ORIGINAL ARTICLE

Prevalence of fungal hyphae in potentially malignant lesions and conditions—does its occurrence play a role in epithelial dysplasia?

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ABSTRACT

Background: Oral cancer is a major public health problem in India. A key factor that has led to lack of improvement in prognosis of oral cancer over the years, is delay in diagnosis and treatment. In many instances, a significant proportion of oral squamous cell carcinomas develop from premalignant lesions and conditions. Identification of such lesions and conditions is very important in order to prevent malignant transformation. The role of fungal infections has been studied and holds promise as an indicator to predict malignant transformation. So we designed a study to analyze the prevalence of fungal hyphae in biopsies of patients with clinically diagnosed cases of premalignant lesions and conditions. **Aims and Objectives:** To determine and compare the prevalence of fungal hyphae in biopsies of patients with clinically diagnosed cases of potentially malignant lesions and conditions and to assess the possible association between the degree of epithelial dysplasia and presence or absence of fungal hyphae. **Materials and Methods:** Clinically suspected and histopathologically diagnosed 70 cases of potentially malignant lesions and conditions (29 leukoplakia, 16 submucous fibrosis and 25 lichen planus) made up the study group. Three tissue sections (5μm) of each were stained with hematoxylin and eosin (H and E), periodic acid–Schiff's reagent (PAS) and Grocott's methenamine silver (GMS) and evaluated for fungal hyphae. The data collected was statistically analyzed by using Chi-square test and Statistical Package for Social Sciences (SPSS) software. **Results:** The estimated prevalence of fungal hyphae in cases with or without dysplasia in leukoplakia was 41.4%, lichen planus 36% and submucous fibrosis 25%. There was a significant association between degree of epithelial dysplasia with presence or absence of fungal hyphae in all the study groups. **Conclusion:** Presence of fungal hyphae in potentially malignant lesions and conditions may prove to be a useful indicator in predicting malignant transformation.

Key words: Epithelial dysplasia, fungal hyphae, malignant transformation, potentially malignant lesions and conditions

INTRODUCTION

Head and neck cancer is a major problem that occurs in Asia, especially in the Indian subcontinent. More than 2 lakh new cases of head and neck cancers are diagnosed each year. India contributes up to 7.8% of the global cancer burden and 8.33% of global cancer deaths.[1] Lack of improvement in prognosis over the years is due to the fact that a significant proportion of oral squamous cell carcinoma (OSCC) is not diagnosed or treated until they reach an advanced stage. It is presumed that such delays are longer for asymptomatic lesions. Although arising *de novo* in many instances a significant proportion of OSCC develop from premalignant lesions and conditions such as leukoplakia, oral submucous fibrosis and lichen planus.[2] The strong association between cancers of the oral cavity and pharynx with use of cigarette smoking, snuff and chewing

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tobacco is well established. Alcohol consumption has a synergistic role and dietary factors such as low intake of fruits and vegetables may also be related to an increased cancer risk. Few studies have also indicated that presence of candidal infection increases the risk of malignant transformation in premalignant lesions.[3] Identification of these premalignant lesions is very important in order to prevent malignant transformation. In order to do so, we must develop criteria which indicate the potential for malignant transformation in individual lesions. The role of fungal infections has been studied in this respect and holds much promise as such an indicator.[4]

Certain strains of *Candida Albicans* and other yeasts play a causal role in the development of oral cancer by means of endogenous nitrosamine production.[5] The association of Candida with various precancer and cancer lesions has been reported as a causative agent.[6]

The association of Candida with premalignant states has been studied extensively and many authors have shown an increase in Candida colonization in these lesions as compared to controls.[7,8] Further, this persistent infection along with other cofactors may also induce epithelial atypia and dysplasia leading to malignant change.[9] It appears that the risk of carcinoma developing in these lesions and conditions depends on whether the lesion is homogenous or nonhomogeneous, the presence and degree of epithelial dysplasia and possibly the method of management adopted.[3] But, literature shows very few studies linking the association of dysplasia and related lesions with Candida. Hence, we designed a study to evaluate the prevalence of fungal hyphae in most common oral potentially malignant lesions and conditions such as leukoplakia, lichen planus and submucous fibrosis and to find out a correlation, if any with presence or absence of fungal hyphae with epithelial dysplasia.

