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"Expression of p16 in oral leukoplakia and oral squamous cell carcinoma and correlation of its expression with individual atypical features"

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1. Introduction

Tumor suppressor $p16^{INK4A}$ (p16), one of the INK4 class members of cell cycle inhibitors. Its expression retains Rb-family proteins in a hypophosphorylated state, which promotes binding of E2F to achieve G_1 cell-cycle arrest. Hyper methylation of p16 promoter is associated with gene silencing; moreover, in oral squamous cell carcinoma (OSCC), it is more common than are genetic alterations. Studies showed that p16 hyper methylation is frequent in pre-cancerous oral lesions and lesions with a hyper methylated p16 promoter tend to transform into oral cancers.

Expression of p16 serves as a marker for oral mucosal dysplasia and malignant transformation.[1–3] In oral premalignant and malignant lesions, both increased and decreased expressions of p16 have been reported. Interestingly, it has been shown that p16 was consistently found at areas of micro invasive and at superficial margins of OSCC, whereas it was heterogeneously expressed in moderate to severe epithelial dysplasia and carcinoma in situ (CIS).[1–3]

This present study is an attempt to determine as to which of the individual dysplastic features could probably be more predictive of malignant transformation by correlating p16 expression, with individual atypical morphological features seen in hematoxylin and eosin (H&E) stained sections of dysplastic epithelium under light microscope.

2. Material and methods

After obtaining ethical clearance from Institutional Ethical Committee, 50 archival formalin fixed paraffin embedded tissue blocks formed part of the study. 20 blocks were of oral leukoplakia diagnosed histopathologically as either grades of dysplasia according to WHO 2005 classification of oral epithelial dysplasia[4]; 20 cases of OSCC cases graded on the basis of differentiation as - well, moderately & poorly differentiated formed part of the study. 10 normal mucosal specimens as controls were taken from buccal mucosa of healthy volunteers.

2.1. Immunohistochemistry

4 μm thin sections were deparaffinised & placed on organosilane-pretreated slides. To detect p16 protein an IHC assay was performed. (Super Sensitive Polymer HRP IHC detection system by Biogenix, CA) Primary antibody used was p16INK4A from immunoglobulin fraction diluted in PBS, (clone: G175-405, Mouse Monoclonal, Biogenix, CA). Incubation time of primary antibody was 40 min at room temperature. All the slides were labelled with the pathology number and the marker protein to be stained. Antigen retrieval was done by immersing the sections in 10 mM sodium citrate buffer, pH 6.0, and boiled for three cycles of 10 min at 800°, 420° & 320°F respectively, in a domestic microwave oven. Jar with slides & buffer was kept at room temperature to cool down. Then slides were washed with Tris buffer. In order to block the non-specific background staining, sections were incubated with 3% hydrogen peroxide for 5 min at room temperature. Without washing after blocking, step sections were incubated with pre diluted specific monoclonal primary antibody against p16 protein for 40 min at room temperature in moist chamber after that sections were washed twice with Tris buffer at pH 7.2 for about 5 min each. Super Enhancer was applied to cover section & incubated for 20 min at room temperature. The sections were incubated in species-specific secondary antibody for 30 min at room temperature in moist chamber and were washed twice with Tris buffer at pH 6 for about 5 min each. Tissue sections were covered with DAB substrate solution & incubated for 5 min at room temperature and were rinsed with Tris buffer solution. Mayer's hematoxylin was used for counter staining the sections for 3 min.

A section of cervical squamous cell carcinoma was used as the positive control in each run.

2.2. Evaluation of p16 expression

The expression pattern of p16 was studied in the entire length of the epithelium in normal, dysplastic epithelium and in OSCC along with its preceding serial sections stained with H&E. The expression of p16 was taken into consideration in the dysplastic epithelium and the

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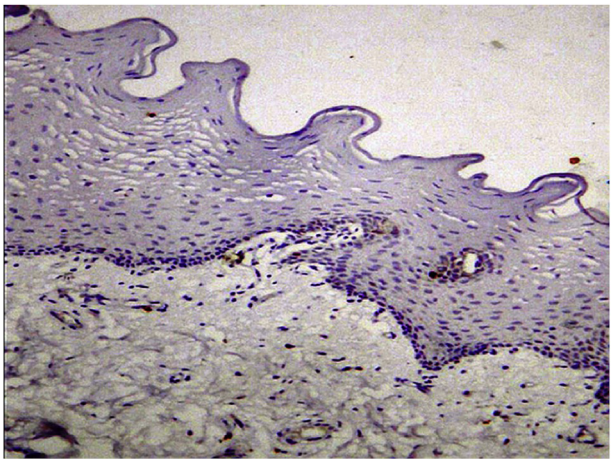


Fig. 1. Normal buccal mucosa negative for p16 expression (10X, IHC). (a) A photomicrograph showing normal buccal mucosa (10X, H&E).

