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Original Article

Immunohistochemistry Analysis of P53 and Ki-67 Proteins in Oral Lichen Planus and Normal Oral Mucosa

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Abstract

Background: Oral lichen planus (OLP) is a relatively common chronic inflammatory muco-cutaneous disease classified among the potentially malignant lesions of oral mucosa. In this study we evaluated tissue expression of p_{53}، ki-67 in OLP compared to normal oral mucosa.

Methods: Specimens (formalin-fixed and paraffin- embedded) of 44 lesions of OLP and 30 controls referred to immunohistochemistry (IHC) analysis for p_{53} and ki-67. Results of immunohistochemistry were statistically evaluated by means of Kolmogorov- Smirnov, Kruskal- Wallis and *t*-test. The level of statistical significance was established at $P< 0.05$.

Results: This study included 44 patients with OLP (27 females and 17 males) and 30 subjects as control group (18 females and 12 males).The mean expression of p_{53} in patients with OLP was greater than subjects in control group. The mean expression of ki- 67 in patients with OLP was more than people in control group. These differences were statistically significant ($P= 0.001$).

Conclusion: Since p_{53} and ki-67 extensively accepted as important biomarkers in diagnosis, prognosis and treatment of malignant and premalignant lesions, therefore high degree of presence of these biomarkers in chronic premalignant lesion like OLP can be a great helpful in its prognosis and suggested treatments.

Keywords: *Oral lichen planus, Immunohistochemistry, P_{53}; ki-67*

Introduction

Oral lichen planus (OLP) is a relatively common chronic inflammatory disease that probably represents a cell-mediated immunological response to an induced antigenic change in the skin or mucosa in predisposed subjects (1). The prevalence of the oral lesions in the general population has been variously reported as being between 0.1% and 4% (2). The mean age of onset is the fifth decade of life, and there is an obvious female predominance. Although OLP may occur at any oral mucosal site, the buccal mucosa is the most common site of involvement. In contrast to cutaneous lichen planus, oral lesions have a prolonged clinical course, and the oral mucosal involvement may persist for many years. OLP can be painful, especially in the atrophic and erosive forms (3-6). Since the first case reported of a squamous cell carcinoma developing from a mucosal lichen planus, the real odds of such transformation, is a matter of discussion. Thus, several studies have shown different proportions of malignant potential of OLP, in general varying between 0.04% and 1.74%. Hence, many authors have accepted the idea that OLP is an actual premalignant lesion (7-11). WHO classifies OLP as a premalignant condition, however, the underlying mechanisms initiating development of cancer in OLP lesions are not understood (6, 12). Histopathologically, lichen planus is characterized by hyperkeratosis, degeneration of the basal cell layer, the appearance of Civatte bodies and a dense lymphocytic infiltration in a band like pattern in the lamina propria (4, 13-15).

The normal p53 gene acts as a tumor suppressor and its wild-type acts inhibiting proliferation and oncogene-mediated proliferation and transformation. Cells that contain p53 genes of the wild type are able to delay cell cycle to allow the repair of damaged DNA, or divert the cell into apoptosis.

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When the protein is mutated or absent, the cells replicate the damaged DNA, which will result in more mutations and chromosome rearrangement. Mutations in the p53 tumor suppressor gene are the most common molecular defects in human malignancies, including Oral squamous cell carcinoma (OSCC) (7). These mutations may result in the formation of defective, highly stabilized protein with an increased half-life in tissues compared with the 20 min for the wild-type protein. This is the basis for the use of immunohistochemistry that can detect mutant p53 products, which are indicative of mutant protein. Other mechanisms may similarly stabilize the protein and so increase the amount detected, while conversely, deletion of p53 gene ("null allele") may produce a false negative result (7, 16).

Many studies correlate immunohistochemical p53 staining with prognosis, histologic gradation and clinical behavior of premalignant lesions, including OLP and lichenoid dysplasia (7).

