

# Demographic study of 366 cases of oral leukoplakia and immunohistochemical analysis – An institutional study

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## Abstract

**Background:** It has been reported that oral squamous cell carcinoma (OSCC) is associated with the presence of potentially malignant disorders (PMDs) in 15%–48% of cases. Among PMDs, oral leukoplakia (OL) is the most common, with 16%–62% of cases associated with OSCC. Hence, in the present study, we have analyzed demographic data and re-evaluated immunohistochemical (IHC) data of OL cases and aimed to correlate the clinical, histopathological and IHC aspects of OL.

**Materials and Methods:** The data of histopathologically diagnosed cases of OL were retrieved from the archives. These data were further evaluated for age, gender, duration, site, size, side, habits, clinical staging and histopathological grading. IHC re-evaluation of OL tissues was done using epithelial cadherin (E-cadherin),  $n = 20$ ; human MutL homolog 1 (hMLH1),  $n = 30$ ; CD1a ( $n = 30$ ); vimentin ( $n = 30$ ); Ki-67 ( $n = 30$ ); heat shock protein-70 (HSP-70),  $n = 30$ ; p16<sup>INK4</sup>,  $n = 20$ ; and mucin-1 (MUC1),  $n = 30$ . All the results and observations were subjected to descriptive statistical analysis.

**Results:** The male: female ratio was 7.5:1; right side and buccal mucosa were more commonly affected. The duration of the lesion ranged from 1 to 30 years. One hundred and twelve patients were habituated to tobacco chewing, while 171 patients came with a combined habit of smoke and smokeless tobacco usage. Clinically, most of the lesions were of stage 2 while histopathologically they were of mild dysplasia. There was a decrease in the immunoexpression of E-cadherin, hMLH1 and CD1a, while there was an increase in the immunoexpression of vimentin, Ki-67, HSP-70, MUC1 and p16<sup>INK4</sup>.

**Conclusion:** The study of different biomarkers such as cytoplasmic, membranous and nuclear in OL will help in better understanding and application of a reliable marker for diagnostic and prognostic purpose.

**Keywords:** Habits, histopathology, oral potentially malignant disorders

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**Submitted:** 13-Jul-2021, **Revised:** 30-Sep-2021, **Accepted:** 24-Oct-2021, **Published:** 11-Jan-2022

## INTRODUCTION

Oral squamous cell carcinomas (OSCCs) account for 90% of the total oral malignancies.<sup>[1]</sup> Globocan in 2018 had

ranked lip and oral cavity malignancies 2<sup>nd</sup> in India and had ranked it 18<sup>th</sup> worldwide.<sup>[2]</sup> In 2017, the WHO replaced it

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**How to cite this article:** Ahire MS, D'Souza ZI, Chettiankandy TJ, Nagar SR, Sinha A, Tupkari JV. Demographic study of 366 cases of oral leukoplakia and immunohistochemical analysis – An institutional study. *J Oral Maxillofac Pathol* 2021;25:478-84.

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#### Quick Response Code:



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#### DOI:

10.4103/jomfp.jomfp\_228\_21

with oral potentially malignant disorders. Furthermore, recently it is grouped as potentially premalignant oral epithelial lesions (PPOELs) which is a broad term to define both histologic and clinical lesions that have the malignant potential.<sup>[3]</sup> Oral leukoplakia (OL) is the most common lesion among the PPOELs, with a reported global prevalence of 2%.<sup>[4]</sup> It shows the highest rate of malignant transformation (0.13% and 34.0%) of all PPOELs.<sup>[5]</sup>

To diagnose PPOELs, Carreras-Torras and Gay-Escoda have enumerated various techniques [Table 1] to be used.<sup>[6]</sup> Among these techniques, immunohistochemical (IHC) methods are widely used molecular technique that is simple, quick and accurate. Here, we have studied IHC markers that are used in OL [Table 2].