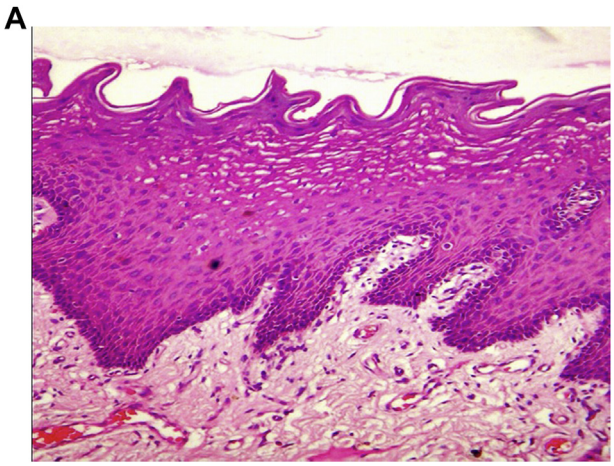


Fig. 1. (*continued*)

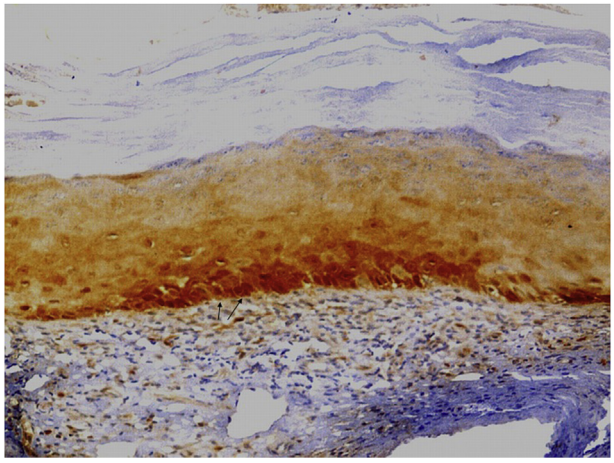
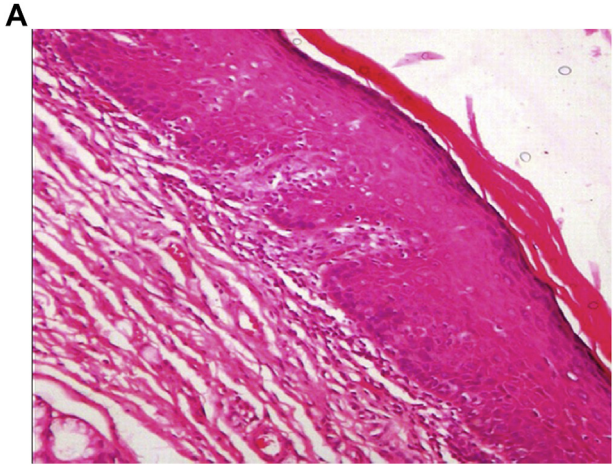


Fig. 2. Detectable p16 expression in the basal and suprabasal layers (arrow) of squamous epithelium with mild dysplasia (10X,IHC). (a) A photomicrograph showing squamous epithelium with mild dysplasia. (10X,H&E).

corresponding H&E sections were reviewed for individual atypical features corresponding to basal and supra basal expression of p16 in IHC slide. Positive cases were defined as having at least five p16 positive stained cells (nucleus and/or cytoplasm). All positively stained epithelial cells in dysplasia were counted in at least five randomly selected fields using 10 × 10 occulometer grid at a magnification of 40X.



