Expression of cdk4 and p16 in Oral Lichen Planus

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ABSTRACT

Objectives: The purpose of this study was to evaluate the expression of cdk4 and p16, the proteins implicated in hyperproliferation and arrest in oral lichen planus and to compare their expression in erosive and non-erosive oral lichen planus and with normal mucosa and oral squamous cell carcinoma.
Material and Methods: Analysis of cdk4 and p16 expression was done in 43 erosive oral lichen planus (EOLP) and 17 non-erosive oral lichen planus (NOLP) cases, 10 normal mucosa and 10 oral squamous cell carcinoma (OSCC) cases with immunohistochemistry.
Results: This study demonstrated a significantly increased expression of cytoplasmic cdk4 (80% cases, cells stained - 19.6%), and cytoplasmic p16 (68.3% cases, cells stained - 16.4%) in oral lichen planus (OLP) compared to normal mucosa. cdk4 was much higher in OSCC in both cytoplasm and nuclei compared to normal mucosa. Also, while comparing OLP with positive control, significant difference was noted for cdk4 and p16, with expression being more in OSCC. While comparing EOLP with NOLP; significant differences were seen for cdk4 cytoplasmic staining only, for number of cases with positive staining as well as number of cells stained.
Conclusions: Overexpression of cytoplasmic cdk4 and p16 was registered in oral lichen planus, however considerably lower than in squamous cell carcinoma. Erosive oral lichen planus demonstrated overexpression of cytoplasmic cdk4 and premalignant nature compared to non-erosive lesion. Therefore there is an obvious possibility for cytoplasmic expression of cdk4 and p16 to predict malignant potential of oral lichen planus lesions.

Keywords: cell cycle checkpoints; immunohistochemistry; lichen planus.

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INTRODUCTION

Lichen planus is a chronic inflammatory disease that affects skin and mucosa [1]. Oral forms of lichen planus are more common, chronic and recalcitrant than cutaneous type causing pain and discomfort leading to significant impairment of quality of life [2]. Oral lichen planus (OLP) has been associated with low but clinically relevant increased risk of oral squamous cell carcinoma (OSCC) [3]. Previous studies have demonstrated increased cdk4 (cyclin-dependent kinase and altered p16 expression being associated with OSCC [4]. Also keratinocytes in OLP have been shown to express increased cdk4 [5], which by forming a complex with cyclin D helps in G1 to S phase progression which is a mechanism to preserve epithelial architecture in response to lymphocytic infiltration [5-7]. Cyclin D-cdk4 complex phosphorylates retinoblastoma proteins (pRb), resulting in dissociation of the pRb-E2F complex and G1 to S phase progression [8]. Keratinocytes in turn express p16 which forms complexes with cdk4, inhibiting the ability of cyclin D-cdk4 complexes to phosphorylate pRb [9] and thus arrests the cell in G1 phase which has been demonstrated to result in basal cell liquefactive degeneration [4,6]. These changes in cell cycle contribute to cancer formation [4,10], supporting the concept of premalignancy in OLP [5].

Expression of cdk4 in nuclei has been observed in normal mucosa while in oral premalignant lesions and OSCCs positive reactions increase mainly in cytoplasm and negative nuclear reactions have been observed in metastatic OSCCs [4]. Thus cytoplasmic positivity is supposed to be associated with hyperproliferative nature and malignant potential of a lesion [4]. In addition to OSCC, cdk4 overexpression has been observed in lung, breast and other body cancers [11,12], as well as in premalignant stage during artificially induced malignant transformation in animal studies [13]. Also p16 mutation and altered expression have been usually seen associated with change in cdk4 expression in cancers [4,14]. Inactivation of p16 has been reported by several mechanisms in head and neck SCC [9,15]. Although p16 may or may not be inactivated in OSCC and in oral premalignant lesions, as shown with the studies reported with overexpression and no alterations in p16 expression [4,16,17]. Thus it can be speculated that cdk4 and p16 play an important role in the malignant transformation of OLP as well as other oral mucosal premalignant lesions and in the pathology of OSCC.