**Table 1: Laboratory techniques used for diagnosis of oral leukoplakia apart from conventional oral examination**

Methods	Methodology
Vital staining	5% acetic acid Toluidine blue Methylene blue Lugol's iodine Rose bengal Iodine staining Tolonium chloride
Light-based detection systems	Tissue fluorescence imaging (Velscope and Identafi 3000) Chemiluminescence (ViziLite Plus, Microlux/DL) Tissue fluorescence spectroscopy (NBI)
Histological techniques	Incisional biopsy Excisional biopsy
Cytological techniques	Oral brush biopsy (Oral CDX) Liquid-based cytology LCMd
Molecular analyses	Gene alterations Epigenetic alterations, loss of heterozygosity and microsatellite instability Viral genome studies Proliferation index and AgNOR analysis Immunohistochemical identification of tumor markers
Imaging techniques	FDG-PET OCT
Other techniques	Onco-chips

LCMd: Laser microdissection, OCT: Optical coherence tomography, FDG-PET: Fluorodeoxyglucose (FDG)-positron emission tomography (PET), AgNOR: SILVER nucleolar organizing region

**Table 2: Stainability and role of different markers**

Marker	Type of antibodies	Stainability of marker	Role in pathogenesis
MUC1	Monoclonal Rabbit	Membranous/cytoplasmic	Promoting receptor tyrosine kinase signaling and potentiating its oncogenic function
CD1a	Monoclonal Mouse	Membranous	Antigenic response and local defense mechanism
Ki-67		Nuclear	Proliferation index
Vimentin		Cytoplasmic	Epithelial-mesenchymal transition
HSP-70		Both (C, N, C/N)	Biological stress and promoting tumorigenesis by suppressing apoptosis
hMLH1		Membranous	MMR - Mutation avoidance and maintaining genomic stability
E-cadherin		Membranous/cytoplasmic	Tumor progression
P16INK4A		Both (C, N, C/N)	CDKN2 Inhibitor (maintenance of cell cycle and inhibition of proliferation)

C: Cytoplasmic, N: Nuclear

## MATERIALS AND METHODS

The present study aimed to analyze the demographic data of OL cases from the institution and also to assess the IHC expression of different markers in OL cases and normal oral mucosa.

From 1981 to 2018, clinical and histopathological data were retrieved from the archives of the Department of Oral Pathology and Microbiology after clearance from the institutional ethical committee. A total of 7432 biopsies were received, out of which 366 cases were of OL. These data were further re-evaluated for age, gender, site, side, habit, histopathological grading and IHC markers. Van Der Waal *et al.* (2000) 4 staging for OLEP was used.<sup>[7]</sup> Different IHC markers, i.e., CD1a, mucin-1 (MUC1), Ki-67, vimentin, heat shock protein-70 (HSP-70) and human MutL homolog 1 (hMLH1), were re-evaluated in mutually exclusive groups of 30 patients while P16<sup>INK4</sup> and epithelial cadherin (E-cadherin) in mutually exclusive groups of minimum 20 cases each of OL, accounting to a total of 220 cases and controls. The localization and type of antibody of these markers are specified in Table 2. All the results and observations were subjected to descriptive statistical analysis and other statistical tests.

## RESULTS

### Demographic data and histopathological findings

In the present study, the annual frequency of OL is 6.6%. The gender distribution showed male predominance with a male: female ratio of 7.5:1. A maximum number of cases were reported in the age group of the 1<sup>st</sup>–8<sup>th</sup> decade of life with a peak in the 4<sup>th</sup>–6<sup>th</sup> decade. Although fewer women were involved than men, both genders showed a peak in the 4<sup>th</sup>–6<sup>th</sup> decade of life with 54.09%. Among the side involved by OL in the oral cavity, 38.79% involved the right side and 37.43% involved the left side while 6.01% involved both sides. In 17.76%, the record of the side was unavailable. Two hundred and twenty-four cases involved the buccal mucosa, 14 cases the labial mucosa and 23 cases the commissures [Figure 1]. Alveolar ridge was involved in nine

cases. We used the OLEP staging provided by Van Der Waal *et al* (2000) to clinically stage oral leukoplakia. 14.25% and 25.74% of cases showed moderate and severe dysplasia, respectively. Out of 366 cases, 112 had habits of tobacco chewing, 32 were habituated to bidi smoking, pan chewing was seen in 35 patients, 12 came with a habit of cigarette smoking and 4 had a habit of mishri usage. A high number of patients, i.e., 171, came with a combination of smoke as well as smokeless tobacco habits in the form of cigarette and tobacco quid. In 69 cases, the habit history was not available [Figure 2 and Table 3].