MATERIALS AND METHODS

The present study was undertaken by retrieving archived records and paraffin-embedded tissue blocks of previously diagnosed cases of 70 potentially malignant lesions and conditions (29 leukoplakia, 16 submucous fibrosis and 25 lichen planus). Relevant information of age, sex and histopathological grading of dysplasias was obtained from the records of the patients and was tabulated [Tables 1 and 2].

Inclusion criteria

Clinically suspected and histopathologically diagnosed cases having hyperkeratosis and different grades of dysplasia.

Exclusion criteria

Poor oral hygiene with chronic generalized periodontitis and diabetes mellitus.

Table 1: Comparison of age and sex distribution between three study groups

Parameters	Group I Leukoplakia (*n*=29)	Group II Lichen planus (*n*=25)	Group III Oral submucous fibrosis (*n*=16)
Age group (years)			
<30	2 (6.9)	6 (24.0)	6 (37.5)
30-49	7 (24.1)	11 (44.0)	4 (25.0)
≥50	20 (69.0)	8 (32.0)	6 (37.5)
Sex			
Male	20 (69.0)	15 (60.0)	13 (81.3)
Female	9 (31.0)	10 (40.0)	3 (18.7)

Table 2: Distribution of grading of epithelial dysplasia between three study groups

Degree of dysplasia	Group I Leukoplakia (*n*=29)	Group II Lichen planus (*n*=25)	Group III Oral submucous fibrosis (*n*=16)
No signs of dysplasia	2 (6.9)	19 (76.0)	14 (87.4)
Mild dysplasia	12 (41.4)	3 (12.0)	1 (6.3)
Moderate dysplasia	9 (31.0)	3 (12.0)	1 (6.3)
Severe dysplasia	4 (13.8)	0	0
Carcinoma *in situ*	2 (6.9)	0	0
P value	0.001 (significant)		

Three sections of 5 μm thickness of each case were stained employing hematoxylin and eosin (H and E), periodic acid–Schiff's reagent (PAS) and Grocott's methenamine silver (GMS). The tissue section of diagnosed case of mucormycosis served as positive control for staining of hyphae.

Statistical analysis

The entire data was entered in MS Excel and analyzed using Statistical Package for Social Sciences (SPSS). Chi-square test of independence of attributes was used to find out the significant differences and linearity trend of proportion was tested using Chi-square test to find out association. The level of significance was taken at $P < 0.05$.

RESULTS

The present study was undertaken to determine the prevalence of fungal hyphae in potentially malignant lesions and conditions such as leukoplakia, lichen planus and oral submucous fibrosis; and to find out association if any, with presence or absence of fungal hyphae with various grades of dysplasia. Fungal hyphae stained with PAS appeared magenta red and with GMS they appeared brownish black [Figure 1a and b].

Demographic details of the patients in Groups I, II and III are shown in Table 1. In all the study groups, majority of patients were men and above 50 years [Table 1].

Statistically significant number of cases of leukoplakia ($n = 27/29$; $P = 0.001$) showed signs of dysplasia, but not in cases of lichen planus and oral submucous fibrosis [Table 2].

In leukoplakia group, 12 out of 29 cases showed presence of fungal hyphae, in lichen planus nine out of 25 were positive for hyphae, but in oral submucous fibrosis group only four were positive for fungal hyphae. These findings were not statistically significant [Table 3 and Graph 1].

The prevalence of fungal hyphae in cases of leukoplakia with dysplasia was 44.44%, in lichen planus was 66.66% and in oral submucous fibrosis, it was 100%. Out of 27 dysplastic cases of leukoplakia group, 12 cases showed presence of fungal hyphae. In lichen planus group out of six cases with dysplasia, four cases showed presence of fungal hyphae and in oral submucous fibrosis group, two cases with dysplasia also showed presence of hyphae. On comparison of the prevalence, with respect to presence of fungal hyphae between cases with dysplasia and cases without dysplasia, we found that cases of lichen planus and oral submucous fibrosis showed proportionately higher prevalence of fungal hyphae in cases with dysplasia as compared to leukoplakia. But the above results were not statistically significant [Table 4 and Graph 2].

