Original Article

Grading of Oral Leukoplakia: Can It be Improvised Using Immunohistochemical Markers p63 and CD31

Abstract

Introduction: Oral squamous cell carcinoma is usually preceded by potentially malignant disorders (PMDs), the most common being oral leukoplakia. A conservative management protocol is followed for milder dysplastic cases, while severe dysplastic lesions are surgically excised. Several classification systems have been developed based mainly on histopathological features with a lot of inter-observer variations. The present study was done to assess the use of immunohistochemical (IHC) markers in grading leukoplakic lesions in addition to histopathological grading. **Aims and Objectives:** To grade leukoplakia using different grading systems and assess if IHC markers can aid in categorizing leukoplakia. **Materials and Methods:** Thirty-five cases of leukoplakia were graded using Ljubljana, 2005 World Health Organization (WHO), and Binary System followed by IHC staining with p63 and CD31. **Results and Statistics:** Variation was noted in 12 cases while using WHO, 11 using Ljubljana, and 7 using Binary System and was significant on Cohen-Kappa statistics, with the least significant variation noted on Binary System. p63 staining assisted to group doubtful cases and even identify variation in cases graded positively on histopathology. In total, 17 cases stained one-third (mild/low), while 15 cases stained one-half or more (higher grade) epithelial thickness. A weak correlation was observed between all grading systems and p63 on Kendall's Tau-b analysis and the weak correlation was significant for the WHO and binary grading systems. Analyzing p63 and CD31 using Kruskal–Wallis test, an increase in mean vessel density (MVD) was noted for mild/moderate cases but decreased for severe cases. **Conclusion:** Addressing histological categorization of PMDs assisted by IHC markers to understand the biological behavior of the tissues is currently essential with studies on more markers to assist in the management protocol.

Keywords: *CD31, dysplasia grading systems, interobserver variation, leukoplakia, p63, premalignant disorders*

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Introduction

International Classification of Diseases Coding Scheme, World Health Organization (WHO) case definitions, and International Agency for Research on Cancer have observed that oral cavity cancer ranks eighth among men and 14th among women all over the globe amid all cancers.[1] The Indian Council of Medical Research has reported that the most common cancer in females is breast cancer and in males is oral cancer, the age range being 30–69 years.[2] Age-adjusted rates of oral cancer are around 20 per one lakh population, accounting for 30% of all cancers in India. Prevailing as the major health issue, oral cancer needs to be detected early for prompt treatment, improvement of cure rate, and prevention of considerable morbidity and mortality.[1] Vast majority of oral cancers are oral squamous cell carcinomas (OSCCs).[3,4] The immediacy of the lymph nodes and the regional variation in the head and neck have increased the frequency of lymph node metastases, and it has been found that once metastases occurs, the 5-year survival rates reduce to as low as 9%.[5]

Majority of OSCC cases follow the occurrence of a series of cellular and tissue alterations that manifest as potentially malignant disorders (PMDs) among which oral leukoplakia is the most common.[5,6] These changes termed oral epithelial dysplasia are restricted to surface epithelium and would be termed as malignant change once the altered cells invade the underlying connective tissue. The malignant transformation rate of epithelial dysplasias is approximately 1%.[5]

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The scale of such cellular and tissue changes is graded using various classification systems. The most commonly used and accepted classification systems include Ljubljana, 2005 WHO, and 2-tier classification (Binary System) by Kujan *et al*.[7-9] These classification systems are mainly based on the architectural and cytological variations observed on hematoxylin and eosin (H and E) stains under light microscopy.[5,6] The pathologists have always found a lot of inter- and intra-observer variations in grouping the dysplastic cases into those which need to be treated and those which require conservative management. The present study was done to know if immunohistochemical (IHC) markers such as p63 and CD31 can assist in grading the leukoplakic lesions.