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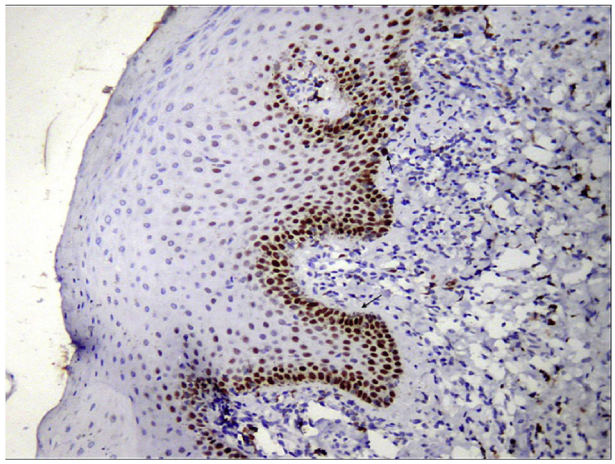


Fig. 3. p16 expression in basal, suprabasal and lower spinous layers of squamous epithelium (arrow) with moderate dysplasia (10X,IHC). (a) A photomicrograph showing squamous epithelium with moderate dysplasia (10X,H&E).

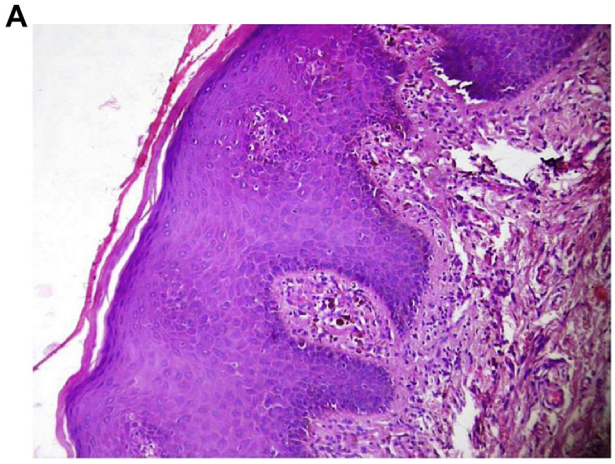


Fig. 3. (*continued*)

The cases in which intense/marked p16 staining corresponded to particular atypical morphologic features seen in the H&E sections were noted. The related figures for each group namely (Figs. 1–6) depicts the p16 expression and the corresponding H& E staining.

To further validate the findings and to look for predictive value of these atypical features, we also studied the islands and surface

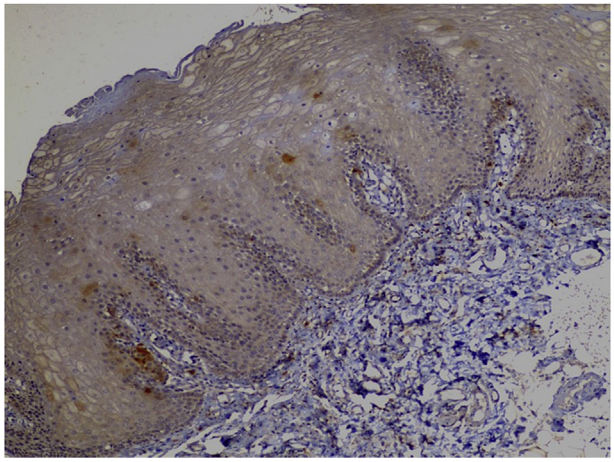
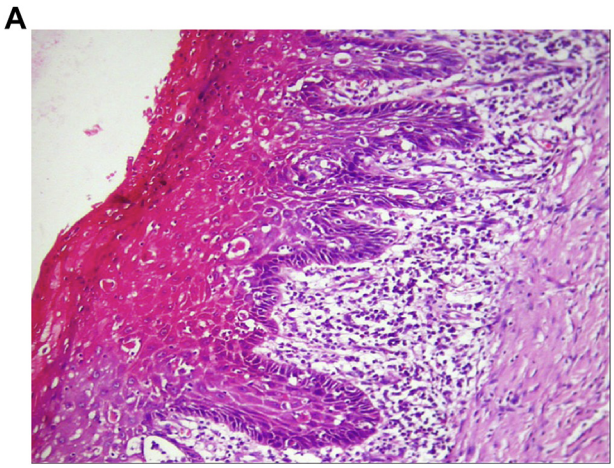


Fig. 4. Severe dysplasia/CIS stained feebly (few cells showed strong cytoplasm positivity) for p16 expression (10X,IHC). (a) A photomicrograph showing squamous epithelium with severe dysplasia/CIS (10X,H&E).



