RESEARCH ARTICLE

Suitability/Unsuitability of Cell Proliferation as an Indicator of Malignant Potential in Oral Lichen Planus: an Immunohistochemical Study

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Abstract

Background: Oral lichen planus (OLP) is categorized as premalignant lesion although its malignant potential is a matter of controversy. The objective of this study was to investigate Ki67 expression in OLP, oral epithelial dysplasia and oral squamous cell carcinoma (OSCC). Materials and Methods: Expression of Ki67 was evaluated through immunohistochemistry (IHC) in groups of A (17 cases of epithelial hyperplasia), B (16 cases of OLP), C1 (10 cases of mild epithelial dysplasia), C2 (10 cases of severe epithelial dysplasia), D1 (10 cases of well-differentiated OSCC), and D2 (10 cases of poorly-differentiated OSCC). Results: There was a significant difference in Ki67 expression based on quantitative analysis among the six studied groups as well as group B compared bilaterally with groups C2, D1 and D2 ($p < 0.0001$). However, there was no significant difference between groups B and C1 or between groups D1 and D2 ($p > 0.05$). Conclusions: Based on the results of the present study it may not be possible to definitely consider malignant transformation potential for OLP. However, expression of Ki67 was significantly higher in OLP than that of epithelial hyperplasia with no significant difference from that of mild epithelial dysplasia. This should be considered by clinicians to carefully and regularly follow up OLP lesions to detect potential subtle changes at an early stage.

Keywords: Immunohistochemistry - Ki67 - oral squamous cell carcinoma - oral lichen planus - oral epithelial dysplasia

Asian Pac J Cancer Prev, **14 (11)**, 6979-6983

Introduction

Oral mucocutaneous diseases (a heterogeneous group of disorders) could involve skin and mucous membranes as one of the most common of them is oral lichen planus (OLP) frequently found in patients visiting dental clinics (Jaafari-Ashkavandi et al., 2011; Lin et al., 2011; Gorugantula et al., 2012).

OLP is a chronic inflammatory and T cell-mediated autoimmune disease related to stratified squamous epithelia having a wide range of clinical manifestations (Roopashree et al., 2010; Georgakopoulou et al., 2012). Its prevalence is about 1 to 2% among adults who are 40 years old or elder mostly occurring during the 6th decade of life with an approximate ratio of 1.4 to 1 for women to men (Roopashree et al., 2010; García-García et al., 2012).

This chronic inflammatory oral mucosal disease has also been considered as a potential precancerous disorder (de Sousa et al., 2009a ;García-García et al., 2012). It was suggested that patients with OLP are at risk of developing oral squamous cell carcinoma (OSCC) although actual malignant transformation potential of OLP remains a matter of controversy (de Sousa et al., 2009a; Jaafari-Ashkavandi et al., 2011; Shen et al., 2011). According to Safadi et al. (2010) some previous studies rate of developing cancer in OLP has been reported from 0.5-12.5% (Safadi et al., 2010). However, other studies failed to show potential malignization of OLP (Rödström et al., 2004; Brzak et al., 2012).

Carcinogenesis is a multistep process whereas carcinoma can be preceded by premalignant lesions although they do not necessarily progress to malignancy (Siar et al., 2011; Dragomir et al., 2012). One of the mechanisms essential for cancer to develop is enhancement of proliferation capacity (de Sousa et al., 2009a; 2009b). Cell proliferation rate can be evaluated through immunohistochemical study of proliferation related antigens (Watanabe et al., 2010; Bologna-Molina et al., 3013).

Ki-67 is a proliferating cell nuclear antigen and one of the most common markers widely used for this propose (Watanabe et al., 2010; Ranganathan and Kavitha, 2011; Bologna-Molina et al., 3013).

