Comparative Investigation of Cyclin D1 Marker in Oral Squamous Cell Carcinoma and Oral Lichen Planus
Mehrnaz Alikhāsi1, Faezeh azmoudeh1, Azadehzeinab Titidej1∗
1Assistant professor of Oral Pathology, Dental caries prevention research center, Qazvin University of Medical Science, Qazvin, Iran.

Abstract: Oral lichen planus (OLP) is a chronic inflammatory disease with unknown etiology, and WHO has classified it as a premalignant lesions that could be transformed to oral squamous cell carcinoma (OSCC). Since uncontrolled cellular proliferation is considered the base of malignancy, the aim of this study is the comparison Cyclin D1 immunohistochemical expression in OLP and OSCC. Cyclin D1 immunohistochemical expression was evaluated in 21 samples of hyperplasia without dysplasia, (group :A), OLP (group: B) and OSCC (group: C). Label index of the Cyclin D1 expression was 13.69±6.006, 28.38±3.35 and 66.94±14.49, in A, B, and C groups respectively. Significant difference was found statistically between 3 groups (p<0.001) and between A and B (p<0.009), B and C (p<0.001), A and C (p<0.001). we have seen a statistically significant difference between groups and each 2 groups in semi-quantitative analysis. (p<0.001). The findings of this study with Cyclin D1 showed that cellular proliferation in the lesions of OLP samples is significantly lower than OSCC samples. This will be a warning to clinicians so that patients with OLP, especially those with increased cell proliferation are always followed by regular periodic examinations and detailed and continuous follow-up to detect the slightest changes in lesions in the early stages and provide appropriate treatment.

Keywords: Cyclin D1, immunohistochemy, oral lichen planus, oral squamous cell carcinoma

INTRODUCTION
Oral squamous cell carcinoma (OSCC) is one of the most common cancers around the world [1, 2]. Also, OSCC is considered as a health problem in the international community which the incidence of new cases per year is increasing [3]. This aggressive malignant tumor is followed by morbidity and mortality significantly for patients. Despite impressive progress, the average its 5-year survival is very low, and in recent decades has not improved [1, 3]. Therefore, early diagnosis is necessary to apply appropriate treatment methods in the early stages of the diseases and improve prognosis of patients [2, 3]. Many of OSCC begin after a pre-cancerous lesion (1 and 4). These potentially malignant oral lesions, though not necessarily become Carcinoma, but they are significantly associated with increased risk of OSCC. Oral Lichen Plan (OLP) is discussed as one of these lesions [2].

OLP is a chronic inflammatory mucous-skin disease and considered as the most common disorders that affect the oral cavity, so that even this position may be the only place for the risk of injury. OLP [5] occurs in 2-3% population and most often in adults over 40 years old, women with a ratio of approximately 1.4 to 1 are affected more than men [5, 6]. Cutaneous lichen planus essentially does not the risk of malignant change. Some studies have shown that patients with OLP are at risk of OSCC [6, 8, 9].

Carcinogenesis (carcinogenesis) is a multi-step process which can occur with the advent of successive mutations and epigenetic abnormalities in multiple genes, which in the meantime, cell cycle control genes are important. Regular progress of cells during cell cycle progression is driven precisely by a protein called Cyclin, which play its role by connecting and activation of Cyclin dependent kinases (CDK) [11].

Cyclin D1 is a 45 KDa protein and is coded by the gene CCND1 located on chromosome 13 q 11 [12]. During the different stages of the cell cycle (G1 → S → G2 → M), Cyclin D1 is a part of a molecular system that plays an important role in necessary settings to pass from G1 to S (checkpoint G1/S) [11, 13].

So that the protein after forming the sets of CDK4 and CDK6, leads to protein phosphorylation of retinoblastoma (RB). With phosphorylating RB, inhibiting the activity of transcription factor E 2F is removed and the cell enters the S phase [11, 13, 14].
Unlike the normal function of Cyclin D1 as a key protein, and a positive regulator of cell cycle progression regularly, its overexpression leads to shorten the G1 phase and less dependency of cell on the growth factors. Such circumstances lead to the loss of normal control of cell cycle, and consequently, reproduction (proliferation) of cells in an uncontrolled manner [11-13].