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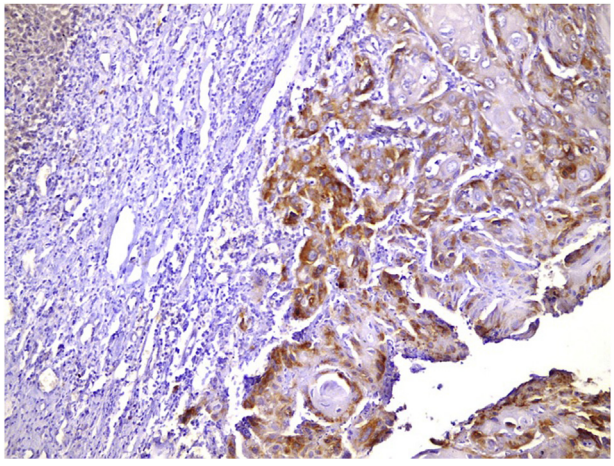

Fig. 5. Intense p16 expression in well-differentiated squamous cell carcinoma (10X,IHC). (a) A photomicrograph showing well-defined epithelial pearls of well-differentiated squamous cell carcinoma (10X,H&E).

epithelium of 20 OSCC cases, by correlating the expression of p16 to atypical features seen in H&E. All analyses were performed on Graph Pad Prism (Windows version 5.0). Mann Whitney U and chi square tests were applied for statistical analysis.

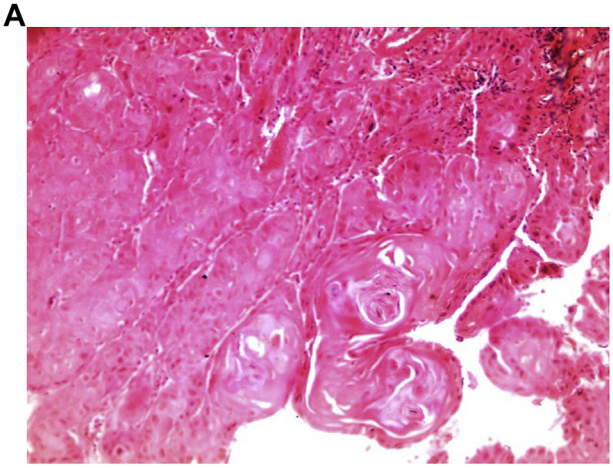

Fig. 5. (*continued*)

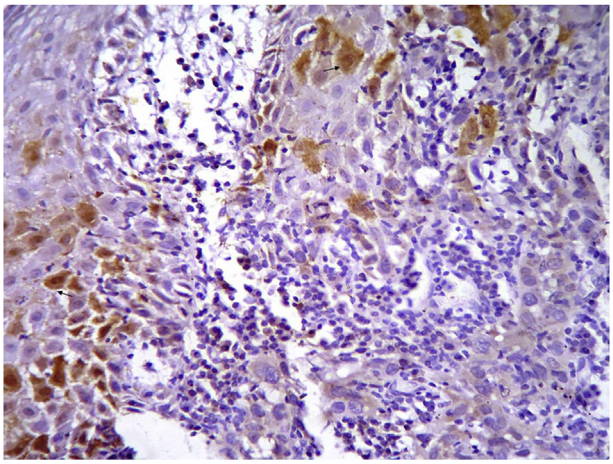

Fig. 6. Moderately differentiated squamous cell carcinoma showing strong nuclear/cytoplasm (arrow) positivity for p16 expression (40X,IHC). (a) A photomicrograph showing moderately differentiated squamous cell carcinoma (40X,H&E).

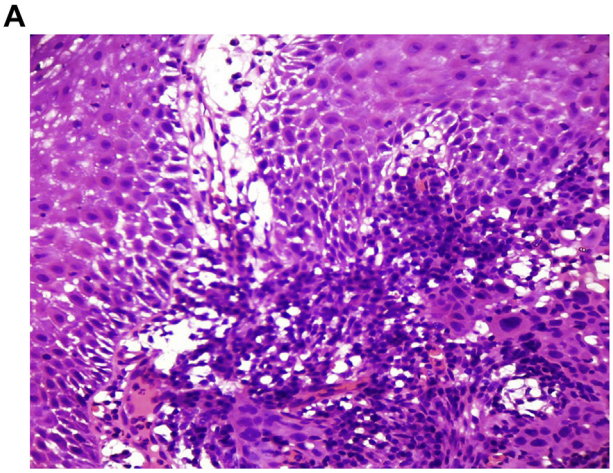

Fig. 6. (*continued*)

3. Results

A total of 50 tissue blocks were assessed with IHC for expression pattern of p16. There were total 20 cases of OL, which were further classified as mild dysplasia (65%) followed by moderate (30%) and

Table 1
Distribution of ol and oscc cases according to histopathological diagnosis.