### Immunohistochemical analysis

#### Mucin-1

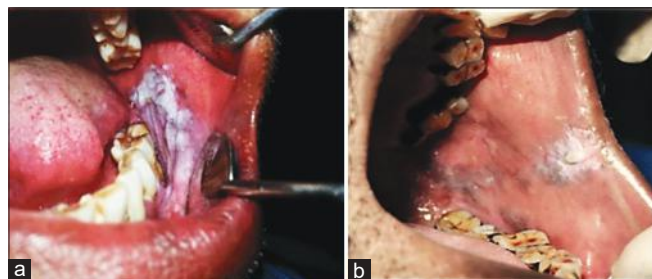
All cases of control group showed negative MUC1 expression. MUC1 was positive in only 26.6% (8/30) of cases, of which 2 cases were of mild dysplasia, 2 cases were of moderated dysplasia and 4 cases were of severe dysplasia [Table 4 and Figure 3].

#### CD1a

All cases of control group showed positive expression. Out of 30 cases of OL, mild dysplasia ( $n = 27$ ) showed the highest CD1a expression with a mean of 30.52 cells/mm<sup>2</sup>, followed by moderate dysplasia ( $n = 1$ ) with a mean of 25 cells/mm<sup>2</sup> and severe dysplasia ( $n = 2$ ) with a mean of 22 cells/mm<sup>2</sup> [Table 4 and Figure 3].

**Table 3: Gender-based age distribution and side distribution of oral leukoplakia**

Variables	Number cases		Percentage (%)
	Male	Female	
Age			
11-20	1	0	0.27
21-40	89	6	25.96
41-60	173	25	54.09
>60	34	2	9.84
Unknown	36		9.84
Total	366		100
Side of lesion			
Right	142		38.79
Left	137		37.43
Both	22		6.01
Unknown	65		17.76
Total	366		100



**Figure 1:** Clinical picture of oral leukoplakia on (a) buccal mucosa and (b) commissural area

#### Ki-67

Ki-67 expression was seen in all 30 cases of control group, the range of expression being 19.1%–46.93%, and the mean value was 27.10% [Table 4]. The expression of Ki-67 staining was seen only in the basal cell layer [Table 4 and Figure 3].

Of the 30 cases of OL, immunopositivity for Ki-67 was seen in 24 cases and 6 cases were completely negative. Expression of Ki-67 staining was seen in the basal and suprabasal layers of the epithelium.

#### Vimentin

All the tissues in the control group gave a negative expression of vimentin in epithelial cells.

On IHC staining of the leukoplakia group, 10 cases of 6 mild dysplasia, 7 cases of moderate dysplasia and 11 cases of severe dysplasia showed positivity for vimentin, weak positivity for Vimentin was seen in (93.3%) cases and 2 (6.7%) cases were negative. The range of expression was 3.2 cells/mm<sup>2</sup>–48 cells/mm<sup>2</sup>, and the mean value was 20.05/mm<sup>2</sup> [Table 4 and Figure 3].

#### Heat shock protein-70

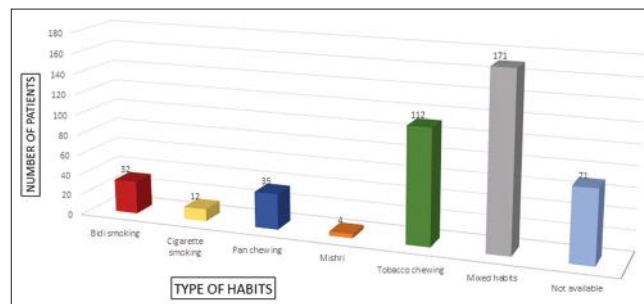
Twenty-seven tissues of the control group showed positivity for HSP-70. Out of 30 cases, 7 cases of mild dysplasia, 12 cases of moderate dysplasia and 11 cases of severe dysplasia showed positivity for HSP-70 [Table 4 and Figure 3].

#### Human MutL homolog 1

hMLH1 was positive in all cases of a control group. Among the study group, 25 cases (83.3%) showed positivity out of 30 cases for hMLH1. Ten were positive in mild, six in moderate and nine in severe grades of dysplasia [Table 4 and Figure 3].