In normal cells, intense p63 nuclear localization is seen in the basal layer of stratified squamous epithelium with a gradual diminution of nuclear intensity in the more terminally differentiated cell layers. The superficial cells show barely detectable p63 levels. The studies done by Ramasubramanian *et al*. and Sinha *et al*. have stated that the expression of p63 increases with increase in the severity of dysplasia with increased expression in the suprabasal cells.[10,11] Studies have shown that p63 is needed to maintain cell proliferation in the basal progenitor cells as well as to initiate the differentiation program by inducing gene set critical for this process. It has been observed that as the severity of dysplasia increases, the proliferation rate increases; however, the differentiation of the cells is compromised. p63 helps to maintain stemness of the cells allowing the cells to behave in a more primitive manner which is a feature of dysplasia. This prompted us to use the marker p63.[12,13]

It has also been observed that as the disease progresses, the number of blood vessels or angiogenesis increases. Solid epithelial tumors cannot grow to large sizes until they develop their own network of blood vessels with the formation of new microvessels from the preexisting vasculature. It is one of the factors that play an important role in tumor growth and metastases by providing nutrition to the expanding tumor. Tumor cells and the host stromal cells such as macrophages and mast cells produce proangiogenic factors that allow endothelial cell proliferation, thus providing nutrition to the budding tumor. Various studies have shown that there is increase in the vasculature as the lesion progresses from normal to dysplastic epithelium. Thus, CD31 which is an angiogenesis marker was used to study the vascular changes in proximity to the epithelium.[4,14] The anti-CD31 antibody stains the endothelial cell membrane and marks the microvessels. CD31 also marks platelets and certain leukocytes (T-cells, B-cells, dendritic cells, natural killer cells, and macrophages).[15] In the present study, the vessel area marked by CD31 subepithelially was identified and the mean vascular density (MVD) was calculated.

Materials and Methods

A total of 32 archival blocks of diagnosed cases of leukoplakia were taken up for the study. The inclusion criterion was diagnosed cases confirmed both clinically and histopathologically as leukoplakia with or without a habit history. Exclusion criterion was those cases which were not confirmed both clinically and histopathologically as leukoplakia and other white lesions which represent reactive lesions. They were graded for dysplasia on H and E sections by three different grading systems: 2005 WHO system, Ljubljana classification, and Binary System by Kujan *et al*.[6,9] by two observers, and an interobserver variation was noted. All the cases were then subjected to IHC staining using anti-p63 (BioGenex, India Pvt. Ltd.) and anti-CD 31 antibodies (Abgenex India Private Limited). Four-to-five μm thick sections were subjected to antigen retrieval using ethelyene-di-amine-tetra-acetic acid at pH 9. The sections were incubated with primary antibodies p63 and CD31 preceded by peroxide and protein blocks, followed by secondary antibody, and the reaction was detected using DAB chromogen.

The sections were analyzed for localization of IHC stains and intensity of staining as compared to normal mucosa which was taken as control. The intensity was graded as mild, moderate, or severe based on the staining in the normal mucosal tissue. The strata of cells which picked up the p63 brown stain and the localization within the nucleus or cytoplasm or both were analyzed. For CD31 staining, three representative areas where the intensity of staining was maximum (hotspot) were considered. The hotspots were determined under ×40 magnification subepithelially, and the number of blood capillaries in each hotspot was counted under ×100 magnification. The mean of the three hotspots thus gave MVD for each case of leukoplakia.[4,10]

Results and Statistics

A total of 32 cases in the age group of 10–70 years were considered for the study. Around 41% of them were females and 59% were males.

Descriptive statistics and inferential statistics were performed using Statistical Package for the Social Sciences for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp.

The interobserver variation/correlation using the WHO classification for 32 cases is presented in Table 1, where two cases were graded as simple hyperplasia, 15 cases as mild dysplasia, three cases as moderate dysplasia, and one case as severe dysplasia. There was disagreement in grading 12 cases. With Ljubljana classification, two cases were graded as simple hyperplasia, 14 cases as abnormal hyperplasia, and five cases as atypical hyperplasia, and 11 cases showed interobserver variation [Table 2]. On Binary System, two cases showed no dysplasia, 19 cases could be categorized as low-risk lesions, and four

Table 1: Correlation/variation between observers using World Health Organization classification