Histopathological diagnosis	Number of cases	(%)
Mild dysplasia	13	65
Moderate dysplasia	6	30
Severe dysplasia	1	5
WDSCC	11	55
MDSCC	9	45
PDSCC	0	0

Inference: Mild > Moderate > Severe & WDSCC > MDSCC.

severe (5%). Similarly, 20 cases of OSCC were further classified as Well Differentiated Squamous Cell Carcinoma (55%), Moderately Differentiated (45%) and Poorly Differentiated (0%) (Table 1).

The total number of cells in OL group ranged from 0 to 678 with mean (± SE) 191.05 ± 52.04 while in OSCC group ranged from 0 to 438 with mean (± SE) 149.70 ± 26.89. The p16 positive cells in OL group ranged from 0 to 435 with mean (± SE) 109.80 ± 32.06 while in OSCC group ranged from 0 to 320 with mean (± SE) 101.50 ± 18.71. *U* test revealed similar p16 positive cells in two groups (109.80 ± 32.06 vs. 101.50 ± 18.71, U = 181.50; p = 0.605) i.e. p16 positive cells did not differ significantly (p > 0.05) between the two groups though it was 7.6% lower in OSCC group (Table 2).

The nucleus positive cells in OL group ranged from 0 to 3 with mean (± SE) 0.15 ± 0.15 while in OSCC group ranged from 0 to 7 with mean (± SE) 0.35 ± 0.35. It was 57.1% higher in OSCC group (Table 2). The cytoplasm positive cells in OL group ranged from 0 to 411 with mean (± SE) 89.15 ± 30.08 while in OSCC group ranged from 0 to 320 with mean (± SE) 80.65 ± 19.24. It was 9.5% lower in OSCC group (Table 2). The nucleus/cytoplasm positive cells ratio in OL group ranged from 0 to 254 with mean (± SE) 20.85 ± 13.14 while in OSCC group (Figs. 2 and 3) ranged from 0 to 150 with mean (± SE) 22.05 ± 9.22. It was 5.4% higher in OSCC group (Table 3). Nucleus, cytoplasm & nucleus/cytoplasm positive cells ratio did not differ significantly (p > 0.05) between the two groups. The individual dysplastic features seen in H&E and p16 expression of OL is summarized in Table 3 and also shown graphically in Graph I, respectively. In OL group, the dysplastic features seen in H&E and p16 expression did not differ significantly (p > 0.05) i.e. found to be almost the same (OL: $\chi^2 = 0.07$; p = 0.964).

3.1. List of dysplastic features

1. Drop Shaped Rete Ridges
2. Irregular Epithelial Stratification
3. Intra Epithelial Keratinization
4. Basilar Hyperplasia
5. Loss of Cohesion
6. Loss of Polarity
7. Nuclear Hyperchromatism
8. Anisocytosis/Nucleosis
9. Prominent Nucleoli
10. Increased N:C ratio

Table 3
Frequency distribution of h & e and p16 expression in ol of individual dysplastic features.

Groups	Dysplastic Features Grading	Dysplastic Features in H & E	p16 expression	χ^2 value (DF = 2)	p value
Leukoplakia (OL)	0–1	15	15	0.07	0.964
	2–5	13	13		
	> 5	16	18		

Inference: p value (< 0.05).

11. Pleomorphism
12. Mitosis (2–4/hpf)
13. Level of mitosis (Restricted to lower half of the epithelium)
14. Bizarre mitosis

4. Discussion

The risk level for malignant transformation of leukoplakia is syndicated with the lesion's histological features. The ability to identify subjects of OL at increased risk of cancer development is crucial to improve control of oral cancer. Upon identification, the highest risk individuals could be offered more aggressive treatment options as well as intensive follow-up. The OL is more than just a potentially malignant disorder but also a marker for increased cancer risk throughout the upper aero-digestive tract.[5]

The challenge within the field of oral pre cancer is to predict which lesions will eventually develop into carcinoma. Conventional clinical and histopathological aspects are not optimal for decisions on management, which is, of course influenced by the perceived risk of malignant development. For the pathologist, epithelial dysplasia/CIS are probably more or less subconsciously defined as “histological changes in which the risk of development of carcinoma is higher than in non-dysplastic epithelium.” Several studies have shown great inter/intra examiner variability in the assessment of the presence or absence and the grade of OED.[6]

