Immunohistochemical expression of Cyclin D1 and NF-KB p65 in oral lichen planus and oral squamous cell carcinoma (Comparative study)

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ABSTRACT

Background: Oral Lichen Planus (OLP) is a chronic inflammatory mucosal disease, presenting in various clinical forms. WHO had regarded OLP as a precancerous condition in 1978 because of its potential with cancer. Both antigen-specific and nonspecific mechanisms involved in the pathogenesis of OLP. Oral Squamous Cell Carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity representing more than 94% of oral cancer. It occurs in different sites and has many etiological factors. Cyclin D1 is a proto-oncogene which consider as the key protein in the regulation of cell proliferation and its overexpression led to the occurrence and progression of malignant tumors. NF-KB p65 is a member of NF-kB family of transcription factors that widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival, also plays a major role in inflammation. The aims of this study were to evaluate the immunohistochemical expression of Cyclin D1 & NF-KB p65 in OLP & OSCC & to correlate the expression of the studied markers with the clinicopathological findings and with each other.

Materials and Methods: Fifty (50) formalin – fixed, paraffin – embedded blocks of both Oral Lichen Planus (25 cases) & Oral Squamous Cell Carcinoma (25 cases) were collected prospectively in this study. Hematoxylin & Eosin stain was performed for each block for reassessment of histopathological examination. An immunohistochemical staining was performed using anti Cyclin D1 and anti NF-KB p65 monoclonal antibodies.

Results: Of twenty five OLP studied cases, positive Cyclin D1 & NF-KB p65 expression was found in (84%) and (88%) of cases respectively. For OSCC, out of 25 studied cases, positive Cyclin D1 & NF-KB p65 expression was observe in (88%) and (96%) of cases respectively. Statistically significant correlation between Cyclin D1immunoreactivity and clinical presentation of OLP was found. Statistically significant correlation of Cyclin D1 immunoreactivity with tumor grade and NF-KB p65 expression with tumor stage in OSCC cases was found. Statistically a highly significant correlation between the expression of two studied markers in OLP and OSCC was found.

Conclusion: A highly significant correlation was seen regarding the expression of both markers with each other, suggesting their cooperative role in the pathogenesis of OLP and OSCC.

Keywords: OLP, OSCC, Cyclin D1, NF-KB p65, Immunohistochemistry. (J Bagh Coll Dentistry 2014; 26(1):80-87).

INTRODUCTION

Oral Lichen Planus is a relatively common chronic inflammatory disease of oral mucosa with a prevalence rate of 0.5% - 2.2% of the population. Clinically, OLP may assume a variety of morphological changes (1). The premalignant potential of OLP is still debatable. Malignant transformation has been estimated to occur in 0.5 – 2.9% of the OLP patients. Currently, there are no prognostic markers to identify which chronic OLP lesions are at a higher risk for progression. Thus, every OLP patient should be monitored carefully to detect early cancer development (2).

Oral Squamous Cell Carcinoma is the most commonly diagnosed oral cancer (3). It is the malignancy of stratified squamous epithelium. It remains a lethal disease in over 50% of the cases diagnosed annually, due mostly to late detection of advanced cancer (4). Studying its molecular pathway may help in searching for molecular markers that might predict the clinical behavior of the tumor, which may not strictly related to TNM staging or histological grade (5).

Cyclin D1, a 45 kD (kilo Dalton) protein encoded by Cyclin D1 gene (CCND1) located on chromosome 11q13, is a part of the molecular system that regulates the cell cycle G1 to S transition (6). Overexpression of Cyclin D1 leads to shortening of G1 phase and less dependency on growth factors resulting in abnormal cell proliferation which in turn might favour the occurrence of additional genetic lesions (7). Cyclin D1 expression has been studied in various carcinomas including oral squamous cell carcinomas. Some studies have been carried out to correlate the expression of Cyclin D1 with histological grading of this neoplasm (8). Nuclear Factor-kappa B is a transcription factor that induces the expression of various genes, leading to inflammatory reactions, embryonic morphogenesis, and anti apoptosis. In recent years studies suggested that NF-kB has been implicated in the regulation of cell proliferation, transformation, and tumor development. NF-kB was found to stimulate transcription of cyclin D1, a key regulator of G1 checkpoint control. NF-kB binding site in the human Cyclin D1 promoter conferred activation by NF-kB as well as by growth factors. Both levels and kinetics of CyclinD1 expression during G1 phase were
controlled by NF-xB. Recent advances in biological research have revealed that NF-xB can stimulate neovascularization\(^9\). More ever continuous NF-xB activity protects cancer cells from apoptosis and in some cases stimulates their growth. Therefore, many current anti tumor therapies seek to block NF-xB activity as a means to inhibit tumor growth or to sensitize the tumor cells to more conventional therapies, such as chemotherapy\(^10\).