This non-histonic nuclear protein expresses in G1,

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S, G2 and M phases (all active phases of cell cycle) but not in G0 phase (resting cells) (Watanabe et al., 2010; Ranganathan and Kavitha, 2011; Bologna-Molina et al., 2013). It is revealed that Ki67expression in premalignant and malignant oral lesions increases according to their degree of malignancy (Montebugnoli et al., 2009; Ranganathan and Kavitha, 2011; Dragomir et al., 2012) and increased cellular proliferation might have a potential to be an initial indicator to malignant transformation potential of a lesion (de Sousa et al., 2009a; 2009b).

Objective of this study is to evaluate and compare cell proliferation rate in OLP , epithelial dysplasia and OSCC through immunohistochemical expression of Ki67.

Materials and Methods

The samples used in this study consisted of 4 groups as follows: *Group A*: 17 cases of epithelial hyperplasia (with no dysplasia); *Group B*: 16 cases of OLP; *Group C*: 20 cases of epithelial dysplasia including 10 mild and 10 severe dysplasia, subgroups C1 and C2 respectively; *Group D*: 20 cases of OSCC including 10 well-differentiated and 10 poorly differentiated OSCC , subgroups D1 and D2 respectively.

Histopathological diagnostic criteria described by Eisenberg, (2000) were used for oral lichen planus diagnosis. Any case exposed to risk factors of oral cancer such as smoking/drinking behaviors were excluded. Additionally, the medical records of samples had no history of use of medications.

Immunohistochemistry

Immunohistochemical staining of Ki67 was performed on 4-µm thick paraffin sections mounted on poly-L-lysine coated glass slides by Envasion TM technique. Deparaffinzed of the sections was conducted using xylene and then rehydrated in descending grades of ethanol, respectively. In order to block endogenous peroxidase the sections were covered in metanol with 3% hydrogen peroxide for 15 minutes and then for antigen retrieval the slides were boiled in citrate buffer (PH=6) for 20 minutes using microwave. The primary antibody used for Ki67 detection was MIB-1 (Clone MIB-1, code N1633, ready-to-use N-series primary antibody,Glostrup, Dako, Denmark) as the slides were incubated for 60 minutes with antibody. After washing in the buffer solution, Envasion was carried out for 30 minutes. Subsequently, the sections were washed with the buffer solution for 5 minutes and then the reaction was revealed through 3,3/-diaminobenzidine tetrahydrochloride (DAB) application. Finally, the sections were counterstained with Harris hematoxylin stain then dehydrated, and mounted.

Immunohistochemical evaluation

A light microscope (Olympus BX41, Tokyo, Japan) was applied to evaluate immunostaining of Ki67.

Ki67 immunoreactivity

Nuclear staining was considered as Ki67 positive immunoreaction among all of the samples and quantitative analysis was realized based on following steps: *i*) Low magnification was applied to identify areas having the highest levels of Ki67 expression; and *ii*) One thousand cells were counted using high magnification (×400) and percentage of positive cells was regarded as labeling index (LI).

Statistical analysis

In this study statistical analysis used were One-way ANOVA and Tukey tests (SPSS, version 16) and $p \leq 0.05$ was considered as statistically significant.

Results

In the present study, the samples evaluated belonged to 13 men (76.47%) and 4 women (23.52%) in group A, 1 man (6.25%) and 15 women (93.75%) in group B, 11 men (55%) and 9 women (45%) in group C, and 11 men (55%) and 9 women (45%) in group D. The mean age in groups A, B, C and D were 46.23±15.19, 38.37±12.77, 57.65±12.03 and 64.45±14.06 years, respectively.

The Ki67 immunostaining findings

Ki67 expression was observed in groups A,B,C and D (Figures 1-5) as the related labeling indices are presented in Table 1. Additionally, Ki67 LI calculated for the subgroups belong to C and D were as follows; *i*) 12.13±1.51% and 26.19±5.56% for the mild epithelial dysplasia (C1 subgroup) and severe epithelial dysplasia (C2 subgroup), respectively; and *ii*) 37.92±6.04% and 44.53±7.21% for the well-differentiated OSCC (D1 subgroup) and poorly-differentiated OSCC (D2 subgroup), respectively.