Grading system	WHO_2				Total
	Simple. hyperplasia	Mild dysplasia	Moderate dysplasia	Severe dysplasia	
WHO_1					
Simple. Hyperplasia					
Count	2	2	0	0	4
Percentage within WHO_2	100.0	10.0	0.0	0.0	12.5
Mild dysplasia					
Count	0	15	6	0	21
Percentage within WHO_2	0.0	75.0	66.7	0.0	65.6
Moderate. dysplasia					
Count	0	3	3	1	7
Percentage within WHO_2	0.0	15.0	33.3	100.0	21.9
Total					
Count	2	20	9	1	32
Percentage within WHO_2	100.0	100.0	100.0	100.0	100.0

WHO: World Health Organization

Two cases of simple hyperplasia, 15 of mild dysplasia and 3 of moderate dysplasia cases showed no inter-observer variation.

Table 2: Correlation/variation between observers using Ljubljana classification

Grading System	Ljubljana_2 (LJ_2)			Total
	Simple	Atypical	Abnormal	
Ljubljana_1 (LJ_1)				
Simple hyperplasia				
Count	2	0	3	5
Percentage within LJ_2	100.0	0.0	15.0	15.6
Atypical hyperplasia				
Count	0	5	3	8
Percentage within LJ_2	0.0	50.0	15.0	25.0
Abnormal hyperplasia				
Count	0	5	14	19
Percentage within LJ_2	0.0	50.0	70.0	59.4
Total				
Count	2	10	20	32
Percentage within LJ_2	100.0	100.0	100.0	100.0

Two cases graded as simple hyperplasia, 14 cases as abnormal hyperplasia, and 5 cases graded as atypical hyperplasia showed no inter-observer variation.

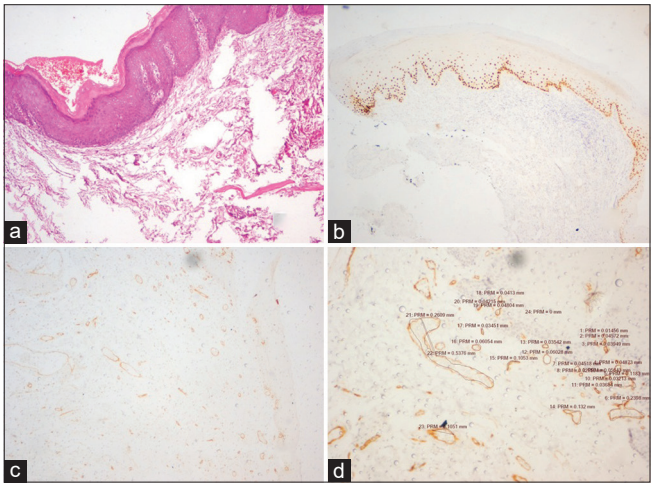

Figure 1: Mild dysplasia: (a) Leukoplakia with mild dysplastic features and hyperkeratinization (H and E stain, ×40); (b) p63 antibody staining 1/3 the epithelial thickness (IHC stain, ×40); (c) hotspot of CD31 stain selected subepithelially (IHC stain, ×100); (d) higher magnification of the ×10 view with morphometric analysis of the vessel walls where mean vessel density is 37 (IHC stain, ×400)

cases could be categorized as high-risk lesions, and an interobserver variation was seen in seven cases [Table 3].

On application of Cohen-Kappa statistics, it was noted that there were fair agreement between the observers for WHO and Ljubljana classification and moderate agreement for binary classification. A *P* value for all the classification systems was statistically significant [Table 4].

The p63 IHC staining was seen in all the cases, was intense and mainly localized in the nucleus of the cells.

Incongruity was that the cases which showed consensus on the WHO grading system between two observers, also showed variation in p63 staining pattern. Of 15 cases graded as mild dysplasia, eight cases showed p63 staining confined to lower one-third of the epithelium [Figure 1]; out of three cases graded as moderate dysplasia, two cases showed p63 staining half the epithelial thickness [Figure 2]; and one case graded as severe showed more than half the thickness of p63 staining [Figure 3] correlating with the histopathological grading. For about six cases graded as mild dysplasia, p63 staining extended till half the epithelial thickness prompting to reconsider the diagnosis. One case showed p63 staining involving two-third the epithelial thickness.