This study aimed to:
- Evaluate immunohistochemical expression of (Cyclin D1) and (NFkBp65) monoclonal antibodies in oral lichen planus and oral squamous cell carcinoma.
- Correlate the expression of the studied markers with the clinicopathological findings of OLP and OSCC.
- Correlate the expression of the studied markers with each other.

MATERIALS AND METHODS

Twenty-five cases, histologically diagnosed as oral lichen planus and other twenty–five cases, histologically diagnosed as oral squamous cell carcinoma were collected pro- and retrospectively from the archives of Oral Pathology Department, College of Dentistry, Baghdad University, Al-shaheed Ghazi hospital and Teaching laboratories Department, Medical city/ Baghdad from (2004 – 2011). The clinicopathological information regarding age, sex, tumor site, clinical presentation, tumor grading and staging was obtained from the case sheets presented with the specimens. The clinicopathological characteristics of OLP and OSCC patients from which the specimens were taken are illustrated in table 1 and 2 respectively.

Four µm thick sections were cut and hematoxylin and eosin slides were prepared for histopathological reassessment. Another 4 µm thick sections were cut for immunohistochemical staining with anti Cyclin D1 and anti NFkBp65 monoclonal (Abcam UK). Negative and positive controls were included in each IHC run. Tissue blocks of small intestine were used for Cyclin D1 and breast adenocarcinoma blocks were used for NFkBp65 (according to antibodies manufacturer). For immunohistochemistry, the sections were mounted on positively charged slides. Slides were baked in hot air oven at 65°C overnight. Sections were sequentially dewaxed through a series of xylene, graded alcohol and water immersion steps.

### Table 1: Clinicopathological findings of OLP cases

<table>
<thead>
<tr>
<th>Age</th>
<th>Frequency %</th>
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<tr>
<td>20-40</td>
<td>9</td>
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<tr>
<td>41-60</td>
<td>14</td>
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<tr>
<td>age&gt;60</td>
<td>2</td>
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<tr>
<td>Total</td>
<td>25</td>
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### Table 2: Clinicopathological findings of OSCC cases

<table>
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<th>Age</th>
<th>Frequency %</th>
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<tr>
<td>20-40</td>
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<tr>
<td>41-60</td>
<td>12</td>
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<tr>
<td>age&gt;60</td>
<td>10</td>
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<tr>
<td>Total</td>
<td>25</td>
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Antigen (Ag) retrieving was done for both markers as recommended by the manufacturer. Then endogenous peroxidase activity was blocked followed by blocking the non-specific staining. Primary Abs (100 ml) were applied for each section. Cyclin D1 and NF kappa B p65 were diluted into 1/1000 & 1/250 respectively.
After an overnight incubation and washing with phosphate buffered solution (PBS), secondary Abs were applied, incubated and rinsed with a stream of PBS. Primary Abs were visualized with 3,3-diaminobenzidine (DAB) chromogen, then counterstained with Mayer’s hematoxyline, dehydrated and mounted.

**Evaluation of Immunohistochemistry Results:**

In both OLP and OSCC, the cells with clear brown nuclear staining were considered positive for Cyclin D1 immunostaining whereas cells with clear brown cytoplasmic staining were considered positive for NF kappa Bp65 immunostaining within a violet-blue tissue section background of Hematoxyline staining. The immunoreactivity in tumor cells was classified and scored as follows: - Cyclin D1, Negative (-),( 1+) < 1% , (2+) 1-25% , 26-50%, (3+) 50-75% and (3+) >75% (11). For NF kappa Bp65, negative (-) no detectable immunostaining or basal immunostaining in<10%, (1+) mild immunostaining 10-30%,(2+) moderate immunostaining 30-50%. (3+) strong immunostaining >50%.(12).

