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; p16INK4, 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 p16INK4.

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

INTRODUCTION

Oral squamous cell carcinomas (OSCCs) account for 90% of the total oral malignancies.[¹] Globocan in 2018 had ranked lip and oral cavity malignancies 2nd in India and had ranked it 18th worldwide.[²] In 2017, the WHO replaced it...
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.[5] Oral leukoplakia (OL) is the most common lesion among the PPOELs, with a reported global prevalence of 2%.[6] It shows the highest rate of malignant transformation (0.13% and 34.0%) of all PPOELs.[8]

To diagnose PPOELs, Carreras-Torras and Gay-Escoda have enumerated various techniques [Table 1] to be used.[9] 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].

**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.[7] 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 P16Ink4a 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 1st–8th decade of life with a peak in the 4th–6th decade. Although fewer women were involved than men, both genders showed a peak in the 4th–6th 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

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

| Methods                       | Methodology                                                                 |
|-------------------------------|-----------------------------------------------------------------------------|
| Vital staining                | 5% acetic acid, Tolidine blue, Methylene blue, Lugol's iodine, Rose Bengal  |
| Light-based detection systems | Tissue fluorescence imaging (Velscope and Identafi 3000)                    |
| 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, Onco-chips                                                    |
| Other techniques              |                                                                             |

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
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 moderate 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/mm², followed by moderate dysplasia (n = 1) with a mean of 25 cells/mm² and severe dysplasia (n = 2) with a mean of 22 cells/mm² [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    |                |

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 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/mm²–48 cells/mm², and the mean value was 20.05/mm² [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 is 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
Ahire, et al.: Demographic study of 366 cases of oral leukoplakia with immunohistochemical analysis

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.\cite{7} 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.\cite{8}

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).\cite{9} The most common site was buccal mucosa (61.2%) which was similar to the findings noted by Napier and Speight\cite{8} and Kumat et al.\cite{10} 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.\cite{10}

### DISCUSSION

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].

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

| Markers | E-cadherin (n=20) | P16INK4A (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 negative | Basal layer increased expression | Basal layer increased expression | Basal layer increased expression | Basal layer increased expression | Basal layer increased expression | Basal layer increased expression |
| Study group | Decreased expression | Increased expression | Increased expression | Increased expression | Increased expression | Increased expression | Increased expression | Increased expression |
| Moderate dysplasia | 3 | 3 | 6 | 22.0 | 1 | 12 | 2 | 4 |
| Severe dysplasia | 7 | 9 | 5 (27.6) | 0 | 2 (27.7) | 12 | 11 | 4 |
| Negative (%) | 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].

\subsection*{P16INK4A} All the control tissues were negative for P16INK4A. P16INK4A 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].

![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](image-url)
CD1a IHC expression was seen in all cases of the control group. The mean value was 31.5 cells/mm². A similar study by Lasisi et al. showed a range of 80.7 ± 66.9 cells/mm², 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/mm² while OL with dysplasia showed a mean of 29.78 cells/mm². This was contrary to a study conducted by Öhman et al., wherein there was not much difference between CD1a immunoeexpression 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. Silva et al. 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. 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. 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.

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). 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. In our study, the leukoplakia group showed 93.3% positivity, while in a similar study carried out by Sawant et al. (2014), 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. Vimentin serves as a marker as well as a driver for an emergency medical technician.

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. 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). The increase of HSP-70 in dysplastic cells has been suggested to play a role in tumorigenesis by suppressing apoptosis. HSP-70 upregulation indicates that the cells of the lesion are under biological stress.

Both increased and decreased expressions of p16 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. and Volgareva et al.

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. 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. Loss/reduced expression of E-cadherin may be due to reduced transcription as a result of hypermethylation of CpG islands in the promoter region [Table 4]. Our findings coincided with those of Costa et al. 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. 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.

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.\[30\] Thus, in cancers, MUC1 is always overexpressed and alteration in glycosylation is associated with the development and progression of malignancy.\[30\]

**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. Prakhar 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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