Ki67 LI had increased from A to B, C and D respectively statistically significant at $p<0.001$. Two-by-

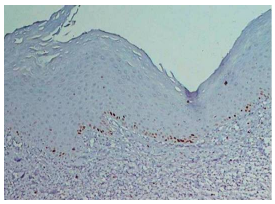

Figure 1. Nuclear Ki67 Immunostaining in Epithelial Hyperplasia with no Dysplasia, ×100

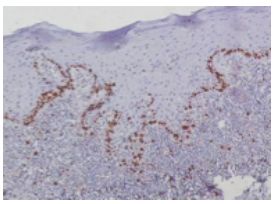

Figure 2. Nuclear Ki67 Immunostaining in OLP, ×100

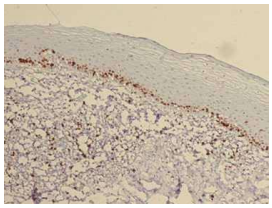

Figure 3. Nuclear Ki67 Immunostaining in Mild Epithelial Dysplasia, ×100

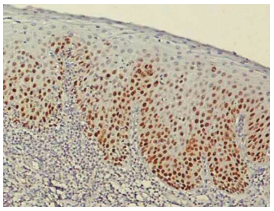

Figure 4. Nuclear Ki67 Immunostaining in Severe Epithelial Dysplasia, ×400

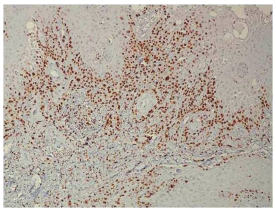

Figure 5. Nuclear Ki67 Immunostaining in OSCC, ×100

Table 1. Quantitative Results of Ki67 Immunostaining (Ki67 LI) in Studied Groups

±SD:(%)	Ki67 LI:(%)	Group:(n)
1.91%	5.14%	A:(17)
2.45%	13.88%	B:(16)
8.23%	19.16%	C:(20)
7.31%	41.22%	D:(20)

two comparisons of the groups although were significant at $p<0.001$ except for B and C comparison ($p=0.077$).

Moreover, LI values differences were compared between the studied groups of A, B, C1, C2, D1 and D2 all significant at $p<0.001$. However, comparison of two-by-two differences for the six mentioned groups was evaluated as no significant for B, C1 ($p=0.720$) and D1, D2 ($p=0.606$).

Discussion

During last decades many studies considered risk of OSCC development for OLP as it was classified as a premalignant condition (de Sousa et al., 2009a; Jaafari-Ashkavandi et al., 2011; Shen et al., 2011).

OLP is a T cell-mediated chronic inflammatory disease of the oral mucosa (Roopashree et al., 2010; Georgakopoulou et al., 2012).

According to the previous studies there is a relationship between chronic inflammation and various cancers as inflammatory infiltration is a potent risk factor for cancer development in inflammatory conditions like ulcerative colitis, Barrett's esophagus and atrophic gastritis. Recently, it has been proposed that OLP can be included in this group (González Moles et al., 2009; Liu et al., 2010; Georgakopoulou et al., 2012). In OLP, secretion of proinflammatory cytokines by T cells will result in the infiltration of mast cells into the lesion and their degranulation. Release of factors such as TNF-α and chymases from mast cells results in the upregulation of T lymphocytes to secrete chemokines once again. Such a cyclical mechanism will not only be followed by chronic inflammation in OLP (Roopashree et al., 2010), but also it might play a role in the initiation of malignant changes and development of cancer in this lesion (Mignogna et al., 2004; Liu et al., 2010). Some molecules and radicals produced by inflammatory cells can act as mutagenic agents for epithelial cells (González Moles et al., 2009; Liu et al., 2010).