Given that the uncontrolled proliferation of cell is considered as one of the most important biological mechanisms of carcinogenesis [15], and changes in expression of proteins associated with it are considered as the important factors of determining the potential for malignant transformation of lesions [12, 16] in the present study, using Immunohistochemistry (IHC)-staining method, expression of Cyclin D1 in lesions OLP and OSCC was compared and evaluated, so that using the results of this study, the possibility of future malignant transformation in the OLP can be predicted.

**MATERIAL AND METHODS**

This study as a retrospective and descriptive-analytical study was performed using cross-sectional method. After reviewing the archives of Pathology, School of Dentistry, Hamadan University of Medical Sciences and Isfahan Alzahra Hospital over a period of 10 years (1380-1390, Perian date) the samples were selected based on the contents of records and pathology reports patients. Then, all samples were reviewed by 2 pathologists. It should be noted that criteria defined were used by Eisenbrg to confirm the diagnosis of OLP lesions. The eligible samples for dysplasia changes, samples suspected in terms of diagnosis, poor stability, bleeding and necrosis were excluded. Also recording confounding factors risk, such as smoking, alcohol and drugs in the file or the simultaneous detection of other lesions (with original samples) were considered as exclusion criteria.

63 samples of epithelial hyperplasia lesions (control group), lichen planus and oral squamous cell carcinoma were selected (21 samples in each group), it should be noted that hyperplasia samples selected didn’t have dysplasia and squamous cell carcinoma group included 13 samples (61.90 %) having the distinction of good to moderate (grade I & II) and 8 samples (38.10%) having poor distinction (grade III). Immunohistochemical staining of samples with mouse monoclonal anti-Cyclin D1 antibody samples were prepared (Denmark, DAKO, ready-to-use code N1687) and was carried out using peroxide anti-peroxide method as follows:

From each sample, a 4 micron section was prepared, and placed on the slides. The slides were placed at a temperature of 58°C in oven for 24 hours and were passed from two Xylenol containers each for 5 minutes to deparaffinize and they were passed from alcohol at different levels (from absolute alcohol up to 70 degrees) for dehydration. After washing with distilled water at a later stage, in order to recycle antigen the slides were placed in a Citrate buffer solution with 9 = PH. This was placed for 5 minutes with a power of 80w and then 15 minutes in the microwave 450w. After washing the slides (for 15 minutes), and drying them to contain internal peroxide of all samples in hydrogen peroxide 3% were incubated for 20 minutes. After this stage, and in between the next steps, including the addition of primary antibodies, secondary antibodies, the chromogenic Diaminobenzidine (DAB) and Haematoxylin for painting and the Phosphate buffered saline: PBS was used for washing. In the final stage, samples were placed in alcohol with different degrees for dehydration and then put Xylenol for clarification and, ultimately, were mounted. In all the examples Mantle cell lymphoma as a positive control to ensure the accuracy of paint was used. At all stages, the Mantle cell lymphoma sample was used as a positive control to ensure the accuracy of staining. The nucleus was used to ensure accuracy of staining. The nucleus of antibody-stained cells (brown nuclei) was used as staining. The nucleus of antibody-stained cells was used as positive staining. For counting cells stained with Cyclin D1, a light microscope (Olympus BX41, Tokyo, Japan) was used. First, the slides were observed at low magnification (x40) and the areas were determined with a maximum staining intensity. Then epithelial was counted, and the percentage of stained cells, labeling index (Label Index: LI) was calculated for each sample (quantitative analysis). Also the risk of the marker Cyclin D1 was examined by semi-quantitative method. Accordingly, the number of stained cells was less than 1% of, 1-10% equal to +1, 10-35% equal to =2and 35-70% equal to +3 and the samples more than 70% were classified as positive four and studied.