Since there was difference noted even in cases graded with less inter-observer variation on histopathology, the overall staining pattern of p63 was considered – out of 32 cases: 17 cases stained one-third, 12 cases half, and three cases two-third of the epithelial thickness. Kendall's Tau-b statistical method was applied to study the correlation

between different grading systems and p63 staining pattern. The correlation was weak for all the methods, with the average significant value being slightly higher for Binary System, indicating a better correlation between p63 staining and Binary System [Table 5]. It was observed that on assistance with p63 staining, 17 cases could be categorized as no dysplasia/low-risk lesions (p63 stained lower one-third of the epithelium) and 15 cases as high-risk lesions (p63 stained half to two-third of the epithelial thickness) using the binary system.

The next step was to correlate the expression pattern of p63 with CD31 staining; it was noted that the MVD increased as the thickness of epithelium stained with p63 increased for mild and moderate cases [Figure 1 and 2], but it decreased for severe cases [Figure 3]. In severe cases, it was noted that even certain leukocytes were marked up by CD31. This was assessed with Kruskal–Wallis test, and the median MVD values for cases stained one-third epithelial thickness (mild) on p63 were 34.0 and for cases stained half (moderate) were 42.0 but decreased (26.0) for cases that stained two-third of the epithelial thickness [Table 6].

Table 3: Correlation/variation between observers using Binary classification

Grading System	Binary_2 (BI_2)			Total
	No dysplasia	Low risk	High risk	
Binary_1 (BI_1)				
No dysplasia				
Count	2	0	0	2
Percentage within BI_2	100.0	0.0	0.0	6.3
Low risk				
Count	0	19	4	23
Percentage within BI_2	0.0	86.4	50.0	71.9
High risk				
Count	0	3	4	7
Percentage within BI_2	0.0	13.6	50.0	21.9
Total				
Count	2	22	8	32
Percentage within BI_2	100.0	100.0	100.0	100.0

Two cases that were graded as no dysplasia, 19 cases that were graded as no dysplasia and 4 cases that were graded as high risk dysplasia showed no inter-observer variation

Discussion

Categorizing leukoplakic lesions mainly as those which needs to be treated and those which do not require treatment is the main aim of a diagnostic pathologist to aid in proper management of the cases by the surgeons. To assess the magnitude of dysplastic changes in PMDs and also management options, various grading systems have been developed. Some of the popular classification systems include Smith and Pindborg classification, 1978 and 2005 WHO classification, Ljubljana classification, and Binary System of classification. These are mainly based on histopathological observations and certain criteria used. This is not completely reliable as there are a lot of inter- and intra-observer variations.[6]

Geetha *et al*.[16] have observed the inter- and intra-observer variability in grading dysplasia cases using WHO, Ljubljana, and Smith and Pindborg classification. They have found that the consensus in grading was poor in Ljubljana and Smith-Pindborg classification and fair in WHO classification. Krishnan *et al*.[17] and Gupta *et al*.[18] have concluded that Binary System has better inter- and intra-observer agreement as compared to WHO and Ljubljana classification. Shubhasini *et al*.[19] have shown that WHO and Binary System are not competent enough to rule out bias in grading dysplasia through their studies.

The Smith and Pindborg classification is mainly based on photographic correlation and is more time-consuming. WHO classification system, though easy to understand, shows inter- and intra-observer variations whereas Ljubljana classification is mainly designed for laryngeal mucosa and is different from oral mucosa in terms of functional trauma exposed to and histology.[6]

Table 4: The 2 interobserver variation/agreement for leukoplakic lesions and statistics

Interobserver agreement between 2 observers for grading leukoplakic lesions under different methods using Cohen's Kappa statistics

Method	Category	Observer-1, *n* (%)	Observer-2, *n* (%)	Cohen's Kappa	*P*
WHO	Simple hyperplasia	4 (12.5)	2 (6.3)	0.28	0.04*
	Mild dysplasia	21 (65.6)	20 (62.5)		
	Moderate dysplasia	7 (21.9)	9 (28.1)		
	Severe dysplasia	0 (0.0)	1 (3.1)		
Ljubljana	Simple hyperplasia	5 (15.6)	2 (6.3)	0.37	0.007*
	Atypical hyperplasia	8 (25.0)	10 (31.3)		
	Abnormal hyperplasia	19 (59.4)	20 (62.5)		
Binary	No dysplasia	2 (6.3)	2 (6.3)	0.51	<0.001*
	Low risk	23 (71.9)	22 (68.8)		
	High risk	7 (21.9)	8 (25.0)		