In addition, in such situations a microenvironment is created based on inflammatory cells and cytokines, which can affect important mechanisms involved in the regulation of cell cycle such as apoptosis, arrest of cell cycle and cell proliferation or differentiation (Mignogna et al., 2004; González Moles et al., 2009; Liu et al., 2010). Evaluation of cell proliferation has provided important information in relation to the diagnosis and prognosis of some cancers. An increase in cell proliferation capacity might be one of the first indicators of malignant transformation and a key event for the development of cancer (de Sousa et al., 2009b).

In the present study, cell proliferation in OLP (group B) was higher than that in the control group (group A) with statistically significant difference between the two. Due to ethical considerations and lack of normal tissue blocks the control group in the present study consisted of epithelial hyperplasia with no dysplasia. It is considerable that result of comparison between groups A and B in this study was similar to other studies in which OLP have been compared to normal mucosa regardless of the evaluation methods and cell proliferation markers applied (Tipoe et al., 1996; Hirota et al., 2002; Taniguchi et al., 2002; Lee et al., 2005; González Moles et al., 2009; Hadzi-Mihailovic et al., 2012). In contrast, Bloor et al. (1999) reported no difference for Ki67 expression between the OLP and normal mucosa (Bloor et al., 1999). Destruction of the basement membrane in OLP results in the exposure of epithelial cells to an abnormal environment at the epithelial-stroma interface (Montebugnoli et al., 2011). In such a condition, epithelial cells are injured by an inflammatory infiltration of submucosal mononuclear cells (Taniguchi et al., 2002; Liu et al., 2010).

The injured cells in OLP target some complex molecular mechanisms in order to arrest cell cycle (to repair their damaged DNA and maintain genomic stability) or in order to activate apoptosis pathway (to eliminate cells with severity damaged DNA) (Hirota et al., 2002; Taniguchi et al., 2002; Bascones-Ilundain et al., 2008; Liu et al., 2010; Georgakopoulou et al., 2012). In addition, these injured cells can enter cell cycle and proliferate (Liu et al., 2010; Georgakopoulou et al., 2012)

Although mechanisms involved in an increase in proliferation are still unknown (Hirota et al., 2002), one of the mechanisms might be related to the release of cytokines and inflammatory mediators from injured keratinocytes or inflammatory cells in OLP (da Silva Fonseca and do Carmo, 2001; Liu et al., 2010; Mattila et al., 2011).

An increase in cell proliferation might result in a suitable ground for malignant transformation (de Sousa et al., 2009b). A high rate of proliferation in OLP is potentially associated with a high risk for the presence of mutated cells during mitosis, which might result in a malignant phenotype in this lesion (González Moles et

al., 2009).

In the present study proliferation rate of OLP lesions was evaluated and compared to epithelial dysplasia and OSCC. While Ki67 LI was 13.88±2.45% in OLP (group B), 19.16±8.23% in epithelial dysplasia (group C) and 41.22±7.31% in OSCC (group D). There was no significant difference between B and C; however, there was significant difference in Ki67 expression between B and D. Similar to the results of the present study Sousa et al. (2009a) reported higher cell proliferation rate (by PCNA marker) in dysplastic group compared to OLP group. However, contrary to the results of the present study, there was significant difference between the two groups (Sousa et al., 2009a).

In addition, similar to the present study, Hadzi-Mihailovic et al. (2012) observed that Ki67 was less expressed in OLP compared with OSCC (Hadzi-Mihailovic et al., 2012).

Similarly, de Sousa et al. (2009a) reported a lower proliferation rate, determined by PCNA marker, in OLP compared to OSCC with significant difference between the two groups.

In another study by de Sousa et al. (2009b), evaluation of cell proliferation in OSCC, dysplastic and OLP cases showed significant difference between OLP and OSCC . However, there was no significant difference between OLP and epithelial dysplasia (de Sousa et al., 2009b), which was similar to those of the present study regardless of the evaluation methods and cell proliferation markers applied.

Also, Lee et al. (2005) reported a lower proliferation rate (PCNA marker) in OLP compared to dysplastic and OSCC lesions (Lee et al., 2005).

