ORIGINAL RESEARCH

Histopathological Assessment of Fungal Infection in the Biopsies of Oral Squamous Cell Carcinoma, Oral Lichen Planus, and Leukoplakia Using Hematoxylin and Eosin and Periodic Acid-Schiff Stains

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ABSTRACT

Background: The prevalence of diseases caused by *Candida* species has increased in recent years, mainly due to an increase in a number of patients who are immune compromised. The coexistence of *Candida* species within humans either as commensals or pathogens has been a subject of interest, among physicians. Furthermore, the association of *Candida* with various precancer and cancer lesions has been reported as a causative agent. A histopathological report of unexpected fungal infection is likely to be acted on clinically and may affect the patient's management and prognosis. Fungal infection especially that attributable to *Candida albicans* has been extensively researched in individual lesions, but the aim of this study was to determine how frequently fungal hyphae are detected by the hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) stain in oral mucosal biopsies submitted routinely for histopathological diagnosis.

Aims and Objectives: The aim and objective of this study is to determine the frequency of fungal infection in biopsies of oral mucosal lesions, namely leukoplakia, oral squamous cell carcinoma (OSCC), and oral lichen planus (OLP), using H and E and PAS stains.

Materials and Methods: Previously diagnosed biopsy specimen of 20 cases each of different grades of OSCC, oral leukoplakia, and OLP was taken, and tissue sections were prepared and stained with H and E and PAS stains for the visualization of fungi. The observation of fungal hyphae in the PAS-stained sections was expressed in terms of presence or absence only. After the evaluation of the slides, the results were compiled and subjected to statistical analysis.

Results: The values obtained were found to be more statistically significant with PAS stain than H and E stain ($P = 0.016$).

Conclusion: The present study shows an increased prevalence of *Candidal* hyphae in OSCC, followed by leukoplakia and OLP suggestive of *Candidal* infection which are more easily visualized with PAS reagent than H and E stain.

Clinical Significance: Our study suggests that whenever oral mucosal lesions with dysplastic features are diagnosed and show features of *Candidal* infection in H and E-stained sections, PAS staining has to performed to confirm *Candidal* infection and antifungal therapy should be considered in the management of these lesions.

Keywords: *Candidal* hyphae, Hematoxylin and eosin, Oral lichen planus, Oral squamous cell carcinoma, Periodic acid Schiff.

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INTRODUCTION

Normal oral flora comprises a diverse array of organisms which includes eubacteria, archaea, fungi, mycoplasmas, and protozoa.[1] Among these, fungi are classified as eukaryotes, and the most important to dentistry belongs to the genus *Candida*. Human infections caused by *Candida albicans* and other related species range from the more common oral thrush to fatal, systemic super infections in patients who are afflicted with other diseases.[2]

Candida species may be recovered from up to one-third of the mouths of normal individuals and are considered inhabitants of the normal flora of oral and gastrointestinal tract.[3] *C. albicans* is the principal species associated with human oral mycoses and is the most virulent among pathogenic *Candida* species.[4]

The advent of the human immunodeficiency virus, the use of wide spectrum antibiotics, immunosuppressive therapy, and increasing incidence of diabetes are some of the global scenarios that have resulted in the increase in immunocompromised individuals. This, in turn, has paved way for the increased incidence of opportunistic infections, and oral candidiasis (OC) is clinically the most relevant among them for dental health-care providers.[5]

Oral cancer is the sixth most common cause of cancer-related deaths and represents 5.5% of all malignancies. The incidence is increasing, and the mortality rate has not improved for decades.[6] The concept of a two-step process of cancer development in the oral mucosa, i.e., the initial presence of a precursor (pre-malignant, pre-cancerous) lesion subsequently developing into cancer, is well established.[7]

The most common premalignant lesion and condition include oral leukoplakia and oral lichen planus (OLP). The foremost purpose of identifying oral premalignant lesions is to prevent malignant transformation. Therefore, it is necessary to identify risk factors that can help predict those patients with premalignant lesion who are most likely to develop frank carcinoma.[8]