No studies have been done till date on the comparison of cdk4 and p16 expression in erosive oral lichen planus (EOLP) and non-erosive oral lichen planus (NOLP). Consequently the purpose of this study was immunohistochemical analysis of cdk4 and p16 expression in oral lichen planus and comparison of their expression between erosive and non-erosive oral lichen planus as well as with normal mucosa and oral squamous cell carcinoma.

MATERIAL AND METHODS

Samples

The proposed study was approved by Research Ethics Committee of University of Delhi. Patients older than 8 years with histologically proven EOLP cases, were being included in the study. Patients were excluded from the study in cases of lichenoid contact reaction due to any medication, mouth rinse, toothpaste or any other agent; any therapy for lichen planus or drugs associated with lichenoid reaction within past 8 weeks; any malignant or viral involvement in mouth; pregnant or nursing women also excluded.

Patient selection was being done based on the clinical characteristic of OLP (bilateral reticular striae with or without atrophic erosive areas) and diagnosis was confirmed with histopathological analysis of the lesional tissue. Informed consent regarding the procedures being performed was taken from all the patients selected.

Biopsy taken from the representative area without loss of epithelium for diagnosis with the characteristic findings on histopathological examination of subepithelial dense lymphocytic infiltrate, basilar vacuolization with apoptosis leading to homogeneous eosinophilic civatte bodies formation. The immunofluorescence technique was utilized to confirm the diagnosis. The results showed positive fibrinogen at the basement membrane zone with or without IgM deposition, in the absence of other immunofluorescence patterns. After application of these criteria 60 OLP cases (32 males and 28 female), with an age range of 19 - 69 years were included in the study. Negative control group of 10 normal mucosa patients undergoing minor surgery as well as healthy volunteers and positive control group of 10 histopathologically proven OSCC cases was selected for comparison. Preservation of the tissue specimen as paraffin blocks for immunohistochemistry procedure to check for expression of cdk4 and p16 markers was done.

Immunohistochemistry

Sections of 4 µm thickness were cut from the formalin

fixed paraffin embedded blocks and mounted on polylysine coated glass slides. Sections were deparaffinised and rehydrated. Endogenous peroxidase activity was blocked with 30 minutes incubation in 3% H_2O_2. Antigen retrieval was performed by heating the sections in a microwave at 800 W for 30 minutes with intermittent cooling after each 5 minutes period in 10 mM citrate buffer (pH 6.0). Sections were thoroughly washed with phosphate buffered saline and treated with primary antibody against cdk4 (Santa Cruz biotechnology; SC260) and p16 (DB Biotech; DB 152-R) and kept for overnight incubation. Washed in PBS (phosphate buffered saline, pH 7.2) supplemented with 0.05% of Tween-20 (buffer A), for 3 x 5 minutes. Secondary antibody applied and left for 30 minutes followed by tertiary antibody applied and left for 30 minutes, at room temperature. Thoroughly washed in PBS and chromogen (DAB) applied and monitored microscopically for development of brown colour. Counterstained in haematoxylin for 5 minutes, dehydrated and mounted in DPX.

For cdk4 as well as p16, cells showing brown staining in the nucleus or/and cytoplasm were considered positive. Five fields were chosen under light microscope (original magnification x400) randomly and photographed. Number of positive cells and total number of the cells were counted in a 10 x 10 grid in each photograph. Percentage of positive cells was counted. Comparison of expression of cdk4 and p16 among different groups (OLP with negative control, OLP with positive control, EOLP with NOLP) was done.

Statistical analysis

Because some variables in the study groups were not normally distributed, nonparametric statistics were applied. Only two-sided P values less than 0.05 were considered as significant. The data were entered in software (SPSS 13 for windows, SPSS Inc, Chicago, III). Continuous data were reported as mean and standard deviation (M [SD]). Mann-Whitney tests were performed to detect a difference between each pair of groups. For discrete data Pearson Chi Square test with continuous correction was used.