In this study, Ki67LI in group B was less than that of in group C, which might indicate less growth potential in OLP lesions, possibly indicating a lower aggregation of genetic mutations compared to dysplastic lesions (de Sousa et al., 2009b). On the other hand, there was no significant difference between OLP and epithelial dysplasia indicating an equal potential for these two groups to develop malignancy. Considering the two points above, there appears to exist a lack of consistency in interpretation of the results. Therefore, Ki67 LI of OLP was separately evaluated and compared to degree and severity of dysplasia of lesions in group C. The obtained results were considerable as Ki67 expression in OLP was very close to that in mild dysplasia (12.13±1.5%) with no significant difference between the two groups. However, proliferation rate in the severe dysplasia group (26.19±5.56) was significantly higher than that in the OLP group.

Ki67 is a good predicative marker for cells which have undergone genetic changes and it has been recently shown that there is a relationship between loss of heterogeneity (LOH) and Ki67LI (Montebugnoli et al., 2009). Therefore, based on the obtained results it might be concluded that the number of cells with genetic alterations in OLP is different from that in severe dysplasia and similar to that in mild dysplasia and it can be suggested that severe dysplastic epithelium has a higher susceptibility to malignant transformation compared to OLP and mild dysplasia cases; in addition, malignant transformation potential in OLP and mild dysplasia is higher than that in epithelial hyperplasia with no dysplasia.

From statistical point of view and regarding to the similar Ki67 expression rate in OLP and mild dysplastic lesions , it might be possible to consider OLP as a premalignant lesion in the current study but review of literature shows it is not possible to express a definitive idea in this regard as: *i*) Some inflammatory features in OLP, appeared due to many growth factors released to epithelial cells, might be similar to what is seen in premalignant and malignant lesions (Sousa et al., 2009b) especially those features indicating an increase in proliferation (Sousa et al., 2009c); Hence, it seems activating factors for some of cellular mechanisms might be different from each other (such as inflammation vs genetic instability) among the studied groups. Nevertheless, activity outcome for those mechanisms which are evaluated by IHC markers might demonstrate similarly, like nuclear staining. *ii*) It is suggested that an increase in proliferation in OLP is an attempt to preservation of epithelial structure in this lesion (Liu et al., 2010) and is a defense mechanism against epithelial ulceration (González Moles et al., 2009). But this cell proliferation mechanism might be under control as normal defense and protective mechanisms of cells are suitably functioning parallel to a proliferation increase . For example, de Sousa et al. (2009a) reported that the higher the proliferation rate, the more the expression of Bax (proapoptic protein) in OLP (de Sousa et al., 2009a). In addition, it has been reported that with an increase in cell proliferation rate due to inflammation in OLP there is a significant increase in the TNF-α receptors of keratinocytes in this lesion, which might have a role in the inhibition of proliferation (da Silva Fonseca and do Carmo, 2001). Mantebugnoli et al. (2011) reported that over expression of P16 in OLP and its relationship with TNF-α are associated with limiting cell proliferation in inflammatory processes so that uncontrolled growth of malignant-like epithelial cells would be prevented (Montebugnoli et al., 2011). Therefore, it seems some mechanisms acting against proliferation in OLP eliminate cells those have sustained irreversible genetic injuries. This results in a decrease of carcinogens' activity in epithelium (de Sousa et al., 2009a).

As a whole, based on the results of the present study it may not be possible to definitely consider malignant transformation potential for OLP. But in this study expression of Ki67 was significantly higher in OLP than that of epithelial hyperplasia with no significant difference from that of mild epithelial dysplasia. This should be consider by clinicians to carefully and regularly follow up OLP lesion to detect potential subtle changes at early stage.

Acknowledgements

The authors would like to thank the Hamadan Dental Research Center, Hamadan University of Medical Sciences for supporting this study with grant number 890804123048 . The authors do not have any conflict of interest.

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