In 1966, Cawson first suggested the role of *Candida* as a promoter of oral mucosal keratoses to carcinoma, and this has remained a highly controversial point to date.[9] Recently, it has been shown that patients with epithelial dysplasia and oral squamous cell carcinoma (OSCC) harbor higher levels of *Candida*.[10]

The occurrence and relevance of *Candidal* infection in potentially malignant disorders are still to be understood. The present study was undertaken to determine how frequently fungal hyphae are detected by the hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) stain in oral mucosal biopsies submitted routinely for histopathological diagnosis.

MATERIALS AND METHODS

The study was conducted using paraffin-embedded tissue blocks with a clinical and histopathological confirmed diagnosis of leukoplakia, OLP, and OSCC. Biopsy specimen of 20 cases each of different grades of OSCC, 20 cases of oral leukoplakia, and 20 cases of OLP were collected. Tissue blocks corresponding to the cases were taken, and tissue sections were prepared for the same for histopathological examinations. The sections were then mounted on glass slides and stained using H and E stain and PAS stains. The exclusion criteria included patients on anti-fungal therapy 3 weeks before undergoing biopsy, immunocompromised patients, and previously treated cases of OSCC, leukoplakia, and OLP.

The sections obtained from the block of leukoplakia, OLP, and OSCC were stained with H and E stain and PAS stain after diastase treatment to identify *C. albicans* if present.

In PAS-stained sections after diastase digestion, the presence of hyphae or pseudohyphae which appeared as pinkish red or magenta color was the diagnostic criteria.[11]

Identification of *Candidal* infection was also done by the examination of H and E-stained sections using the presence of indicative features such as epithelial hyperplasia, hyperkeratosis, superficial abscess formation, and chronic inflammation of the lamina propria and the presence of thin and weak hematoxyphilic appearance of *Candidal* hyphae.[12]

The observation of fungal hyphae in the PAS-stained sections was expressed in terms of presence or absence only. Olympus BX41 research microscope was used, wherein the stained sections were observed under Bright field, and presence of *Candidal* hyphae was noted under ×40 magnification and recorded.

RESULTS AND OBSERVATION

The results obtained was compiled using MS Excel worksheet and statistical analysis utilized Chi-square test along with statistical software SPSS version 17.

The comparison of *Candida* identification levels in leukoplakia, OLP, and OSCC was done using H and E and PAS stain which is shown in Tables 1 and 2. The features suggestive of *Candidal* infection such as epithelial hyperplasia, hyperparakeratosis, superficial

Table 1: Prevalence of *Candidal* hyphae in OSCC, leukoplakia, and oral lichen planus using H and E stain

H and E stain	Group		
	OSCC	Lichen planus	Leukoplakia
Yes			
Count (%)	8 (40.0)	4 (20.0)	6 (30.0)
No			
Count (%)	12 (60.0)	16 (80.0)	14 (70.0)
Total			
Count (%)	20 (100.0)	20 (100.0)	20 (100.0)

χ^2=1.905, *P*=0.386 non-significant

Table 2: Prevalence of *Candidal* hyphae in OSCC, leukoplakia, and oral lichen planus using PAS stain

PAS stain	Group		
	OSCC	Lichen planus	Leukoplakia
Yes			
Count (%)	16 (80.0)	7 (35.0)	11 (55.0)
No			
Count (%)	4 (20.0)	13 (65.0)	9 (45.0)
Total			
Count (%)	20 (100.0)	20 (100.0)	20 (100.0)

PAS: Periodic acid-Schiff, χ^2=8.281 *P*=0.016 significant

microabscess formation, and chronic inflammation of lamina propria were significantly found.