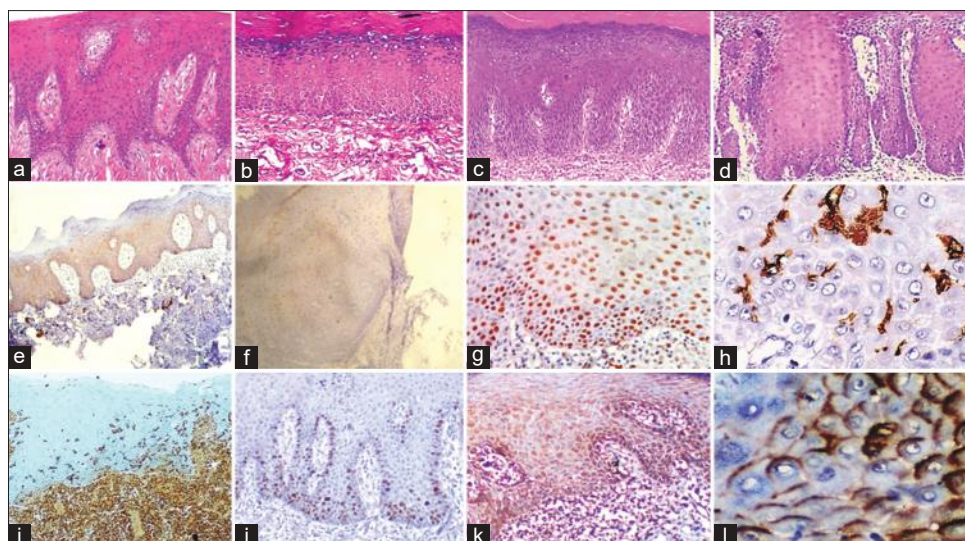
#### Epithelial cadherin

E-cadherin was positive in all cases of a control group. Among the study group, E-cadherin was positive in all



**Figure 2:** Number of patients with the type of habit (graph)





**Figure 3:** The microphotographs depict histopathological features of (a) normal oral mucosa (H and E\*, ×40), (b) mild dysplasia (H and E\*, ×40), (c) moderate dysplasia (H and E\*, ×40), (d) severe dysplasia (H and E\*, ×40), (e) positive immunoexpression for E-cadherin (×10), (f) positive immunoexpression for P16 INK4A (×10), (g) positive immunoexpression for human MutL homolog 1 (×40), (h) positive immunoexpression for CD1a (×40), (i) positive immunoexpression for vimentin (×10), (j) positive immunoexpression for Ki- 67 (×10), (k) positive immunoexpression for heat shock protein 70 (×10), (l) positive immunoexpression for MUC1 (×40). \*Hematoxylin and eosin stain

**Table 4: Immunohistochemical analysis of various markers and their expression in different grades of dysplasia**

Markers	E-cadherin (n=20)	P <sup>16INK4A</sup> (n=20)	hMLH1 (n=30)	CD1a (n=30)	Vimentin (n=30)	Ki-67 (n=30)	HSP-70 (n=30)	MUC1 (n=30)
Control group	Positive	Basal layer positive	Negative	Basal layer weak positive	Negative	Positive	Positive	Positive
Study group	Decreased expression	Increased expression	Increased expression	Increased expression	Increased expression	Decreased expression	Decreased expression	Increased expression
Moderate dysplasia	3	3	6	25.0	7	8	12	2
Severe dysplasia	7	9	9	22.0	11	12	11	4
Negative (%)	0	4 (20)	5 (27.6)	0	2 (27.7)	6 (20.0)	0	22 (73.4)
Total positive (%)	20 (100)	16 (80)	25 (83.3)	30 (100)	28 (93.3)	24 (80.0)	30 (100)	8 (26.6)

E-cadherin: Epithelial cadherin, hMLH1: Human MutL homolog 1, HSP: Heat shock protein, MUC1: Mucin-1

20 cases (100%) that were sampled. However, the staining intensity and the number of cells stained positive were reduced as compared to the control group. Ten cases of mild dysplasia, three of moderate dysplasia and seven in severe dysplasia showed positive expression for E-cadherin [Table 4 and Figure 3].

#### P16<sup>INK4</sup>

All the control tissues were negative for P16<sup>INK4</sup>. P16<sup>INK4</sup> showed positivity in only 16 cases (80%), while 4 cases were negative. Four cases were positive in mild, 3 in moderate and 9 in severe grades of dysplasia. In our study, nuclear staining was negative in all OL samples and cytoplasmic staining was seen in 16 cases [Table 4]. The markers were statistically analyzed using the Chi-square test and Pearson analysis. All IHC markers were statistically significant ( $P < 0.0001$ ) [Table 4 and Figure 3].

