Original Article

Expression of Ki-67, p53, α -SMA and COX-2 in lichen planus and related lesions: A pilot study

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ABSTRACT

Background: Researchers have struggled to understand the natural history of lesions presenting with both lichenoid features and epithelial dysplasia. Thus the present study was designed to differentiate between OLP, OLP with dysplasia, epithelial dysplasia and epithelial dysplasia with lichenoid features based on the expressions of ki-67, p53, COX-2, and α -SMA.

Materials and methods: Formalin-fixed paraffin-embedded archival specimens of OLP, OLP with dysplasia, epithelial dysplasia and epithelial dysplasia with lichenoid features were subjected to immunohistochemical staining with ki-67, p53, COX-2, and α -SMA.

Results: Ki-67 exhibited strong positivity in 100% (6/6) of epithelial dysplasia cases, 71.4% (5/7) of lichenoid dysplasia cases, 57.1% (4/7) of OLP cases and 60% (3/5) of OLP with dysplasia cases. Strong p53 staining was evident in more cases of lichenoid dysplasia [42.8% (3/7)], while moderate staining was more frequent in OLP cases [42.8% (3/7)] and OLP with dysplasia cases [42.8% (3/7)] and mild intensity was more frequent in epithelial dysplasia cases [50% (3/6)] followed by lichenoid dysplasia cases [42.8% (3/7)], OLP cases [28.5 (2/7)] and OLP with dysplasia cases [40% (2/5)]. COX-2 strong positivity was more frequent in cases of epithelial dysplasia cases [57.1% (4/7)] and OLP [50% (3/6)]. Strong α -SMA staining was noted more frequently in lichenoid dysplasia cases [71.4 (5/7)], followed by OLP cases [42.8% (3/7)] and OLP with dysplasia cases [60% (3/5)].

Conclusions: Ki-67, p53, α -SMA and COX-2 expression do not differentiate between OLP, LP with dysplasia and epithelial dysplasia with lichenoid features.

1. Introduction

Krutchkoff and Eisenberg's¹ paper on oral lichenoid dysplasia (OLD) had ignited a spiraling debate as to the malignant potential of oral lichen planus (OLP). They claimed OLD to be a separate histopathological entity, which was responsible for the many false reports of malignant transformation of OLP. Krutchkoff and Eisenberg's conclusion of epithelial dysplasia manifesting with lichenoid features were substantiated in the recent papers published by Patil et al.² and Fitzpatrick et al.³ However, Patil et al.² also observed features of dysplasia in OLP and OLL in their case series, reiterating the malignant potential of OLP and OLL. It is therefore very important for the pathologist to differentiate OLP from epithelial dysplasia with lichenoid features, as it has

significant implications for diagnosis, research and controversies surrounding OLP with regards to its malignant potential. Both genetic and epigenetic factors are implicated in the malignant transformation of dysplastic epithelium. To understand the molecular biology of this transformation one has to analyze the changes induced in both the epithelium and the associated connective tissue.

COX-2 has been reported to be overexpressed in dysplasia compared with normal tissue. Further, the COX-2 expression has been correlated with higher grades of oral epithelial dysplasia and could be an early event in oral carcinogenesis.⁴ Arreaza et al.⁵ reported an increased expression of COX-2 in OLP in contrast to OLL, whereas, Cortes et al.⁶ observed an opposite trend. Overexpression of COX-2 was found to be associated with oncogenic genetic alterations. They also concluded that

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their findings suggested a different etiology and molecular pathophysiological pathways for OLP and OLL.

Chaudry et al. concluded that the presence of myofibroblasts, as measured by the α -SMA expression, increases as the disease progresses from oral mucosa premalignancy to invasive OSCC. Thus, overexpression of myofibroblasts can be used as an indicator of malignant transformation.⁷

OLP with high proliferative index has been associated with an increased risk of malignant transformation. Valente et al.⁸ found a direct correlation between the expression of Ki-67 and malignant potential of OLP. Similar to ki-67, the overexpression of p53 was associated with an increased malignant risk in OLP.⁹

With this view in mind, we evaluated the expressions of ki-67, p53, COX-2 and α -SMA between OLP, OLP with dysplasia, epithelial dysplasia and epithelial dysplasia with lichenoid features. An attempt has been made to differentiate between these lesions based on the expression of these markers.