Data obtained was analyzed by using SPSS software version 16 and statistical test one way ANOVA and Tukey (for comparing the quantitative variables between groups), krurskal-wallis and Mann-Whitney (to compare variables semi-quantitative between groups). In all tests, significance level was <0.05.

**RESULTS**

The results related to the distribution of the sample (gender, age, location of the lesion) are presented in Table 1. X² test showed that in three groups, there was no significant statistically difference in gender (0.169 = p), but there was a significant difference between the three age groups (using one way ANOVA) and location involved in lesions (p<0.001).
In the study of immunohistochemical staining, nuclear staining with a marker Cyclin D1 (positive staining) in all groups (100%) was observed (Figure 1) and cell counting was done in accordance with what was described in the materials and methods. The average rate of the risk of marker Cyclin D1 in epithelial hyperplasia group was 13.69 ± 6.00, in OLP group was 28.38±3.53 and in OSCC group was 66.94±14.49. Since the data haven't had normal distribution and had been towards "skewed right", the logarithmic transformation was used for epithelial hyperplasia group 1.09 ± 1.09, OLP was obtained equal to 1.45±0.06 and OSCC equal to 1.82±0.10 and test conditions of the one way ANOVA was established.

The test showed that there is a significant difference in terms of Cyclin D1 in the groups under study (P<0.001). Also, using Tukey test, there was a significant difference between groups in terms of the average percentage of Cyclin D1 staining intensity (P<0.001). Cyclin D1 expression study findings in the group using Semi-quantitative method are shown in Table 2.

There was a significant difference in ratings among the three groups in terms of Cyclin D1 (0.001 <P, Kurskal Wallis Test). This statistically significant difference was observed between the groups in study two by two (0.05 <p, test, Mann-Whitney).

It is noteworthy that according to Table 2, staining intensity less than 35% was observed in all cases of epithelial hyperplasia and OLP groups (100% sample), which all samples of OSCC group (100% of cases) have showed staining intensity higher than 35%.
DISCUSSION

One of the lesions that have been arisen as pre-malignant conditions is Oral Lichen planus [6, 8, 10]. Accordingly, although OLP clinically has special (typical) [5, 6 and quite recognizable features, but a biopsy of the lesion has been recommended for histopathological studies and rejection of the existence of atypical cells and malignancy signs [6]. However, since only clinical or microscopic features don't determine progress and transforming precancerous lesion into malignant lesions, detecting molecular markers that may introduce such features is necessary [19].

Loss of cell cycle control, and consequently, an increase in cell proliferation is considered as the basis of malignant transformation [20] and is used as one of the indicators of the presence of genetic anomalies associated with the development of premalignant and malignant lesions [12]. The activity of cell proliferation can be examined by markers such as PCNA, Ki67 and Cyclin D1 and using immunohistochemistry method [12] which Cyclin D1 marker was used in this study.

In this study, all samples in the control group (100%) showed staining with Cyclin D1 which was similar to the study of Kotelnikov et al. [18] and Hirota et al. [21]. Contrary to these studies, Bascones-Martinez et al. [22] and Turatti et al. [23], expression of this marker has not reported for any of the samples in the control group.

Also, the rate of cell proliferation of the control group in our study was higher than the study of Hirota et al. [21]. The advantage was observed compared to the proliferation of the control group of some other studies (designated with a marker Ki67) [24-26].

It is noteworthy that, in this study, for ethical reasons and the lack of normal mucosa block in the archive of hyperplasia epithelial samples was used as an alternative of the actual control group (normal mucosa), while in the studies compared the control group has been normal mucosa.

In hyperplasia, the growth factors are by binding to specific receptors on the cell surface enable specific signaling pathways inside the cell code, and stimulate cell proliferation, while, it is in a cell normal tissue in a stable state [27]. This may explain the reason of greater cell proliferation of control group in this study compared to other studies (21 V24-26). In our study, like Hirota et al. report [21], the Cyclin D1 expression was observed in all group samples (100%), but the cell proliferation obtained was greater than OLP group proliferation in the study of Hirota et al. Also, compared with other studies (done with other markers of proliferation), the result of our counting was very close to the lee et al (marker PCNA) [28], and less than the Taniguchi et al (marker ki67) [29].