## DISCUSSION

The frequency of epithelial dysplasia, carcinoma *in situ* or

invasive SCC in leukoplakia varies from 8.6% to 60.0%, and malignant transformation occurred in 13.6% to 36.4% of cases.<sup>[7]</sup> Prevention is better than cure, thus if potentially malignant disorders are identified at an early stage, its transformation into OSCC will also be reduced. The male: female ratio was recorded as 7.5:1 in the present study, following similar studies by Napier and Speight.<sup>[8]</sup> One of the most justified reasons for male predominance would be the most frequent use of tobacco in men than in women. The maximum number of patients was in the age group of 41–60 years per a study of Patil *et al.* (2015) and Markopoulos *et al.* (2012).<sup>[9]</sup> The most common site was buccal mucosa (61.2%) which was similar to the findings noted by Napier and Speight<sup>[8]</sup> and Kumar *et al.*<sup>[10]</sup> The reason could be the most likely placement of tobacco in the buccal vestibule by most of the patients. This was followed by labial mucosa, commissural area, alveolar ridge, dorsum of the tongue, the floor of mouth and gingiva and other sites. The present study and literature review shows a significant association between tobacco use and the occurrence of OL.<sup>[3]</sup>

CD1a IHC expression was seen in all cases of the control group. The mean value was 31.5 cells/mm<sup>2</sup>. A similar study by Lasisi *et al.*<sup>[11]</sup> showed a range of 80.7 ± 66.9 cells/mm<sup>2</sup>, which was CD1a positive in the control group cases. In our study, the expression of CD1a in OL without dysplasia showed a mean of 22.5 cells/mm<sup>2</sup> while OL with dysplasia showed a mean of 29.78 cells/mm<sup>2</sup>. This was contrary to a study conducted by Öhman *et al.*,<sup>[12]</sup> wherein there was not much difference between CD1a immunopositivity in the epithelium of OL with and without dysplasia. However, they reported an increase in the Langerhans cells (LCs) per unit area within the connective tissue of OL with dysplasia concerning OL without dysplasia. Possible mechanisms for this increase in LCs are that in the early stages, the LCs try to eliminate tumor-associated antigens and apoptotic material in an attempt to ward off the dysplastic transformation.<sup>[12]</sup> Silva *et al.*<sup>[13]</sup> suggested that a decrease in LCs could be associated with malignant transformation. This suggestion is reinforced through our findings wherein there is a decrease in LCs with increasing grades of dysplasia.

Expression of Ki-67 staining was seen only in the basal cell layer for the control group. This was in accordance with the study done by Mondal *et al.*<sup>[14]</sup> It is well known that the basal layer of the oral epithelium is the location of the normal proliferating cell compartment, whereas suprabasal layers are only spaces of cellular maturation whose cellular alterations show potential signs of dysplasia.<sup>[15,16]</sup> Ki-67 positivity depicts the aggressiveness as well as the proliferative activity of a lesion. Its gradual increase from normal mucosa to leukoplakia and a subsequent increase in OSCC make it a good prognostic marker.<sup>[14]</sup>

In the control group, all the tissues gave a negative expression of vimentin in epithelial cells. This confirms the study carried out by Sawant *et al.* (2014).<sup>[15]</sup> In our study, a few immunopositive isolated cells were noticed in the suprabasal layer of the epithelium. This can be explained by the fact that nonkeratinocytes such as melanocytes and Langerhans cells normally show positive staining for vimentin.<sup>[15]</sup> In our study, the leukoplakia group showed 93.3% positivity, while in a similar study carried out by Sawant *et al.* (2014),<sup>[15]</sup> immunostaining for vimentin was seen in 44% of leukoplakia samples. MUC1 was positive in only 26.6% (8/30) of cases, of which 2 cases were of mild dysplasia, 2 cases were of moderate dysplasia and 4 cases were of severe dysplasia. A study done by Akhtar *et al.* showed that this increase was seen in cases undergoing a malignant transformation and hence it can be used to predict the malignant potential of oral epithelial dysplastic lesions.<sup>[17]</sup> Vimentin serves as a marker as well as a driver for an emergency medical technician.<sup>[18]</sup>

HSP-70 in the control group was more characteristically stained in the basal epithelial cells; this was in accordance with the study done by Lee *et al.*<sup>[19]</sup> This weak expression may reflect a state of biologic stress or may be associated with a state of increased, cellular activity.