RESULTS

This study demonstrated a significantly increased expression of cytoplasmic cdk4 (80% cases, mean average cells stained - 19.6% [0-80]) and cytoplasmic p16 (68.3% cases, mean average cells stained - 16.4% [0-90]) in OLP (Figure 1A, B) and cdk4 and p16 expression was much higher in OSCC in both cytoplasm and nuclei compared to normal mucosa where only nuclear cdk4 was detected in only 40% of samples (Table 1).

When OLP was compared with negative control, no significant difference was present with respect to cdk4 and p16 nucleus staining although significant difference was seen for cytoplasmic p16 ($P < 0.0001$) and cdk4 ($P < 0.0001$) for average number of cells stained (Mann-Whitney U test). Also significant difference in number of the positive cases in OLP compared to normal mucosa was seen for cytoplasmic

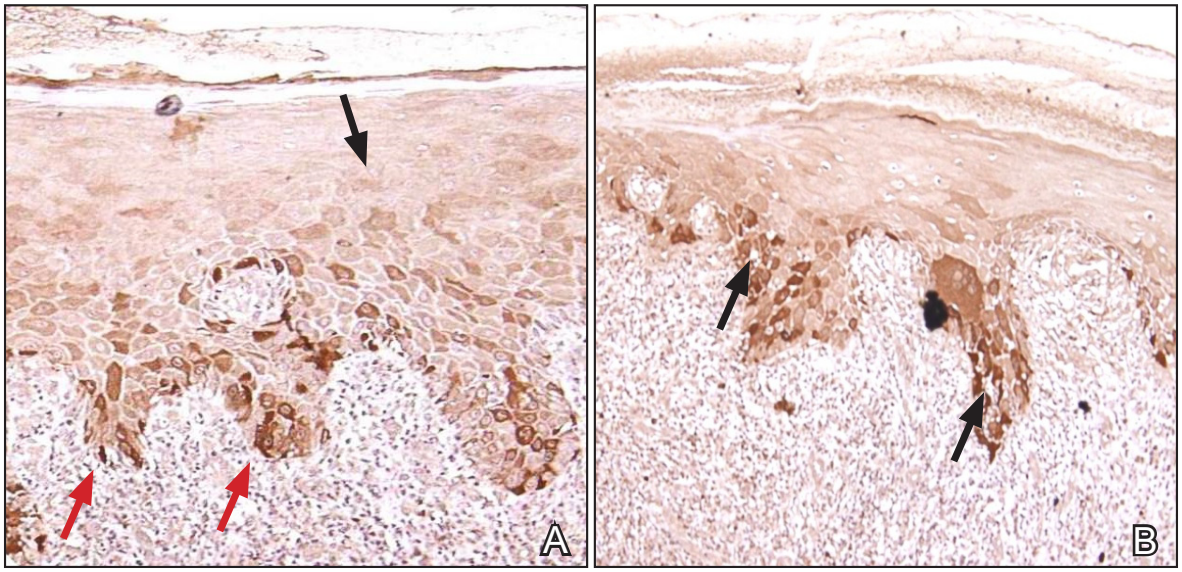

Figure 1. A = cdk4 expression in OLP; dense nuclear and cytoplasmic staining in all layers of the epithelium. The intensity of the staining varies from weak (black arrow) to moderate to intense. Most cells with intense staining are confined to the basal and suprabasal layers (red arrows) (immunostaining, original magnification x400). B = p16 expression in OLP; intense positive cells in the epithelium, again in basal and suprabasal layers (arrows) (immunostaining, original magnification x400).