Ki-67 is a nuclear protein doublet nearly 395kD and can be detected on phases G1, S, G2 and M of the cell cycle, but not on G0 phase, exclusively in the nuclei of cycling cells. This defined period of nuclear expression makes the Ki67 protein a reliable marker of proliferating cells. Located predominantly in the nucleoli, the Ki67 epitope may be associated with nucleolar RNA. The ki-67 labeling index (LI), i.e., the percentage of cells in a tissue staining for ki-67 is an indicator to predict the condition of OLP as a pre-malignant lesion (7, 17).

The aim of this study was to evaluate the expression of p_{53} and ki-67 in OLP compared to normal oral mucosa.

Material and Methods

Sample collection

In this study, we analyzed oral mucosal tissue samples from 78 patients who were clinically diagnosed with lichen planus (presence of bilateral lesions and- Presence of reticular lesions elsewhere in the oral mucosa) In the Oral Medicine Department, School of Dentistry, Medical Sciences, University of Tehran during 2004 and 2006.

As controls, 35 samples of normal oral mucosa were obtained from Implant, Surgery, and Periodontics Departments during surgical operations for implant placement, impacted third molar extraction, and gingival graft.

Written informed consents were obtained from all patients.

Forty four patients with oral lichen planus (27 females, mean age 47.56 and 17 males, mean age 45.24) and a control group of 30 subjects (18 females, mean age 42.67 and 12 males, mean age 45.5) comprised this study. The control and case groups matched in gender and age and this process lasted six months.

Methods

Fresh biopsy was obtained from all subjects with lichen planus after the application of toluidine blue (for the selection of biopsy site) and also from all subjects in control group during above- mentioned surgical procedures.

All tissue specimens (formalin-fixed and paraffin-embedded) were cut with 4 thickness, and mounted on slides. To confirm diagnosis in lichen planus group, one section of each case was stained with Hematoxylin & Eosin (H&E).For Immunohistochemistry, slides were placed in oven with 56° C dry heat for 30 min for deparaffinization, then washed in alcohol and xylol solutions. Slides were placed in citrate buffer in T/T mega instrument with 98° C heats for 5 min for antigen retrieval. Then they were washed in Phosphate Buffered Saline (PBS). For blocking the endogenous peroxidase, sections were incubated in 3% H_2O_2 for 10 min. Slides were placed in distilled water and PBS, then incubated with primary antibody (p_{53} antibody Dakocytomation, RTU and ki-67 antibody zymed company England, diluted, 1:100) for 1 h and washed with PBS. In this stage, the slides were incubated with Biotinylated secondary antibody for 10 min and washed with PBS. Then the slides were incubated with horseradish peroxidase (HRP) for 10 min and washed with PBS. Application of diaminobenzidine hydrochloride chromogen for 10 min and washing with tap water were performed. Slides were counterstained

with hematoxylin and rinsed in tap water and were mounted. For negative controls, the same procedure was carried out with normal serum instead of each antibody. As positive control for p_{53} immunostaining, breast cancer tissue was stained and for ki-67 immunostaining and appendix tissue was also used. The quantitative analysis of positive cells for mutative p53 and Ki67 was accomplished by only one observer. Only cells that presented nuclear brown-colored staining were considered positive. It was only enough to distinguish positive from negative cells to p53 and Ki67 and the intensity of staining was not considered. All of slides were observed by light microscopy x 400 magnification, the selected field for counting being randomly chosen .Counting the percentage of positive nuclei in 400 consecutive epithelial cells of selected areas representative of the lesion gave a semi-quantitative evaluation of the immunohistochemical results. In addition the sites of positive cells (basal and Para basal layers) were also evaluated (Fig.1, 2).

Statistical analysis

Statistical measures (mean and standard deviation) were calculated for continuous variables. For discrete variables frequency distributions were used. Distribution normality assumptions of p_{53}،ki-67 expression and age were assessed by the Kolmogorov- Smirnov test. In some comparisons when normality would not hold, non parametric Kruskal-Wallis test were used. The level of statistical significance was established at $P< 0.05$.