In this study, expression of Cyclin D1 was found in 100% of the OSCC Group. This result is contrary to the Turatti et al. [23] and Bascones-Martinez et al. [22] that the expression of this marker was reported at 66.6% and 46.6% of OSCC samples.

Comparison of to the proliferation of the OSCC Group in this study with existing studies also found that, compared to the Kotelnikov et al. [18] (samples used: SCCs of the head and neck, Cyclin marker D1) and Kurokawa et al. (marker k i67) [25] was greater but similar to the Saito et al. (Marker Ki67 [30]. While taking into account the heterogeneous of tissues examined and sometimes the use of different markers, differences in the studies (especially in cases where the marker used has been similar) may indicate problems in immunohistochemical studies of tissue sections from the paraffin blocks.

It seems that a number of factors, such as the use of non-standard materials and methods for maintenance and fixing tissues and during preparation of paraffin blocks, using various methods of immunohistochemical staining, a type of antibody used (monoclonal/polyclonal) and its manufacturer could affect the results of the review procedure. In this study, the cell proliferation was examined in both groups using quantitative and semi-quantitative method, which indicates a significant increase in proliferation from the control group to OSCC.

In reviewing the two groups bilaterally, the cell proliferation of OLP Group was significantly

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of samples</th>
<th>0 &lt;1%</th>
<th>+1 1-10%</th>
<th>+2 10-35%</th>
<th>+3 35-70%</th>
<th>+4 &gt;70%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial hyperplasia</td>
<td>21</td>
<td>0 (0%)</td>
<td>6(28.5%)</td>
<td>15(71.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Oral Lichen Planus</td>
<td>21</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>21(100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>21</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>11(52.4%)</td>
<td>10(47.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>0 (0%)</td>
<td>6(9.5%)</td>
<td>36(57.1%)</td>
<td>11(17.4%)</td>
<td>10(16%)</td>
</tr>
</tbody>
</table>

Kruskal Wallis test, P<0.001

Table 2: Frequency of the Cyclin D1 marker and its ratings in the groups on the base of the positive epithelial cells

Available online at http://sassociety.com/sasjm/
higher than the control group, the findings were consistent with the results of existing studies (Of course with a control group of normal mucosa and use one of the markers of proliferation). [21, 28, 29 V31-34]. In this study, the greater number of the proliferation in OLP group compared to the proliferation of epithelial hyperplasia may be due to chronic inflammation, and specific release of inflammatory mediators and specific cytokines from inflammatory cells in these lesions. In OLP, some pro-inflammatory cytokine (chemokines) is secreted by T lymphocytes cells, which can absorb mast cells into the lesion, and stimulate their degranulation [5]. Degranulation of mast cells is associated with the release of TNF-α and kinase, which in turn increases UP-regulation of T cells to re-secretion Chemokines [5], so, while hyperplasia epithelium is considered as cell compatibility, whereby, but a situation changed with the new stability has been, achieved [27]. In OLP, a vicious cycle is being repeated while the chronic inflammation seems can affect epithelial cells reproduce [5, 31, 35].

Many studies have investigated the relationship between cancer and inflammation, and findings refer to the relationships between chronic inflammation and cancer positively [36-38]. Inflammatory infiltrate is considered as a strong risk factor for cancer in chronic inflammatory conditions such as ulcerative colitis, Barrett’s esophagus, atrophic gastritis, and recently it has been suggested that, OLP also can be added to this group [3].

Inflammatory cells and their different inflammatory mediators such as the cytokine and chemokine are produced through facilitating angiogenesis, increased production of matrix metalloproteinase, the destruction of the base membrane and increased cellular proliferation of microenvironment for starting malignant transformation and cancer [39]. Increased proliferation is considered as one of the first malignancy indicators and a key event in cancer [20].

