002; Agha-Hosseini et al., 2011).

In the present study, the most frequent age group was in their sixth decade of life, and women had more incidence than men, with no significant difference in each of them. These findings are in accordance with other studies

reporting that the high-risk groups are older age groups and female patients (Sugerman and Savage, 2002; Hwang et al., 2020), although much lower mean ages have also been documented (Mahboobi et al., 2010), and it appears that the risk is higher in women than in men (Shen et al., 2011; Bombeccari et al., 2011). But other researchers said that men were more likely to get sick in their fourth and fifth decades of life than women. This may be because different studies used different sample sizes when they did their research (Van der Meij et al., 2003; Raju et al., 2005; Tripathi et al., 2018).

In terms of Cyclin D1 expression, our findings showed that there were no significant differences among various clinical categories of OLP. Such findings were similar to earlier studies that found increased Cyclin D1 overexpression in the erosive subtype and deemed the erosive group of OLP to be at a greater risk of malignant alterations, although this variation did not reach statistical significance (Kurago, 2016; Hwang et al., 2020). Furthermore, Rezaee et al. observed that the erosive and atrophic OLP epithelium seems to be more prone to cancer development and is more vulnerable to cancerous exposures than the usual oral mucosa (Rezaee et al., 2013). On the other hand, Gandolfo et al. indicated that the hypothesis of a greater link between atrophic-erosive or plaque forms to cancer progression had been based on isolated case reports as well as no controlled investigations (Gandolfo et al., 2004).

The findings of this investigation revealed that cyclin D1 levels were substantially higher in OLP than in normal mucosa. According to earlier authors who reported that cyclin D1 upregulation is correlated with cellular proliferation of oral epithelial tissues, a large rise in the cyclin D1 index might promote generative conditions in OLP epithelium (Gambichler et al., 2011; Ghallab et al., 2017; Sharada et al., 2018). The overexpression of Cyclin D1 distinguished in OLP caused increased cell proliferation due to shortening in the cell cycle G1 phase (Rezaee et al., 2013; Tripathi et al., 2018).

The present study found that OLP patients had positive Cyclin D1 expression in all OLP samples, which is consistent with prior research (Ghallab et al., 2017), whereas other researchers suggest that not all OLP cases have positive expression. This discrepancy is determined by the kind and conditions of their study (Zhang et al., 2010; Gambichler et al., 2011).

The proliferative capacity of epithelial cells of OLP is associated with changes in the cell cycle regulatory system, suggesting that this highly proliferative state in OLP is a protective technique of the epithelium to preserve its architecture in the presence of vigorous lymphocyte attack(González-Moles et al., 2006).

The increased cell proliferation in OLP promotes the accumulation of cell cycle genetic alterations, which may be regarded as a significant signal of malignant potential in OLP (Rezaee et al., 2013; Ghallab et al., 2017).

Oncogenic effects acting on proliferating cells may induce a malignant cell pattern in OLP individuals. This result supports recent findings that imply amplification of CCND1 at both the protein and gene expression levels in OLP may impair normal cell cycle control, allowing cancer cells to develop (Yao et al., 2007; Abid and Merza, 2014).

The current data indicates that Cyclin D1 inactivation is a recognizable early event in oral carcinogenesis and precancerous oral conditions. Therefore people who had oral lichen planus (OLP) had more CyclinD1 staining than people who had normal oral mucosa.

5. Conclusions

The findings of this study revealed that greater cyclinD1 expression is related to an increased risk of cancer. Due to the absence of an appropriate biomarker that might predict OLP malignancy, various markers should be performed. In the future, more research on Cyclin D1 should have bigger sample sizes and better designs, as well as gene-reduction tests. OLP patients should be closely watched for predictive factors of malignant progression into OSCC. It is very important to know what causes OLP, what causes it to become malignant, and how to properly treat it.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and material
Not applicable.

Competing interests
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Authors' contributions
MM contributed to writing and grammar correction of the manuscript. FD contributed in spelling and punctuation correction of the manuscript. All authors have read and approved the manuscript.

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