In case of oral leukoplakia, 6 cases showed the presence of hematoxyphilic hyphae [Figure 1] and 11 cases (55%) were positive for *Candidal* hyphae in PAS [Figure 2]. Among 20 cases of OLP,H and E showed positivity for Candida in four cases [Figure 3] while PAS showed positivity in seven cases (35%) as represented in Figure 4. In 20 cases of OSCC, 16 cases were found positive for *Candidal* hyphae in PAS stain [Figure 5] as compared to H and E which showed positive in 8 of the OSCC cases [Figure 6].

The values obtained were found to be more statistically significant with PAS stain than H and E stain (P = 0.016). The present study showed that the prevalence of *Candidal* hyphae was more in OSCC, followed by leukoplakia and OLP as represented in Graphs 1 and 2. The present study also showed that the *Candidal* hyphae which were suggestive of *Candidal* infection were more easily visualized with PAS reagent than H and E stain.

DISCUSSION

Cancer is a major cause of disease and death throughout the world. Oropharyngeal cancer is the fifth most common cancer worldwide in men and the seventh in women, but there are marked geographical variations.[13] Although most oral cancers probably arise in clinically normal mucosa, some are preceded by a precancerous lesion, which indicates an increased risk of cancer development at a particular site.[6]

Potentially malignant disorders of the oralmucosa are site specific predictors and indicators of the risk of future malignancies.[14] A variety of precancerous lesions and conditions affect oral mucosa. Leukoplakia and OLP are the most common potentially malignant disorders of the oral mucosa.[15]

Oral leukoplakia occurs in 3–4% of the adult population, and if untreated, 5–10% of the cases will develop into carcinoma.[16] According to Neville *et al*., the prevalence of cutaneous lichen planus is approximately 1%, whereas the prevalence of OLP is between 0.1% and 2.2%.[17]

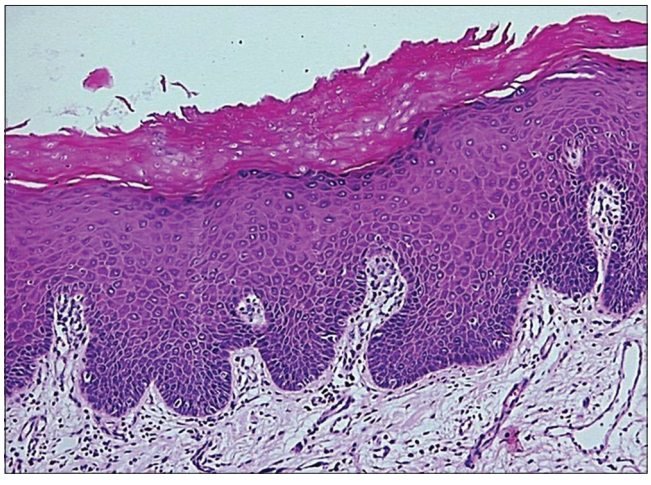


Figure 1: Hematoxylin- and eosin-stained section of leukoplakia with epithelial dysplasia (×10)

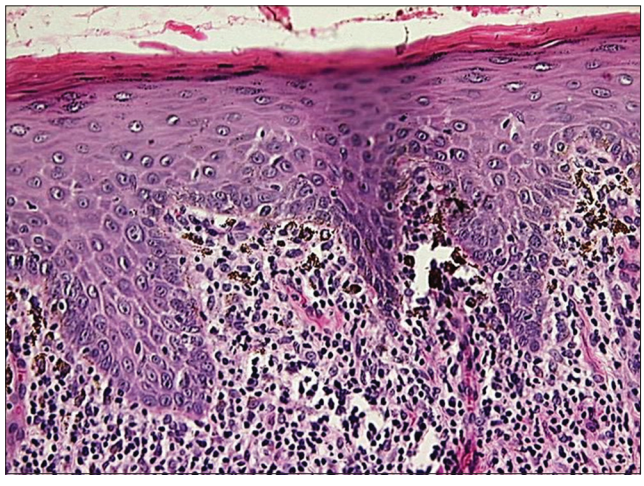


Figure 3: Hematoxylin- and eosin-stained section of oral lichen planus (×40)