2. Materials and methods

The study was approved by the scientific and ethics committee of the institution. Slides of oral lesions diagnosed as oral epithelial dysplasia (OED) and OLP were retrieved from the archives of the Department of Oral Pathology, Faculty of Dental Sciences, M.S.Ramaiah University of Applied Sciences. Cases of OED were re-evaluated for the presence of lichenoid features and OLP were re-confirmed and were checked for dysplastic features. Subsequently, formalin fixed paraffin embedded (FFPE) blocks of the corresponding slides; OLP (n = 7), OLP with dysplasia (n = 5), OED (n = 6) and OLD (n = 7) were retrieved, sectioned and stained with the IHC markers ki-67, p53, COX-2, and α -SMA. The slides were compared and evaluated for the positivity and stronger expressivity of the IHC markers. Following criteria were used for the diagnosis of the lesions:

OLP: Well-defined subepithelial inflammatory band predominantly consisting of lymphocytes, signs of basal cell liquefaction degeneration and absence of epithelial dysplasia with a clinically consistent diagnosis of OLP (bilateral reticular pattern, no habit history, and no medications).

OLP with dysplasia: Well-defined subepithelial inflammatory band predominantly consisting of lymphocytes, signs of basal cell liquefaction degeneration and presence of epithelial dysplasia with a clinically consistent diagnosis of OLP (bilateral reticular pattern, no habit history, and no medications).

OED: Dysplastic features were assessed using the WHO 2005 criteria¹⁰ in cases diagnosed clinically as leukoplakia/erythroplakia.

OLD: A histopathological diagnosis of OED using WHO 2005 criteria with a clinically consistent diagnosis of leukoplakia/erythroplakia. Additionally, histopathology may reveal either a well-defined subepithelial inflammatory band predominantly consisting of lymphocytes or diffuse inflammation consisting predominantly of lymphocytes or lymphocytes admixed with plasma cells.

The demographic data for the archival slides were extracted from the corresponding case records and are presented in Table 1.

2.1. Immunohistochemistry

Section of 3–4 μ m thickness was taken from the Formalin-fixed blocks on the PLL-coated slides and kept in a Hot air oven for 20 min at 75°, following which the sections were deparaffinized by immersing them in xylene for 10 min (two changes). The slides were subjected to a series of graded alcohols of decreasing concentration (100%, 100%, 95% and 70%) followed by exposure to tap water. Further dehydration of the slide was prevented. The slides were subjected to antigen retrieval by placing them in EDTA (pH8) for α -SMA, Ki67, Cox-2 and Sodium Citrate (pH6) for p53. Following antigen retrieval, the temperature of the slides was reduced to the room temperature, following

Table 1
Demographic Data of the cases.

Demographic Data		OED (n = 6)	OLD (n = 7)	OLP (n = 7)	OLP with dysplasia (n = 5)
Age	< 40	1	3	2	–
	> 40	5	4	5	5
Gender	Male	6	3	3	3
	Female	–	4	4	2
Localization	Buccal mucosa	5	6	7	5
	Labial mucosa	1	–	–	–
	Gingiva	–	1	–	–
	Tongue	–	–	–	–
Evolution time	≤ 6 months	5	7	4	2
	> 6 months	1	–	3	3
Habits	Smoking	5	1	2	1
	Smokeless tobacco	3	3	–	–
	Both	2	–	–	–
	smokeless and smoked tobacco	–	–	–	–

OED: oral epithelial dysplasia, OLD: oral lichenoid dysplasia, OLP: oral lichen planus, n: number of cases.