Hence, perhaps it can be considered as one of the changes that even in the absence of clinical and histopathologic features of a malignant neoplasm, it represents the initial phase of Synojenesis work, and shows that a seemingly benign lesion has affected by malignant transformations [4]. In this study, cell proliferation in OSCC was significantly higher than the OLP, which was similar to the results of the study of de Sousa et al. [9, 20] and Lee et al. [28] (marker used in the study of PCNA). Compared with OSCC, less number of OLP cellular proliferation in this study may indicate that in this group of Lesions, still some protective mechanisms are active and play their role properly and efficiently. For example, cell proliferation in OLP is discussed as an attempt to maintain the thickness of the epithelium and prevent scarring it [34, 35], but it seems mechanisms protecting prevent cellular proliferation; one of them is removing cells through the activation of the apoptotic pathway (apoptosis cell). For example, Fan et al. [40] demonstrated that overexpression of Bax protein is associated with apoptosis of epithelial cells in OLP.

De Sousa et al. [9] also reported that, with higher cell proliferation in the lesions of OLP, more Bax expression was also observed. This is especially authorized to remove the cells, which have irreversible genetic damage, and by the way, carcinogenic activity on epigenetic is reduced [9].

Another mechanism to deal with increased cell proliferation caused by inflammation in OLP may be significantly an increase in receptors TNF-α of the epithelial cells of this lesion which can play an important role to inhibit the proliferation.

On the other hand, there is a direct relationship between the amount of P16 (protein inhibitor of cell cycle), and the degree of tissue inflammation. Overexpression of this protein in chronic inflammatory stimulations (such as OLP) is associated with high levels of TNF-α, and is done in order to limit the proliferation of the cell to prevent the uncontrolled growth of Malignant-like epithelial cells [41].

Overall, it can be seen that, in the aftermath of cell proliferation in OLP, the process has been controlled and there is a balance between positive and negative regulators. But it should be noted that these results do not necessarily reject the malignant changes in the OLP. It is important to point out that in chronic inflammatory lesions the products are produced by inflammatory cells that by damage cellular DNA, they can play an important role in the protective response of dependent on P53 (tumor suppressor gene) and accumulation of oncogenic mutations act as oncogenic mutation-causing agents for epithelial cells [39].

Thus, with increasing rate of cell proliferation in oral lichen planus, the possibility of presence of mutated cells has increased, which can cause malignancy of the lesion [34]. In this context, the findings of the semi-quantitative review on expression of marker Cyclin D1 in samples of OLP and OSCC in this study are important.

In all the samples OLP studied, the expression of Cyclin D1 was less than 35%, while in OSCC Group, all samples showed greater staining intensity than 35%. Based on this result, the Cut-off level of OLP and OSCC lesions cellular proliferation from each other can be considered as the staining intensity of epithelial cells by 35% with marker Cyclin D1 indicator, and it can be suggested that, in the OLP samples, with less incidence of than 35%, likely mechanisms of protection properly perform their tasks and have the capacity to remove cells with mutation, and genetic variations, and
therefore the genome stability of these lesions is maintained. However, if cell proliferation is more than 35% (crossing points Cut-off), because of overlap and similarity with the rate of cellular proliferation of OSCC lesions, the possibility of mutant cells and defects in the protective mechanisms has increased and the possibility of malignant transformations of these lesions OLP will be increased.

Of course, it should also be noted that, although the increase in cell proliferation has been identified as one of the most important carcinogenesis symptoms, but confirmation and the use of Cut-off suggested taking into account the limitations of this study, such as small sample size and lack of a similar study requires more and more widespread research.

CONCLUSION
Although it has been suggested that patients with OLP are at risk of more OSCC, but there have been differences of opinion about the relationship between OLP and OSCC and malignant potential of OLP is still not approved. The findings of this study with Cyclin D1 showed that cellular proliferation in the lesions of OLP samples is significantly lower than OSCC samples. This will be a warning to clinicians so that patients with OLP, especially those with increased cell proliferation are always followed by regular periodic examinations and detailed and continuous follow-up to detect the slightest changes in lesions in the early stages and provide appropriate treatment.

REFERENCES