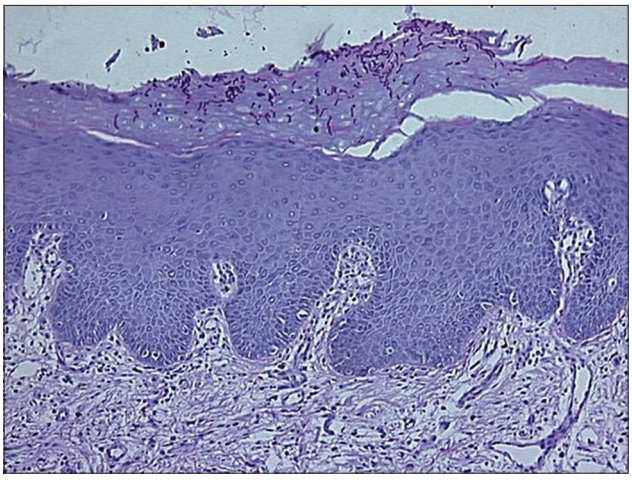


Figure 2: Periodic acid-Schiff'-stained section of leukoplakia with epithelial dysplasia (×10)

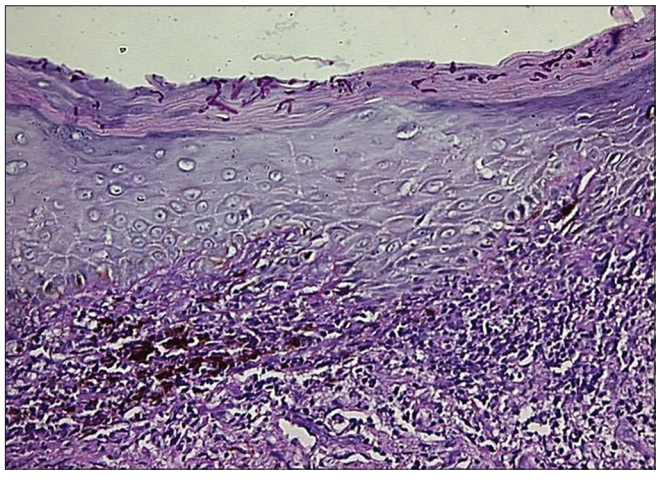


Figure 4: Periodic acid-Schiff-stained section showing *Candidal* hyphae in oral lichen planus (×40)

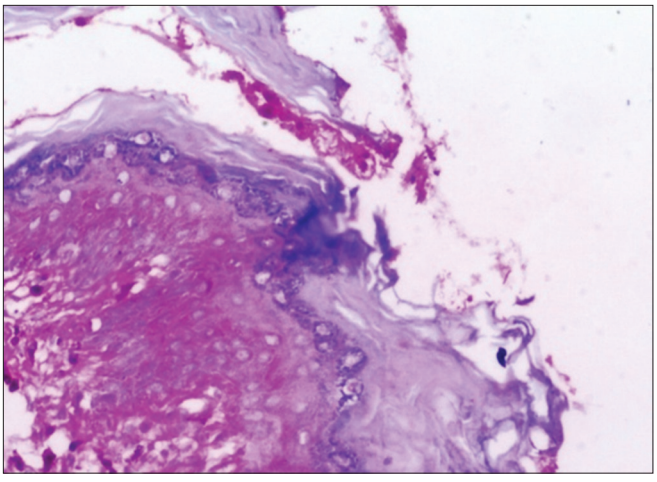


Figure 5: Periodic acid-Schiff-stained section showing *Candidal* hyphae in oral squamous cell carcinoma (×40)