Results

The group of patients with OLP comprised 27 women and 17 men with a mean age of 46.66 yr. The control group comprised 18 women and 12 men with aged within the age range of cases. The majority of the lesions were located on the buccal mucosa (43.2%), followed by the tongue (24.7%), gingivae (16%), lip (12.3%), and floor of mouth (3.7%). Also, most of the lesions were erosive (61.4%), followed by atrophic (38.6%).

The mean of p_{53} expression in males with OLP (19.53) was greater than males in the control group (2.55), this was also in females with OLP (26.31) compared with females in the control group (2.11), and in general, in patients with OLP (23.89) was greater than subjects in control group (2.28). These differences were statistically significant (P= 0.026, 0.001, 0.001, respectively) (Table1).

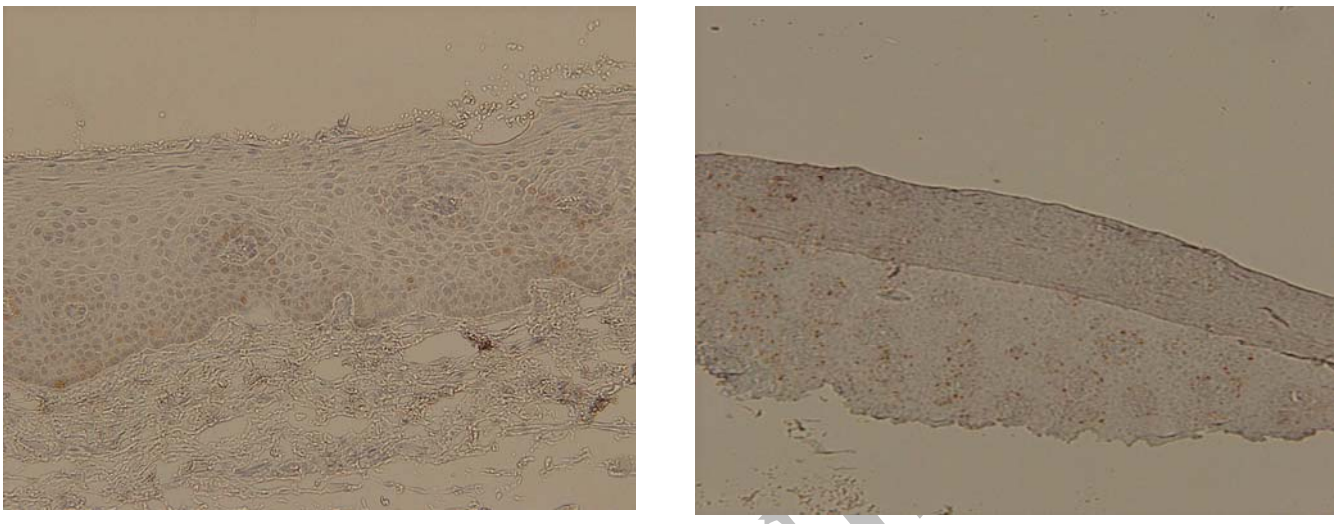
The 95% confidence interval for mean of p_{53} expression was 16.35 to 31.43 in OLP group and 0.698 to 3.85 in control group.

The mean of ki-67 expression in males with OLP (51.29) was more than males in control group (6.6), also in females with OLP (53.54) compared with females in control group (7.81), and in general, in patients with OLP (52.67) than subjects in control group (7.33). These differences were statistically significant (P= 0.001) (Table1).

The 95% confidence interval for mean of ki-67 expression was 45.15 to 60.18 in OLP group and 3.22 to 11.42 in control group.