In present study, p16 expression pattern was studied in various grades of dysplastic epithelium. Mild epithelial dysplasia expression was mainly in the basal, suprabasal and a few cells in the lower spinous cell layer, in moderate dysplasia expression was not only found in the basal and suprabasal but also prickle cell layers. Similar results were seen in other studies as well.[7]

It has been proposed that p16 expression serves as a marker for oral mucosal dysplasia and malignant transformation.[2,3,8] In oral pre-malignant and malignant lesions, both increased and decreased expressions of p16 have been reported.[7] Some of the previous studies evaluated only nuclear staining,[9,10] whereas others utilized both nuclear and /or cytoplasmic staining[11] to study the p16 expression. Cytoplasmic staining was found to be negative probably due to p16 inactivation. In the present study nucleus positive cells were 57.1% higher in OSCC group than OL group though the increase was not statistically significant (p > 0.05). The cytoplasm positive cells also showed an increase of 9.5% in OSCC group as compared to OL, though

Table 2
p16 Positive cells IN OL AND OSCC.

	Oral Leukoplakia	Oral squamous cell carcinoma	% mean change	U value	p value
Total no of p16 positive cells	109.80 ± 32.06 (0–435)	101.50 ± 18.71 (0–320)	7.6%	181.50	0.605
Nucleus p16 positive cells	0.15 ± 0.15 (0–3)	0.35 ± 0.35 (0–7)	57.1%	199.50	0.971
Cytoplasm p16 positive cells	89.15 ± 30.08 (0–411)	80.65 ± 19.24 (0–320)	9.5%	170.00	0.395
Nucleus and cytoplasm p16 positive cells	20.85 ± 13.14 (0–254)	22.05 ± 9.22 (0–150)	5.4%	177.00	0.465

Numbers in parenthesis represents the range (min-max).
U-Mann Whitney *U* test.

the increase was not statistically significant ($p > 0.05$). The nuclear and/or cytoplasm positive cells showed increase of 5.4% in OSCC group as compared with OL, though it was also not significant ($p > 0.05$).

Bradley et al. in their study concluded that p16 expression is not useful in differentiating dysplastic from non-dysplastic oral lesions although decreased p16 expression with increasing severity of dysplasia was observed.[12] Buajeeb W et al.[7] studied the frequency of p16 expression in10 cases of normal mucosa, 15 cases of OL without dysplasia, 15 cases of OL with dysplasia and 16 cases of OSCC and found positive staining in OL without dysplasia (26.7%) and OSCC (12.5%) with no statistically significant difference in the frequency of p16 expression among OSCC, OL with and without dysplasia, and normal mucosa. This is consistent with our findings as no significant difference was observed in the expression of p16 among normal mucosa, OL and OSCC.

Overexpression of p16 in premalignant and malignant oral lesions has been found to be associated with high-risk HPV infection.[13,14] The HPV E7 onco protein interacts and induces proteolytic degradation of p Rb leading to increased expression of p16.[15,16] Therefore, positive cases may be associated with HPV infection. Similarly, a low prevalence of HPV has been reported in OSCC patients older than 60 years.[17] As we did not evaluate the presence of HPV in the specimens, it is uncertain about HPV infection in this population. Moreover, it should also be noted that overexpression of p16 in OSCC might not be associated with HPV.[18]

As our study is an attempt to determine as to which of the individual dysplastic features could probably be more predictive of malignant transformation by correlating p16 expression, with individual atypical morphological features seen in H&E stained sections of dysplastic epithelium, most common atypical features observed in H&E sections as compared with p16 expression were drop shaped rete ridges, prominent nucleoli, increased nucleus-cytoplasmic ratio, basilar hyperplasia, loss of polarity, prominent nucleoli, mitosis and loss of cohesion. Statistics showed no significant difference (OL: $\chi^2 = 0.07$; $p = 0.964$). Except, the percentage of prominent nucleoli, which was marginally on higher scale. Hence, additional emphasis should be given to prominent nucleoli in dysplastic epithelia and should be viewed with greater caution. Additional studies with larger sample size and long-term follow up would further help to assess the significance of p16 in oral carcinogenesis.

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