In cases of leukoplakia, the degree of epithelial dysplasia was significantly associated with presence of fungal hyphae [Table 5]. Out of twelve cases of mild dysplasia three were positive for fungal hyphae by PAS and GMS staining. [Figure 2a and b] Out of nine cases of moderate dysplasia, five were positive for fungal hyphae by PAS and GMS staining. [Figure 3a and b] In severe dysplasia; out of four cases, three were positive for fungal hyphae [Figure 4a and b and Table 5].

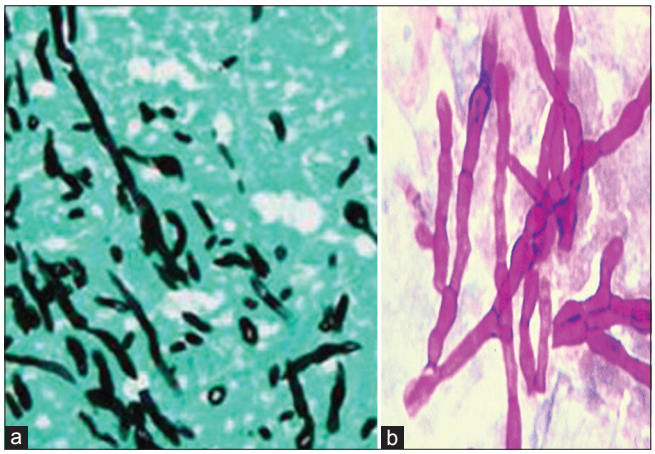


Figure 1: (a) Dark brown to black fungal hyphae in tissue section (GMS stain, x400). (b) Magenta red colored fungal hyphae in tissue section (PAS stain, x400). PAS = Periodic acid–Schiff, GMS = Grocott's methenamine silver

In lichen planus, the degree of epithelial dysplasia was not significantly associated with presence of fungal hyphae. Out of three cases each of mild and moderate dysplasia, two each were positive for fungal hyphae by PAS and GMS [Figure 5a and b and Table 6].

In oral submucous fibrosis, the degree of epithelial dysplasia was significantly associated with presence of fungal hyphae. One case each with mild and moderate dysplasia showed presence of fungal hyphae by PAS and GMS [Figure 6a and b and Table 7].

The association of degree of epithelial dysplasia with presence or absence of fungal hyphae was statistically significant (P - value 0.003) in all three study groups [Table 8 and Graph 3].

DISCUSSION

Oral cancer is the sixth most common cancer in the world and is largely preventable. It accounts for approximately 4% of all cancers and 2% of all cancer deaths worldwide.[10] In India, oral cancer is the commonest malignant neoplasm, accounting

Table 3: Distribution of prevalence of fungal hyphae in each study group with or without dysplasia in total cases

Fungal hyphae Overall (PAS or GMS)	Group I Leukoplakia (n=29)	Group II Lichen planus (n=25)	Group III Oral submucous fibrosis (n=16)
Positive	12 (41.4)	9 (36.0)	4 (25.0)
Negative	17 (58.6)	16 (64.0)	12 (75.0)
P value	0.353	0.162	0.046

PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver

Table 4: Distribution of prevalence of fungal hyphae in cases with dysplasia between three study groups

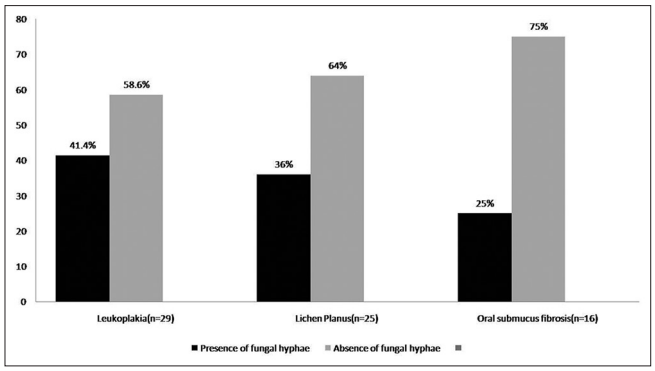
Fungal hyphae Overall (PAS or GMS)	Group I Leukoplakia (n=27)	Group II Lichen planus (n=6)	Group III Oral submucous fibrosis (n=2)
Positive	12 (44.44%)	4 (66.66%)	2 (100.0)
Negative	15 (55.55%)	2 (33.34%)	0
P value	0.564	0.414	0.847

PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver

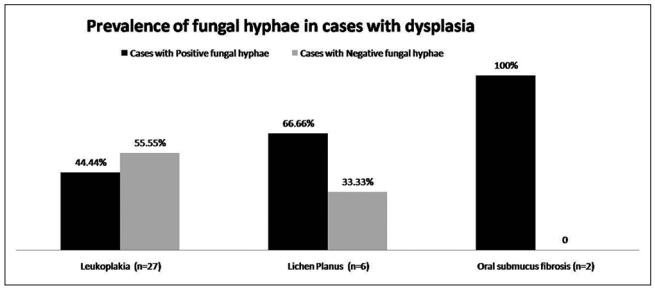
Table 5: Association of degree of epithelial dysplasia with presence of fungal hyphae in cases of leukoplakia (Group I)

Degree of dysplasiain leukoplakia (n=29)	Group I Fungal hyphae positive (PAS or GMS)	P value for trend
No dysplasia (2)	0	0.050 (significant)
Mild dysplasia (12)	3 (25%)	
Moderate dysplasia (9)	5 (55.55%)	
Severe dysplasia (4)	3 (75%)	
Carcinoma *in situ* (2)	1 (50%)	

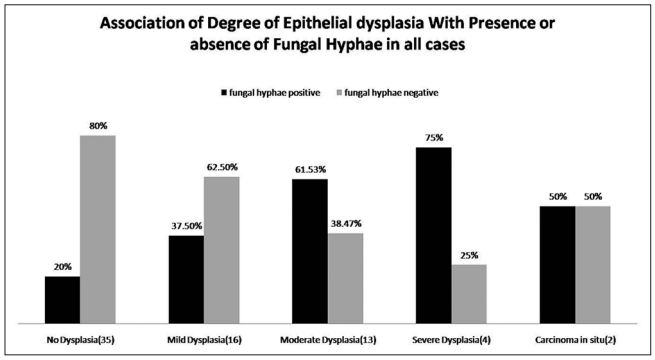
PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver



Graph 1: Distribution of prevalence of fungal hyphae in each study groups in total cases



Graph 2: Distribution of prevalence of fungal hyphae in cases with dysplasia between three study groups



Graph 3: Association of degree of epithelial dysplasia with presence or absence of fungal hyphae in all cases

Table 6: Association of degree of epithelial dysplasia with presence of fungal hyphae in cases of lichen planus (Group II)

Degree of dysplasia in lichen planus (*n*=25)	Group II	*P* value for trend
	Fungal hyphae positive (PAS or GMS)	
No dysplasia (19)	5 (26.31%)	0.100
Mild dysplasia (3)	2 (66.66%)	
Moderate dysplasia (3)	2 (66.66%)	
Severe dysplasia (0)	0	
Carcinoma *in situ* (0)	0	

PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver

Table 7: Association of degree of epithelial dysplasia with presence of fungal hyphae in cases of oral submucous fibrosis (Group III)

Degree of dysplasia inoral submucous fibrosis (*n*=16)	Fungal hyphae positive (PAS or GMS)	*P* value for trend
No dysplasia (14)	2 (14.28%)	0.017 (significant)
Mild dysplasia (1)	1 (100%)	
Moderate dysplasia (1)	1 (100%)	
Severe dysplasia (0)	0	
Carcinoma *in situ* (0)	0	

PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver

Table 8: Association of degree of epithelial dysplasia with presence or absence of fungal hyphae in all cases