*Statistically significant. κ<0 as indicating no agreement, 0.01-0.20 as none to slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.00 as almost perfect agreement. WHO: World Health Organization

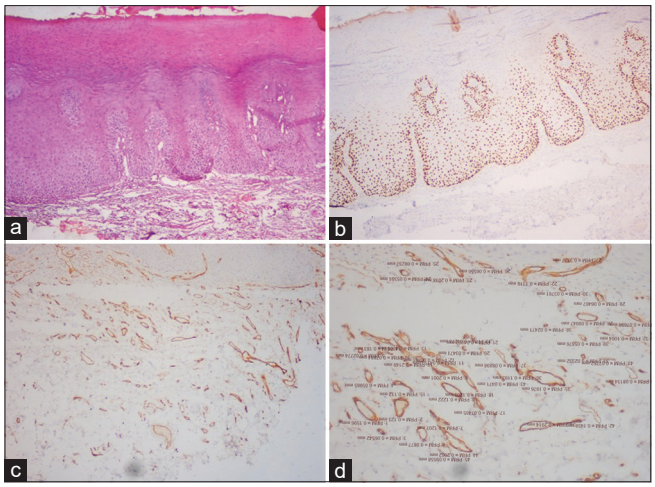

Figure 2: Moderate dysplasia: (a) Leukoplakia with dysplastic features involving half the thickness of epithelium-moderate dysplasia (H and E stain, ×100); (b) p63 antibody staining 1/2 the epithelial thickness (IHC stain, ×100); (c) hotspot of CD 31 stain selected subepithelially (IHC stain, ×100); (d) higher magnification of the × 10 view with morphometric analysis of the vessel walls where mean vessel density is 52 (IHC stain, ×400)

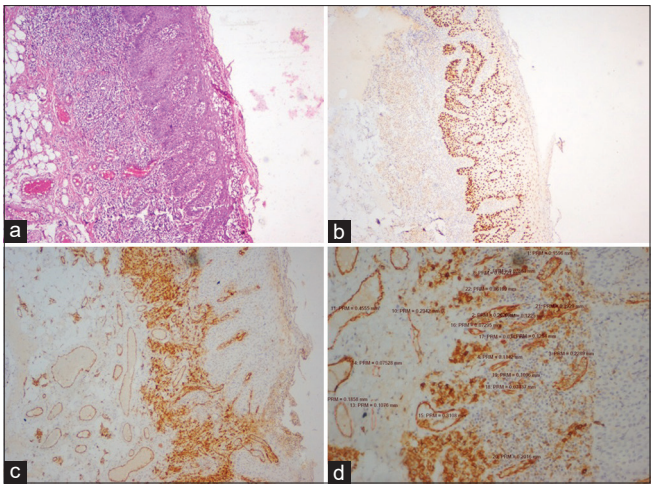

Figure 3: Severe dysplasia: (a) Leukoplakia with dysplastic features involving more than half the epithelial thickness (H and E stain, ×40); (b) p63 stain extending to involve more than half the thickness of the epithelium (IHC stain, ×40); (c) hotspot of CD 31 stain selected subepithelially where along with blood vessels few leukocytes are also stained with CD 31 (IHC stain, ×100); (d) higher magnification of the ×10 view with morphometric analysis of the vessel walls where mean vessel density is 26 (IHC stain, ×400)

Table 5: Correlation between the histopathological grading and p63 staining pattern

Correlation between the different grading systems and p63 biomarker

Methods	Marker	Values	Observer _1	Observer _2
WHO	p63	τb	0.35	0.36
		P	0.04*	0.03*
		n	32	32
Ljubljana	p63	τb	0.13	−0.24
		P	0.45	0.16
		n	32	32
Binary	p63	τb	0.30	0.39
		P	0.08*	0.02*
		n	32	32

*Statistically Significant. τb - Kendall's correlation coefficient, Minus sign denotes negative correlation. Correlation coefficient range: 0.0 - No correlation, 0.01-0.20 - Very weak correlation, 0.21-0.40 - Weak correlation, 0.41-0.60 - Moderate correlation, 0.61-0.80 - Strong correlation and 0.81-1.00 - Very strong correlation

The current study was aimed at comparing the most accepted grading systems such as 2005 WHO grading, Ljubljana, and Binary System for grading leukoplakia. The other aim was to analyze if addition of IHC markers would help better grade the epithelial maturation pattern, associated angiogenesis and assess the dysplastic situation.