Table 1. Comparison of cytoplasmic and nuclear cdk4 and p16 expression in oral lichen planus and control group tissue samples

		cdk4		p16	
		Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
Oral lichen planus	Cases with positive staining/total no. (%)	48/60 (80)	19/60 (31.7)	41/60 (68.3)	11/60 (18.3)
	Mean of % no. of cells stained (SD)	19.6 (22)	3 (7.5)	16.4 (22.6)	3.9 (13)
	Median (range)	11 (0 - 80)	0 (0 - 42)	5 (0 - 90)	0 (0 - 65)
Negative control (normal mucosa)	Cases with positive staining/total no. (%)	0/10 (0)	4/10 (40)	0/10 (0)	0/10 (0)
	P-value[a]	0.0001	0.06	0.0002	0.31
	Mean of % no. of cells stained (SD)	0 (0)	1.3 (0.2)	0 (0)	0 (0)
	Median (range)	0 (0 - 0)	0 (0 - 4)	0 (0 - 0)	0 (0 - 0)
	P-value[b]	0.0001	0.17	< 0.0001	0.16
Positive control (OSCC)	Cases with positive staining/total no. (%)	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
	P-value[a]	0.23	0.0001	0.03	0.0001
	Mean of % no. of cells stained (SD)	74 (6.8)	74 (6.8)	80.9 (3.1)	80.9 (3.1)
	Median (range)	74 (65 - 83)	74 (65 - 83)	81 (75 - 85)	81 (75 - 85)
	P-value[b]	< 0.001	< 0.001	< 0.001	< 0.001

[a]P value between oral lichen planus and normal mucosa group, Pearson Chi Square test.
[b]P value between oral lichen planus and oral squamous cell carcinoma group, Mann-Whitney U test.
P < 0.05 was considered statistically significant.
OSCC = oral squamous cell carcinoma; no. = number; SD = standard deviation.

cdk4 (P = 0.0001), cytoplasmic p16 (P = 0.0002) only while difference was no significant for nuclear cdk4 (P = 0.06) and nuclear p16 (P = 0.31) (Pearson Chi-square test). While comparing OLP with positive control (OSCC), significant difference was noted for cytoplasmic cdk4, p16, nuclear cdk4 and p16 (P < 0.001), with expression being more in OSCC (Mann-Whitney U test).

In OLP cases when cdk4 and p16 expression was correlated, 43 of 60 samples expressed p16 antigens with cdk4 positive nuclei and/or cytoplasm. Statistically a significant positive correlation was seen between cdk4 and p16 expression (P < 0.05) for OLP cases.

Significant differences were seen between EOLP and NOLP, with respect to percentage of OLP cases with Cdk4 cytoplasmic staining positivity only (P = 0.01) and no significant difference was seen in cdk4 nuclear and P16 cytoplasmic as well as nuclear staining; with Pearson Chi-Square test (with continuity correction) (Table 2, Figure 2). Also when comparison was done with respect to average of percentage number of cells stained, significant differences

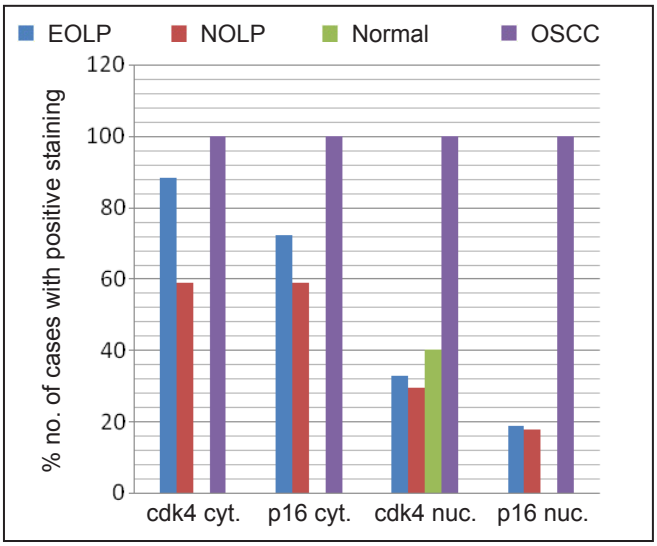

Figure 2. Cdk4 and p16 expression in nucleus and cytoplasm in EOLP, NOLP, normal mucosa and OSCC (% no. of cases with positive staining). In EOLP group, only cytoplasmic cdk4 is frequently expressed; compared to NOLP and normal mucosa (green invisible has a value of zero).
EOLP = erosive oral lichen planus; NOLP = non-erosive oral lichen planus; OSCC = oral squamous cell carcinoma; cyt. = cytoplasmic; nuc. = nuclear.