which the slides were placed in Buffer solution for 5 min (PBS, phosphate buffer solution) which in turn was followed by application of peroxide block (0.3% hydrogen peroxide in methanol) for 5 min. Rinsed in buffer solution 2 times for 2 min. Then power block was applied for 10 min, rinsed in buffer solution 2 times for 2 min. Primary antibody for ki-67, antihuman p53 protein for p53 (1:50), α -SMA (1:100) and COX-2 (1:100) (Biocare Medicals, USA) were added and incubated for 1 h, following which the slides were subjected twice to wash buffer for a period of 2 min. The buffer washed slides are subjected to the Poly HRP (horse radish peroxidase enzyme-polymer, Biocare Medicals, USA) reagent for a period of 40 min, following which the slides were subjected thrice to wash buffer for a period of 2 min. The slide was subjected to Diacetyl bromoacetic acid (DAB) for a period of 10 min, following which the slides were subjected to wash buffer. The slides were stained for 2 min with hematoxylin, following which the slides were subjected to water wash for 5 min. The slides were subjected to ascending concentration of alcohol for dehydration. Following dehydration, the slides were exposed to xylene. Finally, diphenylene phthalate xylene was used to mount the slide. Simultaneously along with the study specimens, the positive and the negative controls were run. For positive control of Ki-67 and p53 squamous cell carcinoma cases were used, for α -SMA blood vessel walls served as positive internal control and for COX -2, ulcerative colitis case was used. For the negative control, the non-immune mouse serum was used instead of the primary antibody.

2.1.1. Evaluation of the IHC staining

The lesions to be studied were categorized into 4 groups: ED, OLD, OLP, and OLP with dysplasia.

Several parameters including the expression pattern, staining intensity and the labeling index (LI) were used to analyze the expression of p53 protein and ki-67 antigen.

The color of the IHC stain was used to evaluate its intensity as follows: negative (–, no color), mild (+, light brown color), moderate (+ +, dark brown color) or strong (+ + +, very dark brown color). Based on the level of involvement of the epithelium, the staining distribution was assessed as follows: basal layer, basal and suprabasal layers, all epithelial layers. Epithelial cells with a clear brown nuclear stain were considered positive. P53 expression pattern was semi-quantitatively assessed from the number of positive cells per 100 basal cells and it was expressed in percentage as follows: 1 = 0–25%; 2 = 26–50%;

Fig. 1. Photomicrograph of epithelial dysplasia group showing (1a) H&E staining, (1b) Ki67-nuclear staining, (1c) p53-nuclear staining, (1d) COX2- cytoplasmic staining and (1e) alpha SMA-cytoplasmic staining. (Total magnification 40 $\times$).

3 = 51–75%; 4 = 61–99%. The proportion of Ki67 positive cells in the basal layer was assessed as follows: 1 (10–30%), 2 (30–50%) and 3 (> 50). A specific area was selected in each section based on the maximum number of positive cells.

2.2. α -SMA

The stromal cells (non-inflammatory and non-endothelial) were assessed for the percentage of α -SMA immunopositivity. The intensity of staining was assessed as follows: No staining, Positivity under 40 $\times$ – 1, Positivity under 10 $\times$ but not under 4 $\times$ – 2 and Positivity under 4 $\times$ – 3.

The frequency of positive cells in specific areas was scored as 0% (no positive cells), 1 (1–33% positive cells), 2 (34–66% positive cells), and 3 (67–100% positive cells).

The staining index of each specimen was calculated by multiplying their percentage and intensity scores. The staining index was assessed as follows: zero (0), low (1–2), moderate (3–4); and high (6–9).¹¹

2.3. COX- 2

A combination of both the staining intensity & extent was used to assess the COX-2 immunostaining, which was scored as 0 (negative), 1 (mild), 2 (moderate), and 3 (strong).

Staining extent was scored as 0 (0%), 1 (< 10%), 2 (10–50%), and 3 (> 50%), according to the percentage of positively stained cells. The weighted COX-2 score for each case was calculated by multiplying the scores of the staining intensity and extent (maximum possible, 9). COX-2 immunostaining was considered positive if at least a moderate staining intensity (2 or 3) existed in a minimum of 10% tumor cells (weighted score of ≥ 4 out of 9).¹²

3. Results and observation

Ki-67 exhibited strong positivity in 100% (6/6) of epithelial dysplasia cases, 71.4% (5/7) of lichenoid dysplasia cases, 57.1% (4/7) of OLP cases and 60% (3/5) of OLP with dysplasia cases. The moderate intensity in lichenoid dysplasia was noted in 28.5% (2/7) of cases and mild intensity was noted in 14.2% (1/7) of lichen planus cases [Fig. 1b, 2b, 3b and 4b].