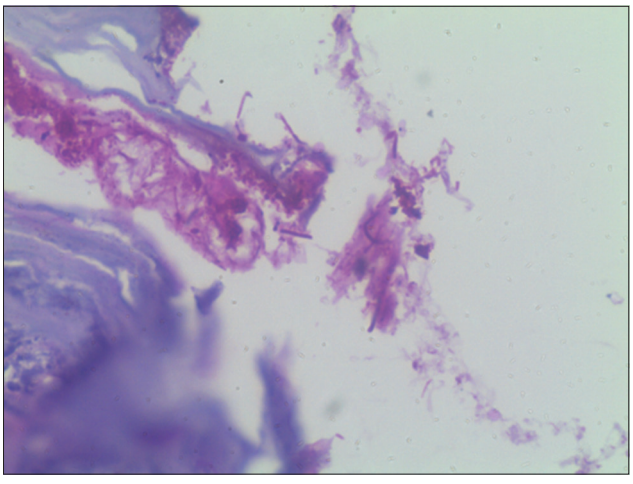
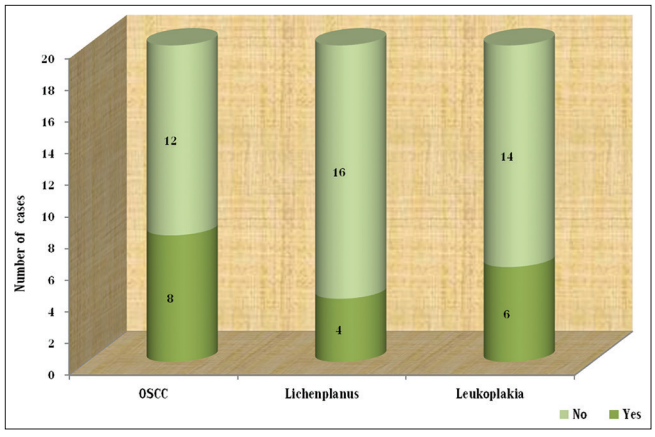
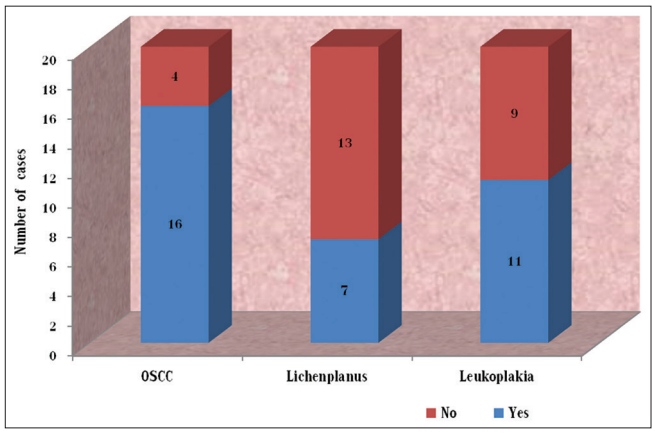


Figure 6: Hematoxylin- and eosin-stained section of oral squamous cell carcinoma showing *Candidal* hyphae (×40)



Graph 1: Hematoxylin and eosin



Graph 2: Periodic acid-Schif

The presence of pathogenic and non-pathogenic microorganisms including fungi in the oral cavity is an established fact. Systemic and local factors which reduce the individual's resistance and factors related to depression of cell-mediated immunity are believed to promote the transition from commensalism to parasitism of fungi.[11,18]

Yeasts such as *Candida* species are common members of the oral microflora and are generally regarded as being commensals. However, they are able to cause a range of opportunistic infections, referred to as candidiasis. The prevalence of diseases caused by *Candida* species has increased in recent years, mainly due to the increasing number of immunocompromised patients. *C. albicans* is still the predominant species isolated, and it has the potential to infect virtually any tissue within the body.[19]

The present study was conducted to determine the frequency of fungal infection in biopsies of oral mucosal lesions, namely leukoplakia, OSCC, and OLP. Biopsy specimen of 20 cases of OSCC, 20 cases of oral leukoplakia, and 20 cases of OLP was included in the study.