Overall, it was noted that the Binary System of grading which categorizes PMDs as low risk or high risk showed least interobserver variation on H and E-stained sections and was statistically significant.

The IHC markers used in the study were p63 and CD31. p63 is mainly involved in assessing the maturation pattern of the epithelium. The nuclear expression of p63 is strong in the basal and parabasal layers in the normal mucosa and fades away as it reaches the superficial layers. Thus, expression of p63 in the superficial layers speculates improper maturation and differentiation pattern of the epithelium, a feature of dysplastic condition.[13]

Various studies have shown that as the dysplastic features in the epithelium increases, the MVD also increases.[4,14] In the present study, IHC with CD31 was carried out to analyze MVD in each of the leukoplakic cases and correlate with p63 staining,

In general, out of 32 cases, histopathological assessment for grading varied between two observers for a maximum of 12 cases and was least for Binary System (seven cases). p63 staining assisted in grading these 12 cases, and it was noted that certain cases in which there was agreement between the observers, p63 staining pattern differed. This was true especially while judging mild dysplastic to moderate dysplastic cases. On the whole, 17 cases stained one-third (mild), 12 cases half (moderate) and 3 cases two-third of the epithelial thickness (severe). Using the Binary System, 17 cases could be grouped as no dysplasia/ low-risk cases and 15 cases as high-risk cases.

Disparity in staining pattern was noted in cases presenting with atrophic epithelium with parakeratinization and orthokeratinization (total of 10 cases). Four cases expressed p63 in half or more than half the thickness of the epithelium, and also, the median value of MVD was more than 40 [Figure 4]. However, the dysplastic changes were minimal and they were categorized as mild dysplastic lesions, with the assistance of IHC markers; abnormality in the differentiation pattern of these four cases was thus assessed. The rest of the cases with atrophic epithelium (six

Table 6: Correlation between p63 and CD 31 IHC markers

Comparison of CD31 marker levels with p63 biomarker expressions for various leukoplakic lesions using Kruskal-Wallis test						
P63	**Median**	**IQR**	**Minimum**	**Maximum**	***H***	***P***
Mild dysplasia	34.0	15.0	19	49	2.065	0.36
Moderate dysplasia	42.0	16.5	20	60		
Severe dysplasia	26.0	28.0	21	49		

IQR: Interquartile range

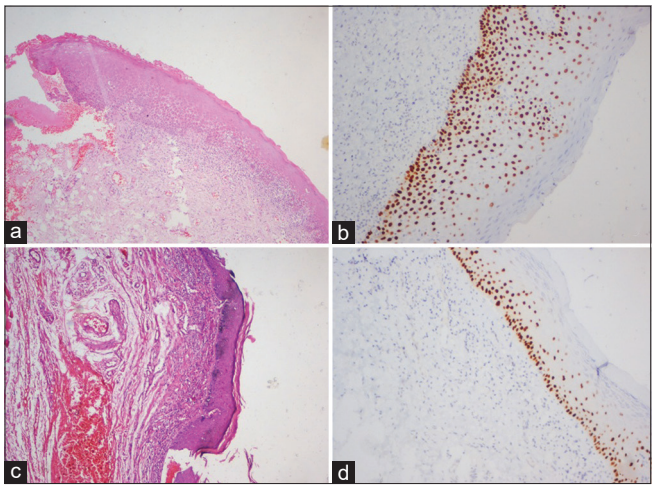

Figure 4: (a) Atrophic parakeratinized epithelium with minimal dysplastic features (H and E stain, ×40); (b) atrophic parakeratinized epithelium showing more than half the thickness of epithelium showing p63 staining (IHC stain, ×200); (c) atrophic orthokeratinized epithelium with minimal dysplastic features (H and E stain, ×40); (d) atrophic orthokeratinized epithelium showing half the thickness of epithelium stained with p63 (IHC stain, ×100)