Strong p53 staining was evident in more cases of lichenoid dysplasia [42.8% (3/7)], while moderate staining was more frequent in OLP cases [42.8% (3/7)] and OLP with dysplasia cases [42.8% (3/7)] and mild intensity was more frequent in epithelial dysplasia cases [50% (3/6)] followed by lichenoid dysplasia cases [42.8% (3/7)], OLP cases [28.5% (2/7)] and OLP with dysplasia cases [40% (2/5)] [Fig. 1c, 2c, 3c and 4c].

COX-2 strong positivity was more frequent in cases of epithelial dysplasia cases [57.1% (4/7)] and OLP [50% (3/6)]. Moderate staining was more frequent in OLP with dysplasia cases [60% (3/5)] when compared to the other group of lesions [Fig. 1d, 2d, 3d and 4d].

Strong α -SMA staining was noted more frequently in lichenoid dysplasia cases [71.4 (5/7)], followed by OLP cases [42.8% (3/7)] and OLP with dysplasia cases [60% (3/5)] [Fig. 1e, 2e, 3e and 4e].

Although subtle variations were observed in the staining intensity of each marker among the 4 examined entities, the variations were not of any statistical significance as depicted in Table 2.

4. Discussion

Malignant transformation of lesions is associated with significant changes in the expression of proteins involved in apoptosis and cell proliferation.¹³ The obtained results from epithelial dysplasia, lichenoid dysplasia, OLP and OLP with dysplasia indicated that cell proliferation index in these four groups of lesion favors the risk for malignant

Fig. 2. Photomicrograph of lichen planus showing (2a) H&E, (2b) Ki67-nuclear staining, (2c) p53-nuclear staining, (2d) COX2- cytoplasmic staining and (2e) alpha SMA-cytoplasmic staining. (Total magnification 40 $\times$).

transformation. Ki-67 is often used as an adjunct marker to assess the proliferative activity of potentially malignant lesion.¹⁴ Pigatti et al. studied the expression of Bcl-2 and Ki-67 in OLP and leukoplakia with epithelial dysplasia. They found that expression of Ki-67 in most OLP sections (42.9%) and leukoplakia sections (64.3%) indicated positivity in > 50% of cells. None of the samples of normal oral mucosa showed positivity in > 50% of cells.¹⁴ Acay et al. studied the Ki-67 and p53 expression in OLP and OLL. They did not find any statistical difference in Ki-67 staining but p53 had a significant higher index in OLP than OLL. The intensity of the inflammatory infiltrate was considered as a possible reason for the difference in p53 expression.¹⁵

In our study intensity of staining for Ki-67 was strong in epithelial dysplasia (6/6) followed by lichenoid dysplasia (5/7), OLP (4/7) and OLP with dysplasia (3/5) suggesting of high proliferation rate of epithelial cells; moderate intensity in lichenoid dysplasia (2/7) and mild in one of seven case of lichen planus. Although Ki-67 immunoexpression was not statistically different between ED, LD, OLP, and OLP with dysplasia.

In our study according to the intensity of staining, strong expression of p53 was more in lichenoid dysplasia (3/7) compared to the other group of lesions suggesting of high proliferation rate of epithelial cells, moderate intensity was more in OLP (3/7) and OLP with dysplasia (3/7) and mild intensity was more in epithelial dysplasia (3/6), lichenoid dysplasia (3/7), OLP (2/7) and OLP with dysplasia (2/5). ED, LD, OLP, and OLP with dysplasia did not reveal any statistical difference in the immunoexpression of p53. Oliveira Alves et al. evaluated p53, MDM2, and SUMO-1 expressions in OLP. The results showed OLP, oral epithelial dysplasia and, to a lesser extent, OLP and oral squamous cell carcinoma (OSCC) to have similar expression of p53 and MDM2.¹⁶ de Sousa et al. evaluated the immunohistochemical expression of PCNA, p53, Bax, and bcl-2 in OLP and epithelial dysplasia and found that no

statistically significant differences between the expression of p53 and bcl-2 in OLP and epithelial dysplasia.¹³

The COX-2 enzyme is often expressed during inflammation. It aids in tissue repair, angiogenesis, cell proliferation and differentiation, but chronic, persistent inflammation as noted in OLP and OLR can lead to detrimental effects.⁵