Table 2. Comparison of cases with cytoplasmic and nuclear cdk4 and p16 expression in EOLP and NOLP tissue samples

Group	Cases with cdk4 positive staining/total no. (%)		Cases with p16 positive staining/total no. (%)	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
EOLP (n = 43)	38/43 (88.4)	14/43 (32.6)	31/43 (72.1)	8/43 (18.6)
NOLP (n = 17)	10/17 (58.8)	5/17 (29.4)	10/17 (58.8)	3/17 (17.6)
P-value	0.016[a]	0.535[b]	0.243[b]	0.623[b]

[a]Statisticaly significant, Pearson Chi Square test.
[b]Statisticaly no significant, Pearson Chi Square test.
EOLP = erosive oral lichen planus; NOLP = non-erosive oral lichen planus.

Table 3. Comparison of number of cells having positive cytoplasmic or nuclear expression of cdk4 and p16 in EOLP and NOLP tissue samples

Group	% no. of cells with cdk4 staining				% no. of cells with p16 staining			
	Cytoplasmic		Nuclear		Cytoplasmic		Nuclear	
	Mean (SD)	Median (range)	Mean (SD)	Median (range)	Mean (SD)	Median (range)	Mean (SD)	Median (range)
EOLP	24.5 (23.4)	16 (0 - 80)	3.7 (8.6)	0 (0 - 42)	18.6 (23.9)	6 (0 - 90)	5.1 (15.2)	0 (0 - 65)
NOLP	7 (10.6)	3 (0 - 40)	1.4 (2.3)	0 (0 - 5)	10.8 (18.5)	2 (0 - 60)	0.7 (1.6)	0 (0 - 5)
P-value	0.001[a]		0.832[b]		0.127[b]		0.761[b]	

[a]Statisticaly significant, Mann-Whitney U test.
[b]Statisticaly no significant, Mann-Whitney U test.
EOLP = erosive oral lichen planus; NOLP = non-erosive oral lichen planus; SD = Standard deviation.

between EOLP and NOLP were seen only with respect to cytoplasmic cdk4 positivity (P = 0.001); with Mann-Whitney U test (Table 3, Figure 3).

DISCUSSION

This was the first study to compare the cdk4 and p16 expression between EOLP and NOLP. Study results revealed a significantly high expression of cdk4 and p16 in cytoplasm in OLP compared to normal mucosa, for percentage number of cells stained as well as for number of cases having positive staining. cdk4 nuclear expression was not significantly different between OLP and normal mucosa as nuclear cdk4 was detected in 40% of the normal mucosa samples. These findings were contrary to a previous study where cdk4 showed significantly higher expression in nuclei only, in lesional tissue of OLP [5]. Similarly cdk4 and p16 expression was significantly higher in OSCC in both cytoplasm and nuclei, finding supported by the previous studies [4,10]. In normal mucosa, cdk4 expression seen was confined to the nucleus only, suggesting that although cdk4 being a component of nuclear proteins [9], its positivity registered in the cytoplasm in OLP and OSCC cases was not due to a technical error.

A significant positive correlation was seen between cdk4 and p16 expression in OLP (P < 0.001), supported by the previous studies with similar findings [4,5,8,9]. This could be explained by a positive feedback loop between p16 and cdk4 which is regulated by pRb (retinoblastoma protein). p16 negatively regulates the cdk4, causing cell cycle arrest to promote DNA repair process [4,8,9]. On the other hand if the cells are severely damaged and cannot be repaired, senescence may be another alternative [16]. Therefore p16 induced cell arrest or senescence may be one of the mechanisms that prevent epithelial cells of OLP to develop cancer.