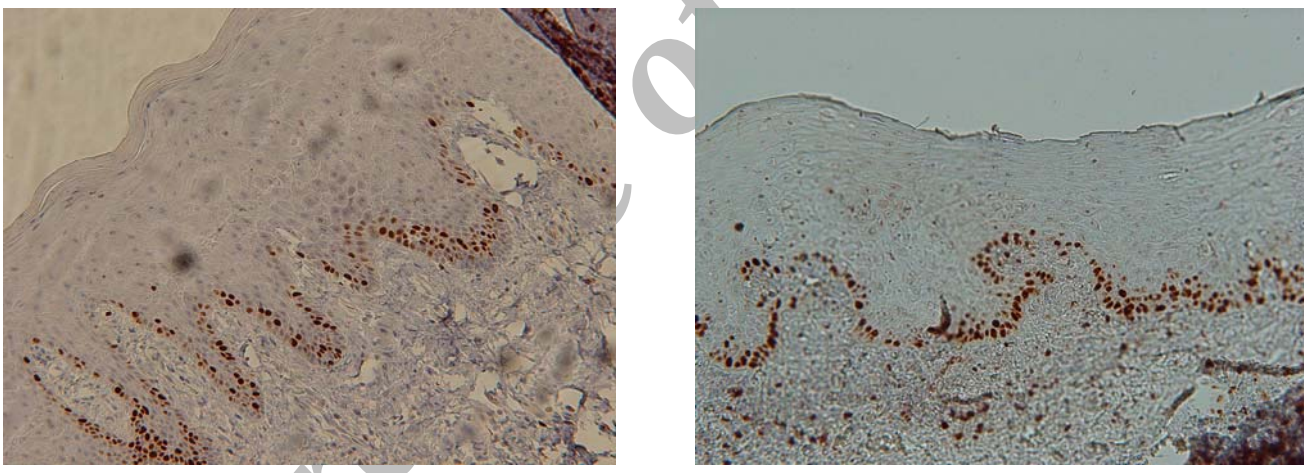
Table1: Indicators of p53 and ki-67 in relation to demographic variable (gender) of case and control groups

Variable	Sex	Group	Number	Mean ± SD	*P*
(%) p_{53}	Female	Control	18	2.11 ± 2/85	.001
		Case	27	26/31 ± 25.78	
	Male	Control	12	2/55 ± 5/85	.026
		Case	17	19/53 ± 21/2	
	Total	Control	30	2/28 ± 4/15	0.001
		Case	44	23/89 ± 24/2	
(%) K67	Female	Control	18	7/81 ± 11/45	0.001
		Case	27	53/54 ± 26/24	0.001
	Male	Control	12	6/6 ± 10/67	0.001
		Case	17	51/29 ± 22/84	
	Total	Control	30	7/33 ± 10/97	0.001
		Case	44	52/67 ± 24/73	



1A 1B

Fig. 1: A. p53 immunostaining of control mucosa (original magnification X200) and **B.** P53 immunostaining of olp mucosa (original magnification X100)



2A 2B

Fig. 2: A. Ki-67 immunostaining of control mucosa (original magnification X200) and **B.** Ki-67 immunostaining of olp mucosa (original magnification X200)

Discussion

The assessment of changes at the molecular level may become the primary means of diagnosis and may guide management since these changes mediate morphologic changes that occur after genetic changes, and knowledge of current morphologic changes is based on the subjective assessment of clinical and histopathologic changes (4).

OLP is a chronic immunologic mucocutaneous disease. The mean age at the time of diagnosis is approximately 45 yr, and is more common in females than males. In this study, the mean age of patients with OLP was 46.66 yr and the number of females with OLP was more than males. This is consistent with previous findings (4, 13).

OLP consists of various clinical forms. In our study, according to internationally accepted criteria, the clinical forms were characterized by reticular keratosis plus erythema (atrophic variety) and/or erosion/ulcerations (erosive variety) (18).

It has been believed that the atrophic, erosive, and ulcerative forms predispose the mucosa to damage from carcinogenic agents (19). In our study, the number of patients with erosive and atrophic lesions were 27 (61.4%) and 17 (38.6%), respectively. Similar studies did not discuss the clinical forms of OLP (15, 20, 21).