Inflammation is strongly associated with carcinogenesis, including the development and progression of oral cancer.^{17,18} Cancer susceptibility of an individual is related to the differential expression of COX-2, which in turn is a result of polymorphisms at the COX-2 promoter level. Of all the potentially malignant disorders of the oral cavity, the malignant transformation of OLP is most perplexing.⁶

COX-2 overexpression is often associated with epithelial carcinogenesis. Several studies including that of Arreaza et al. found a higher COX-2 expression in OLP than OLR, with Chankong et al., observed a direct correlation between OLP's COX-2 expression and its clinical severity.¹⁹ In contrary, Cortes et al. found a higher COX-2 expression in OLR than in OLP.⁵ According to the results of the current study, staining intensity of COX-2 was strong in epithelial dysplasia and OLP compared to the other group of lesions suggesting high cell proliferation in dysplasia and chronic inflammatory reaction in lichen planus. Moderate in epithelial dysplasia and OLP with dysplasia.

According to the results of the current study, staining intensity of α -SMA was strong lichenoid dysplasia, followed by OLP and OLP with dysplasia compared to epithelial dysplasia, suggesting the presence of more number of myofibroblasts. Joshi et al. evaluated stromal myofibroblasts in epithelial dysplasia and oral squamous cell carcinoma using α -SMA and found that, among 20 cases of OED, 6 (30%) cases were positive and 14 (70%) were negative for α -SMA expression.²⁰

We failed to demonstrate the value of p53, ki-67, COX-2, and α -SMA to distinguish clearly between epithelial dysplasia, lichenoid dysplasia,

Fig. 3. Photomicrograph of lichen planus with dysplasia showing (3a) H&E staining, (3b) Ki67-nuclear staining, (3c) p53-nuclear staining, (3d) COX2- cytoplasmic staining and (3e) alpha SMA- cytoplasmic staining. (Total magnification $40\times$).

Fig. 4. Photomicrograph of lichenoid dysplasia showing (4a) H&E staining, (4b) Ki67-nuclear staining, (4c) p53-nuclear staining, (4d) COX2- cytoplasmic staining and (4e) alpha SMA- cytoplasmic staining. (Total magnification $40\times$).

Table 2

The intensity of Ki-67, p53, α -SMA and COX-2 immunohistochemical staining in different groups.

Marker	Lesions	n	Intensity of staining				p value
			Mild	Moderate	Strong	Negative	
Ki-67	OED	6	–	–	6	–	0.151
	OLD	7	–	2	5	–	
	OLP	7	1	–	4	2	
	OLP with dysplasia	5	–	–	3	2	
p53	OED	6	3	2	–	1	0.060
	OLD	7	3	1	3	–	
	OLP	7	2	5	–	–	
	OLP with dysplasia	5	2	3	–	–	
α -SMA	OED	6	2	1	2	1	0.259
	OLD	7	1	1	5	0	
	OLP	7	2	1	3	1	
	OLP with dysplasia	5	0	1	3	1	
COX-2	OED	6	0	3	3	0	0.223
	OLD	7	3	2	2	0	
	OLP	7	2	1	4	0	
	OLP with dysplasia	5	1	3	0	1	

OED: oral epithelial dysplasia, OLD: oral lichenoid dysplasia, OLP: oral lichen planus, n: number of cases.

OLP and OLP with dysplasia. Long-term prospective studies with periodic follow-up are vital in identifying any potentially malignant changes.

Within the limitations of this study, immunohistochemistry demonstrated expression of important proteins (p53, Ki-67, α -SMA and COX-2) related to regulatory mechanisms of proliferation, inflammatory changes and stromal myofibroblasts in epithelial dysplasia, lichenoid dysplasia, OLP and OLP with dysplasia, suggesting that the environment may be favorable to malignant transformation. However, the expression of p53, Ki-67, α -SMA and COX-2 was not significantly different between the four lesions, suggesting that alterations of these markers do not give a clear value to differentiate these lesions using these markers. To our knowledge, there are no studies in the literature investigating the expression of p53, Ki-67, α -SMA, and COX-2 in ED, lichenoid dysplasia, OLP and OLP with dysplasia, and further studies with larger sample size are necessary to distinguish these lesions and their malignant potential.

Conflict of interest

None declared.

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