**Statistical Analysis**

Numerical values were used in this study for describing the variables which includes: No. mean, SD for age, cyclin D1 and NF kappa Bp65. Categorical variable which includes: sites, grade, gender and clinical presentation were described using no. and percentage. Pearson correlation coefficient of correlation (r) was used to find the relation between two markers. ANOVA test (analysis of variance) was used to detect differences for age and two markers .Chi-square test the relationship between categorical variables. Statistical analysis was done using SPSS (statistical package for social sciences) V17 (2008).

**RESULTS**

Immunostaining of Cyclin D1 was detected as a brown staining in the nucleus of target antigen cells, in OLP cases positive IHC Cyclin D1 expression was found in 21 cases (84%),of which, 9cases (36%) showed score 2 immunostaining, 6 cases (24%) with score 3 , 4 cases (16%) score1 and 2cases (8%) score 4. Figures (1,2). In OSCC positive Cyclin D1 IHC expression was found in 22 cases (88%), of which, 7cases (28%) with score 2 immunostaining, followed by 6 cases (24%) score 3, 5 cases (20%) score 4and 4cases (16%) with score1. Figures (3, 4, 5)

Correlating the positive expression of Cyclin D1 with the clinico-pathological findings of OLP revealed statistically significant correlation with clinical presentation (p value= 0.025) , whereas non significant correlation was observed with other clinico-pathological parameters. Table(3). In OSCC, results revealed statistically significant correlation regarding Cyclin D1 expression in relation to tumor grade (p value =0.021),Table(4).While, nonsignificant correlation was observed with other clinico-pathological parameters.

![Figure 1: Positive brown nuclear immunostaining of Cyclin D1 in OLP (400X)](image-url)

**Figure 2: Percentage frequency distribution of Cyclin D1 expression in OLP cases**
Immunostaining of NF-kBp65 was detected as a brown staining in the cytoplasm of target antigen cells. In OLP cases, positive IHC expression was found in 22 cases (88%), of which, 6 cases (24%) showed strong immunostaining followed by 9 cases (36%) showed moderate immunostaining and 7 cases (28%) with mild immunostaining Figures(6,7). In OSCC, Positive NF-kBp65 IHC expression was found in 24 cases (96%), of which, 6 cases (24%) showed strong immunostaining followed by 10 cases (40%) with mild immunostaining and 8 cases (32%) showed moderate immunostaining. Figures (8, 9, 10)
Figure 8: Positive brown cytoplasmic immunostaining of NF-kBp65 in well differentiated OSCC (400X)

Figure 9: Positive brown cytoplasmic immunostaining of NF-kBp65 in moderately differentiated OSCC (400X)

Figure 10: Percentage frequency distribution of NF-kBp65 expression in OSCC cases

Concerning the correlation of Cyclin D1 and NF-kBp65 with each other, results of present study observed statistically a highly significant correlation between the expression of two markers in both OLP (p value =0.007) and OSCC (p value =0.003). Tables (6,7).
owed variable results; these transformation and...This find tokines, HLA molecules, occurrence, progression.

In OSCC and carcinogenic process of OLP. This finding suggests that expression in (71.67%) and (82%) respectively.

Agree with previous studies reporting positive expression of Cyclin D1 in concordance with previous studies.

Cyclin D1 is thought to provide the tumor cells with a tumorogenesis pathways enhancing the cell transformation and the association of strong immunostaining with increased rate of malignant progression of dysplasia might shed light on molecular basis of gene therapy of oral cancer.

In Oral Lichen Planus, 22 (88%) of OLP studied cases showed positive immunoreactivity to NF-kBp65. This finding was agree with (13,18) who found NF-kBp65 positive expression in (85%) and (93%) of OLP cases respectively. Similarly, (19) found positive NF-kBp65-expression in (68%) of oral premalignant cases. In previous Iraqi study, positive NF-kappa Bp65 expression was found in 83.8% of the studied oral dysplastic lesions with low expression score observed in normal oral mucosa indicating the role of this factor in transition from normal oral mucosa to premalignant lesions mostly through prevention of apoptosis (20).

In Oral Squamous Cell Carcinoma, the results of present study showed positive immunoreactivity to NF-kBp65 in 24 of 25 cases (96%). This finding come in accordance with (20,21), who found that NF-kBp65 was expressed in (100%) and (80%) of OSCC cases respectively. Moreover, (19,22) also found that NF-kBp65 was expressed (68%) & (78.38%) of OSCC cases respectively. Increased activation of NF-kB has been shown to occur in premalignant dysplastic lesions and approximately 85% of HNSCCs, indicating it is an early event in carcinogenesis and the association of strong immunostaining with increased rate of malignant progression of dysplasia might shed light on molecular basis of gene therapy of oral cancer (23,24). The variations observed in the immunopositivity rate among different studies could be due to the antibodies used and the processing techniques performed, as well as the heterogeneity of OLP&OSCC which could be attributed to the epidemiological or biological differences between countries and population.