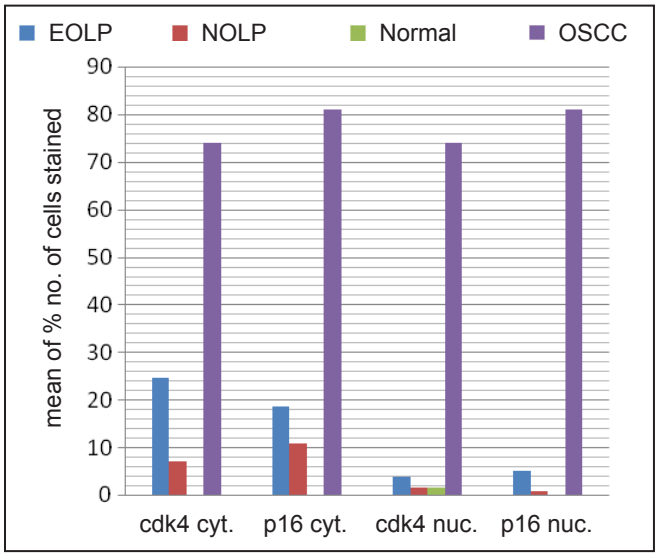

Figure 3. Cdk4 and p16 expression in nucleus and cytoplasm in EOLP, NOLP, normal mucosa and OSCC (mean of % no. of cells stained). In EOLP group, only cytoplasmic cdk4 expression is significantly increased; compared to NOLP and normal mucosa (green invisible has a value of zero), similarly as in Figure 2. EOLP = erosive oral lichen planus; NOLP = non-erosive oral lichen planus; OSCC = oral squamous cell carcinoma; cyt. = cytoplasmic; nuc. = nuclear.

It is accepted that malignant transformation rate in OLP is lower than that of oral leukoplakia where overexpression of cdk4 occurs but no changes in p16 observed [10,17]. In this context it can be postulated that loss of p16 expression may be an initial sign of malignant transformation in OLP with high cdk4 expression. Thus the combination of cdk4 and p16 may be a useful tool in predicting malignant transformation in OLP.

We observed a significantly higher levels of cytoplasmic cdk4 in EOLP compared to NOLP, in pattern (P = 0.001) (Table 3, Figure 3) as well as prevalence (P = 0.016) (Table 2, Figure 2) of positivity. However difference in the prevalence (P = 0.016) of positivity was not clinically acceptable between the EOLP group (38/43 [88.4%]) and NOLP

group (10/17 [58.8%]). According to a previous study, cdk4 was detected in nuclei of the normal and hyperkeratotic tissues while in all groups of oral premalignant lesions and OSCCs positive reactions increased in intensity significantly mainly in cytoplasm ($P < 0.05$) and interestingly almost all the metastatic OSCCs revealed negative nuclear reactions [4]. Thus cytoplasmic positivity points towards hyperproliferative and premalignant nature of EOLP suggesting the requirement of judicious treatment and long term follow-up. Expression of nuclear cdk4 was not significantly increased in EOLP compared to NOLP (Tables 2 and 3), similarly as in case of OLP being compared with normal mucosa. Hence it cannot be suggested as a sensitive marker of abnormal proliferation, as it being present in 40% of normal mucosa as well. Also expression of cytoplasmic p16 and nuclear p16 in EOLP was higher than NOLP but it was not achieving statistical significance (Tables 2 and 3). Increased expression of these markers in OLP reveals that there is an obvious possibility for these markers to predict malignant potential of the OLP. However manual staining was done in this study, while automated procedures could decrease the variability of staining among the batches of IHC. Also molecular methods and image analysis could help in objective evaluation of staining, avoiding the subjective variability. Hence further studies with larger cohort and automated procedures with objective evaluation are needed in future to further substantiate the results and to further establish the association of these molecular markers with the disease pathogenesis.

CONCLUSIONS

Overexpression of cytoplasmic cdk4 and p16 was registered in oral lichen planus, however considerably lower than in squamous cell carcinoma. Erosive oral lichen planus demonstrated overexpression of cytoplasmic cdk4 and premalignant nature compared to non-erosive lesion. Therefore there is an obvious possibility for cytoplasmic expression of cdk4 and p16 to predict malignant potential of oral lichen planus lesions.

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