IHC evaluation revealed that there was an increase in HSP-70-positive percentage cells of OL in relation to the control group; this was similar to the study done by Patil *et al.* (2015) and Markopoulos *et al.* (2012).<sup>[9,20]</sup> The increase of HSP-70 in dysplastic cells has been suggested to play a role in tumorigenesis by suppressing apoptosis.<sup>[9]</sup> HSP-70 upregulation indicates that the cells of the lesion are under biological stress.<sup>[21]</sup>

Both increased and decreased expressions of p16<sup>Ink4a</sup> have been reported in oral premalignant and malignant lesions. In the present study, all controls were negative and p16 positivity increased with increasing grades of dysplasia. This is under studies published by Klaes *et al.*<sup>[22]</sup> and Volgareva *et al.*<sup>[23]</sup>

It was observed that there is the reduction in E-cadherin in oral epithelial dysplasia as the severity of dysplasia is increased. In the mild and moderate degree of dysplasia, loss of E-cadherin was less as compared to the severe degree of dysplasia. The loss of E-cadherin-mediated cell adhesion correlates with the loss of the epithelial morphology. The E-cadherin expression in mild epithelial dysplasia was present in a suprabasal and basal area similar to the normal epithelium.<sup>[24]</sup> In moderate epithelial dysplasia, E-cadherin expression was present in suprabasal while reduced in the basal cell layer. This loss confers an invasive property by the basal cell.<sup>[25]</sup> Loss/reduced expression of E-cadherin may be due to reduced transcription as a result of hypermethylation of CpG islands in the promoter region<sup>[26]</sup> [Table 4]. Our findings coincided with those of Costa *et al.*<sup>[27]</sup> where E-cadherin staining was reduced in poorly differentiated OSCCs.

hMLH1 is a vital part of the mammalian mismatch repair system which is responsible for maintaining genomic stability during duplication.<sup>[28]</sup> A decrease in hMLH1 was seen in the present study from mild to severe dysplasia. hMLH1 reduces due to hypermethylation of its gene by free radicals, peroxides and other carcinogens from tobacco; this results in oxidative stress. Reactive oxygen species then damage the DNA and its repair proteins.<sup>[29]</sup>

Mucins are high molecular weight glycoproteins that play a major role in cell growth, differentiation and cell signaling. Cancer cells use mucins for proliferation,

survival, invasion, metastatic growth and protection against innate immunity.<sup>[30]</sup> Thus, in cancers, MUC1 is always overexpressed and alteration in glycosylation is associated with the development and progression of malignancy.<sup>[30]</sup>

## CONCLUSION

The demographic data suggest that there is an urgent need for community awareness regarding the detrimental effects of smokeless and smoking tobacco to detect lesions at an early stage as it will reduce the future burden of oral cancer. It also emphasizes the importance of proper maintenance of biopsy records and nationwide standardization of case history and biopsy record form at the institutional and community level. It also indicates that there is a need to create a national (central) registry of PPOELs. This will help obtain the geographical prevalence of PPOELs and habits responsible for the same as well as provide evidence for advanced clinical epidemiological and health services research.

Based upon the IHC expression of different markers in the present study and literature review, we conclude that within PPOELs, there is an upregulation and downregulation of numerous molecules which can assist in the prognostication of the lesion. Treatment modality should be based on a holistic approach, in which there is a correlation between the genetics, epigenetic, physical constitution, nutrition, mindfulness, clinical and histopathological findings along with the earliest molecular changes occurring in the affected tissue.

## Acknowledgement

We acknowledge Dr. Monal Yunathi, Dr. Avadhoot Avadhani, Dr. Narendra Choudhary, Dr. Prakhari Agrawal, Dr. Pravin Shinde, Dr. Rashmi, Dr. Anuradha Lokare, Dr. Sayli Jadhav Ex-Postgraduate students and all staff members who had contributed in this study till now. Dr. M.G.Pawar, Ex-Dean, GDC & H, Mumbai.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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