Diagnosis of a number of primary OC is based on microbiological methods.[20] The most appropriate method of sampling from the oral cavity will depend on the nature of the lesion being investigated. Imprint cultures or swabs are most appropriate where distinct localized lesions are present. An oral rinse is favored in situations where there is widespread mucosal involvement or the symptoms are vague with absent mucosal change, while a biopsy is essential for the diagnosis of hyperkeratotic form of candidiasis.[21] Biopsy examination can resolve confusion between a neoplastic, inflammatory, and infectious disease.[22]

The presence of blastospores and characteristic pseudohyphae or hyphae in the superficial non-viable epithelial tissue enables the histopathologists to identify a fungus as a species of *Candida* and to make a diagnosis of candidosis.[23]

Different stains used for the demonstration of fungal elements in histopathological sections are H and E stain, Gridley's or Grocott's methenamine silver stains, and PAS stain. Other specialized stains are Calcofluor white technique, wright's stain, periodic acid-basic fuchsin-light green, acridine orange, fluorescein-conjugated lectins, and Woolfast pink RL-methylene blue.[23,24]

The present study made use of H and E stain and PAS stain for the detection of fungal hyphae in biopsies of oral mucosal lesions such as oral leukoplakia, OLP, and OSCC.

The present study showed a positive association between *Candidal* species and leukoplakia. The prevalence of *Candida* in oral leukoplakia in this study was 55%. This finding was correlated with other studies, where the prevalence range was found to be 7–60%.[23,25] Among 20 cases of oral leukoplakia, 11 cases showed *Candidal* hyphae and 9 cases were negative for *Candida*. These values are in agreement with previous studies which have shown a significant association between fungal infection and oral leukoplakia.[11,26]

The association of oral leukoplakias with *Candida* infections was first reported by Cernea *et al*. (1965) and Jepsen and Winther (1965). However, Lehner (1964; 1967) recognized the presentation of chronic *Candidal* infection in the form of leukoplakia and introduced the term "*Candidal* leukoplakia."[23]

Krogh *et al*. through his study found that yeast was present in the lesions of 82% of leukoplakia patients. *C. albicans* was the dominating species in lesions of leukoplakia, constituting 82% of all yeasts in the leukoplakia lesions.[16]

Dany *et al*. assessed the role of *Candida* infection in different stages of leukoplakia and concluded that, as the stage of the lesion increases, the presence of *Candida* in the lesion also increases.[27] Sahay in her study findings implicated a possible role of *C. albicans* in the transformation of untreated leukoplakia into malignancy.[28]

The present study showed a significant association between fungal infection and leukoplakia. The present study cannot resolve the question of whether pathogenic fungi cause epithelial dysplasia or merely infect the altered tissue but can confirm an increased frequency of fungal infection in the potentially malignant situations.[26]

In the present study, of 20 cases of OLP, 7 (35%) cases showed *Candidal* hyphae, with a prevalence range of 26.3–44.3%. The study showed a higher prevalence range compared to other studies, which were also biopsy-based studies and reported *Candidal* colonization rates ranging from 0% up to 17.7%, without a preference for a particular clinical presentation.[18,25]

To support to the findings of our study, *Candida* infection was evident in several studies which reported 2 (22.22%) cases of *Candidal* hyphae in 9 OLP histological sections,[11] 24 (16.6%) positive *Candidal* hyphae in cases of 145 OLP biopsies,[25] 1 (2.32%) of the 43 OLP biopsies,[29] and 3 (7.6%) of 39 histological sections of OLP.[18]

The comparatively high prevalence of *Candidal* hyphae in OLP biopsies suggests that OLP could be predisposing condition to *Candidal* infection.[18] OLP is a result of cell-mediated immunologic response to antigenic changes in the basal layer of epithelium. This alteration may change the cell-mediated immunologic response against *C. albicans* as well. In contrast to the findings of our study several, other studies have not shown an association of *Candidal* infection with OLP.[16] In one of the studies on histochemical analysis of pathological alterations in OLP, 20 sections of OLP were stained with PAS and none of the histological sections showed *Candidal* hyphae.[30]