In our study, the buccal mucosa (43.2%) was the most common site, followed by the tongue (24.7%), gingivae (16%), lips (12.3%), and floor of mouth (3.7%). In one study, all samples were obtained from the buccal mucosa (15), and in another study, the samples were provided from the buccal mucosa (n=14), tongue (n=12), and floor of mouth (n=2) (22). Other studies, did not explicit the locations of OLP (20, 21).

The possible malignant transformation of oral lichen planus (OLP) is the subject of an ongoing and controversial discussion in the literature. The main criticism of studies on this subject relates to the lack of sufficient data to support the initial diagnosis of OLP in cases that finally developed into squamous cell carcinoma (19). The occurrence of squamous cell carcinoma in most series ranges from 0.4 to 2.0% per 5 yr observation period (23). The most recent and extensive study reported a rate of 1.5% for patients observed during 7.5 yr (24). The presence of dysplasia in OLP increases the risk of malignant transformation, mandating management and close follow-up (9). Potentially malignant lesions are those occurring in a morphologic altered tissue. In order to analyze the malignant potential, it is important to consider that oncogene activation and inactivation of tumor suppressor genes occur precociously in carcinogenesis (7).

P_{53} is a tumor suppressor gene, whose activity stops the formation of tumors. Alteration in p_{53} expression and/or absence of activity of wild-type p_{53} has important roles in cancer development in humans (25). Most authors agree that anti-p53 antibody D07 shows priority targeting to the wild form of p53 protein. Some authors have believed that the antibody used to detect p53 protein (clone DO7), reacts only with its mutant form, thus revealing the presence of mutation in the protein (7).

The results of the present study show the frequent expression of p53 based on the use of monoclonal antibody D07.

The mechanism underlying p53 over-expression in OLP is also subject to controversy. The expressions of p53 and active proliferation status in OLP have also been reported by a few authors (26). A number of authors estimate that p53 over-expression constitutes a form of cell response to the hyper-proliferative state frequently seen in OLP (27).

Warnakulasuriya and Johnson suggested that the presence of p_{53} gene mutation may be used as a marker of risk in a high proportion of malignant and potentially malignant oral lesions (25). The results of their study evaluated various malignant and premalignant lesions other than OLP is consistent with our finding and previous report (7).

Valente et al could not conclude about the molecular pathway leading to neoplastic transformation of OLP, or about the role of p_{53}, the results of their study (The number of subjects in the case and control groups is less than our study) indicated that immunohistochemical evaluation of p_{53} expression may be a practical tool to select cases of OLP with high risk of neoplastic transformation (28). Of course we cannot rule out the possibility that some of the cytogenetic nonrandom anomalies observed represent early steps in cancer development (7).

Ki-67 is the protein that plays a pivotal role in maintaining cell proliferation. This protein is used as a prognostic marker in many tumors. The proliferative index determined by the number of cells stained by Ki67/MIB-1 per number of tumoral cells counted has proven to be a prognostic factor in several neoplasms (11). However, in potentially premalignant lesions its value is still being analyzed (7).

The increased proliferative activity of the epithelium in our patients in case group stressed by the results of Ki67 and p53 analysis, which showed significantly higher mean values of both indices than control group. Over- expressions of Ki67 and p53 in OLP patients have been reported in other studies and OLP has been postulated to have a secondary proliferative disorder probably due to

repeated breakdown of the cycling cells leading to an increased state of proliferation (27).

In another study was found a strong correlation between p_{53} over- expression and cell proliferation (ki-67) (29). This result is consistent with our result; however, this study evaluated various malignant and pre-malignant lesions other than OLP.

In conclusion, since p_{53} and ki-67 extensively accepted as important biomarkers in diagnosis, prognosis and treatment of malignant and pre-malignant lesions, high expression of these biomarkers are useful for the identification of OLP lesion with a more aggressive pattern and with a major tendency to OSCC development

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The authors declare that there is no conflict of interests.

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