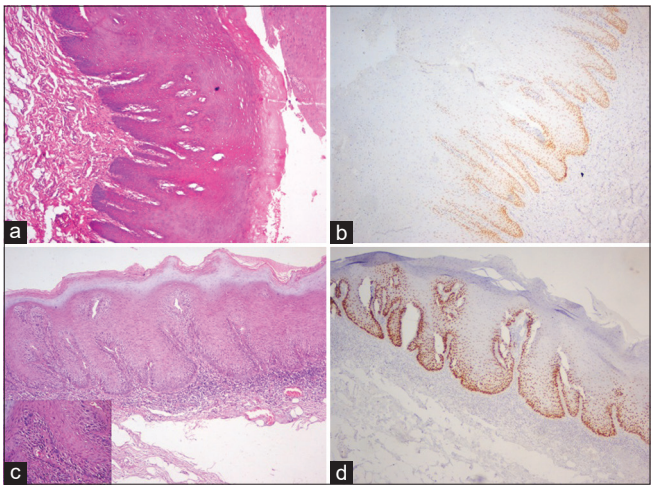

Figure 5: (a) Leukoplakia with hyperplastic epithelium, acanthosis, and hyperkeratinization, suggestive of mild dysplastic features (H and E stain, ×100); (b) p63 stain involves only basal and parabasal cell layer, indicating it to be simple hyperplasia (IHC stain, ×100); (c) photomicrograph showing bulbous rete ridges, altered nuclear cytoplasmic ratio, increased mitoses in the basal layer of cells (Inset: H and E stain, ×400), and hyperparakeratosis, suggestive of moderate dysplasia (H and E stain, ×40). (d) Photomicrograph showing p63 staining mainly basal–parabasal cell layers and few cells in the spinous layer, suggestive of mild dysplasia (IHC stain, ×40)

cases) showed p63 expression in one-third of the epithelial thickness, but MVD was less than 40 in four cases, whereas two cases showed increase in MVD. In the current grading systems, a note on the atrophic epithelium is lacking. More studies have to be done to arrive at a conclusion regarding the behavior of atrophic epithelium with hyperkeratinization and minimal dysplastic features.

One case presented with hyperplastic epithelium with acanthosis but showed p63 expression mainly in the basal and parabasal layers, suggesting that the differentiation pattern was still maintained in the epithelium. Whereas another case showed bulbous rete ridges with hyperparakeratinization and moderate dysplastic features; however, no great increase in p63 staining pattern was noted and was mainly confined to the basal and parabasal layers [Figure 5].

On correlating p63 and CD31 staining for all 32 cases, it was observed that MVD showed a gradual increase from mild to moderate cases where p63 stained one-third and half the epithelial thickness, respectively, but decreased for severe cases. This variation is in correlation with the studies done by Jyothsna *et al.*, who found that the mean vessel area decreased when the dysplastic features were severe.[14]

Interobserver bias in grading leukoplakic lesions can be assisted and improvised with IHC markers. The current study suggests the use of markers in predicting the biological behavior of the PMDs. In association with histopathological features and staging of leukoplakic lesion, IHC markers lend a hand in grouping the lesions which require surgical management and those which require conservative management.

Conclusion

The histopathological assessment in grading oral dysplastic lesions shows interobserver bias. The system which shows least bias on H and E stained sections is Binary System. This assisted with IHC markers can better categorize the PMDs as to those that require definitive treatment and those that fall under a "wait-and-watch" category after the removal of the etiological agent. The validity of these markers on various grounds needs to be assessed on large-scale studies in association with follow-up. The present study gives a clue about the assistance of IHC markers in grading PMDs and elusive or occult progression of the disease can be detected. Correlation of p63 with

MVD subepithelially (CD31 staining pattern) did add value to categorize leukoplakic lesions in low and moderate dysplastic cases.

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Conflicts of interest

There are no conflicts of interest.

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