The difference between the findings of our study and the above study can be explained by the fact that non invasive hyphae and fungi diagnosed in the smears and in the culture could be lost during the laboratory handling of the biopsy specimen, leading to a negative result in histopathologic examination with PAS staining.[11,26] This is in accordance with the opinion of Barrett et al. who stated that the fungal infection as assessed by PAS staining is lower than those obtained by culture, and using the PAS stain, there is a 13% chance of missing fungal infection, particularly if hyphae are scarce or only one section is analyzed.[26]

According to several authors, *Candida* infection not only causes epithelial hyperplasia but also may also induce epithelial atypia, leading to malignant change.[31] In our study, among 20 cases of OSCC, 16 (80%) cases showed positivity for *Candidal* hyphae. The prevalence range was 62.36–97.4%. The present study showed that the prevalence of *Candidal* hyphae was more in OSCC, followed by leukoplakia and OLP. The following studies supported our study.

Barrett *et al*. studied biopsies of oral mucosal lesions and found statistically significant association between histologically determined fungal infection and epithelial dysplasia. PAS staining was recommended whenever oral epithelial dysplasia was diagnosed.[26]

Preeti and Susmita in their study compared and quantified the presence of *C. albicans* in precancerous and cancerous oral mucosal lesions. The frequency of oral yeast carriage was significantly greater in the malignant lesion group than the precancerous lesions as well as precancerous conditions. Results suggested a close correlation of *Candida* infections to leukoplakias and SCC, supporting an association between *Candida* species and oral neoplasia.[32]

Canković *et al*. investigated the presence of *Candida* species in thirty patients with OSCC and compared it to the control subjects. Results showed that the prevalence of *Candida* was significantly higher in oral cancer patients than in control subjects.[33]

Francesca *et al*. assessed the presence of *Candida* species in lesions of the oral cavity in a sample of 103 patients

with precancer or cancer of the mouth. This study supported the frequent presence of *Candida* species in cancer and precancerous lesions of the oral cavity.[19]

In the present study, the comparison of *Candida* identification levels in leukoplakia, OLP, and OSCC was done using H and E and PAS stain. On comparison of the features of *Candidal* infection in H and E with PAS staining, the features which were frequently seen were the presence of hematoxyphilic hyphae and chronic inflammatory changes. In PAS-stained sections after diastase digestion, the presence of hyphae or pseudohyphae which appeared as pinkish red or magenta color was the diagnostic criteria.[11]

In the present study, 6 cases of leukoplakia showed the presence of hematoxyphilic hyphae and 11 cases (55%) were positive for *Candidal* hyphae in PAS. In OLP, H and E showed positivity for *Candida* in 4 cases, while PAS showed positivity in 7 cases (35%). In OSCC, 16 cases were found positive for *Candidal* hyphae in PAS stain as compared to H and E which showed positive in eight of the OSCC cases. The values obtained were found to be more statistically significant with PAS stain than H and E stain (P = 0.016). The above-mentioned studies supported our studies which were conducted using both H and E and PAS stains.

CONCLUSION

The present study showed that the prevalence of *Candidal* hyphae was more in OSCC, followed by leukoplakia and OLP. The present study also showed that the *Candidal* hyphae which was suggestive of *Candidal* infection was more easily visualized with PAS reagent than H and E stain.

Hence, our study suggest that whenever oral mucosal lesions with dysplastic features are diagnosed and show features of *Candidal* infection in H and E-stained sections such as epithelial hyperplasia, hyperparakeratosis, superficial abscess formation, and presence of hematoxyphilic hyphae, PAS staining has to performed to confirm *Candidal* infection and antifungal therapy should be considered in the management of these lesions.

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