**Assessment of Cyclin D1 Immunohistochemistry**

Cyclin D1 is a positive regulator of cell cycle, and it exerts its effects on the Rb pathway, lead to release the E2F transcription factors thereby allowing the cells to enter the S phase. Overexpression of Cyclin D1 may lead to shortening of G1 phase, increased cell proliferation and reduced dependency on growth factors. This may contribute to disturbance in the normal cell cycle control and mitogenic signaling pathways enhancing the cell transformation and tumorigenecity. Thus, overexpression of Cyclin D1 is thought to provide the tumor cells with a selective growth advantage (13).

**In Oral Lichen Planus**, results of present study showed positive Cyclin D1 expression in 21 cases (84%) of OLP studied cases. This finding was agree with (13,14) who found Cyclin D1 positive expression in (71.67%) and (82%) respectively. This finding suggests that Cyclin D1 may play important role in the occurrence, progression and carcinogenic process of OLP (14).

**In OSCC**, 22 of 25 cases (88%) showed positive immunoreactivity to Cyclin D1. This finding was in concordance with previous studies (15,16) who reported positive expression of Cyclin D1 in (85.7%) and (95%) respectively. These studies support the role of Cyclin D1 as a potential marker of proliferation and oncogenesis.

**Assessment of NF-kB Immunohistochemistry**

NF-kB is a signal transcription factor found to play an important role in aberrant gene expression and the malignant phenotype of SCC, other studies revealed that expression of a very large number of genes is stimulated by NF-kB. These genes encode members of various classes of proteins that are involved in the inflammatory response such as cytokines, HLA molecules, tumor necrosis factor, cellular adhesion molecules, acute phase proteins, and growth factors (17).

**In Oral Lichen Planus**, 22 (88%) of OLP cases respectively. Increased activation of NF-kB was found to stimulate transcription of cyclin D1, a key regulator of G checkpoint control. Two NF-kB binding sites in the human Cyclin D1 promoter conferred activation by NF-

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**Table 6: Correlation of Cyclin D1 with NF-kBp65 expressions in OLP**

<table>
<thead>
<tr>
<th>CyclinD1</th>
<th>Pearson Correlation</th>
<th>Sig. (2-tailed)</th>
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**Correlation is significant at the 0.01 level (2-tailed).**

**Table 7: Correlation of Cyclin D1 with NF-kBp65 expressions in OSCC**

<table>
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**Correlation is significant at the 0.01 level (2-tailed).**

**DISCUSSION**

Concerning the epidemiological parameters, including age, sex, site, clinical presentation, studies showed variable results; these inconsistent findings among different studies could be credit with the fact that the current study and some of the others are not an epidemiological type of studies, therefore the limited number and the random selection of the cases according to what is available preclude for definitive clinical findings.

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κB as well as by growth factors. Both levels and kinetics of Cyclin D1 expression during G phase were controlled by NF-κB. Moreover, inhibition of NF-κB caused a pronounced reduction of serum-induced cyclin D1-associated kinase activity and resulted in delayed phosphorylation of the retinoblastoma protein (25).

It has been reported that on one hand Cyclin D1 can inhibits NF-κB transcriptional activity through a co repressor function (26), and on the other hand, NF-κB is activate the transcription of cyclin D1 gene, which then increase the expression of Cyclin D1 protein (27).

Furthermore, (28) found that the regulation of Cyclin D1 transcription by Ral GTPases is dependent on NF-κB activation and is mediated through an NF-κB binding site in the Cyclin D1 promoter. Despite variances in mechanism, it is well established that NF-κB are strong inducers of Cyclin D1 gene expression (29,31). The present study showed statistically significant correlation between Cyclin D1 and NF-κB in OLP & OSCC cases. Similarly, (22) also found a correlation between Cyclin D1 and NF-κB in oral leukoplakia and oral squamous cell carcinoma.

Moreover he reported that there was a significant correlation between the expression of NF-κB and Cyclin D1 during mammary gland carcinogenesis in TA2 mice with spontaneous breast cancer. Journal of Experimental and Clinical Cancer Res 2010; 22: 29–36.