Degree of dysplasia in all cases (*n*=70)	All cases (PAS or GMS) (%)		*P* value for trend
	Absence of fungal hyphae	Presence of fungal hyphae	
No dysplasia (35)	28 (80)	7 (20)	0.003 (significant)
Mild dysplasia (16)	10 (62.5)	6 (37.5)	
Moderate dysplasia (13)	5 (38.47)	8 (61.53)	
Severe dysplasia (4)	1 (25)	3 (75)	
Carcinoma *in situ* (2)	1 (50)	1 (50)	

PAS: Periodic acid–Schiff, GMS: Grocott's methenamine silver

for 20–30% of all cancers with a death: Registration ratio of 0.45. More than 80% of oral cancers are squamous cell carcinomas arising from the oral mucosa with the tongue being the commonest site.[11] Invasive oral cancer is preceded by a potentially malignant lesion or condition. Potentially malignant lesions are those in which the oral mucosa locally has undergone sufficient cytological change as to render it more likely than normal tissue to become malignant. The majority, if not all potentially malignant conditions are systemic disorders that result in atrophy of the oral mucosa resulting in increased penetrance of carcinogens. At the present time it is not possible to differentiate the lesions that will ultimately progress to invasive carcinoma and as such warrant early aggressive treatment from those that will regress and can safely be left alone.[12] Reibel in 2003 reviewed the concept of a two-step process of cancer development in the oral mucosa. The terms 'precancer', 'precursor lesion', 'premalignant', 'intraepithelial neoplasia' and 'potentially malignant' have been used in the international literature to broadly describe clinical presentations that may have a potential to become malignant. Oral leukoplakia is the best-known precursor lesion. Follow-up studies have shown that between <1 and 18% of oral premalignant lesions will develop into oral cancer and few clinical subtypes of leukoplakia are at a higher risk for malignant transformation than others. The presence of epithelial dysplasia is more important in predicting malignant development than the clinical characteristics.[4]

The clinical concept of oral mucosal premalignancy is now more than 150 years old. Sir James Paget is credited with the first description of an association between an oral lesion (which he

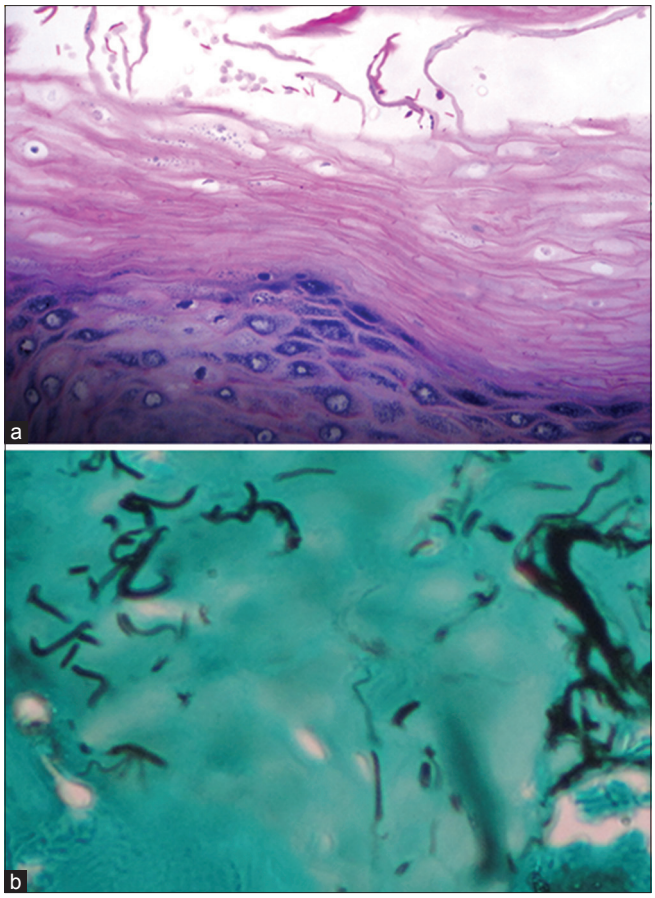


Figure 2: (a) Superficial colonization of fungal hyphae in cornified layer in mild dysplasia (PAS stain, x200). (b) Superficial colonization of fungal hyphae in cornified layer in mild dysplasia (GMS stain, x400)

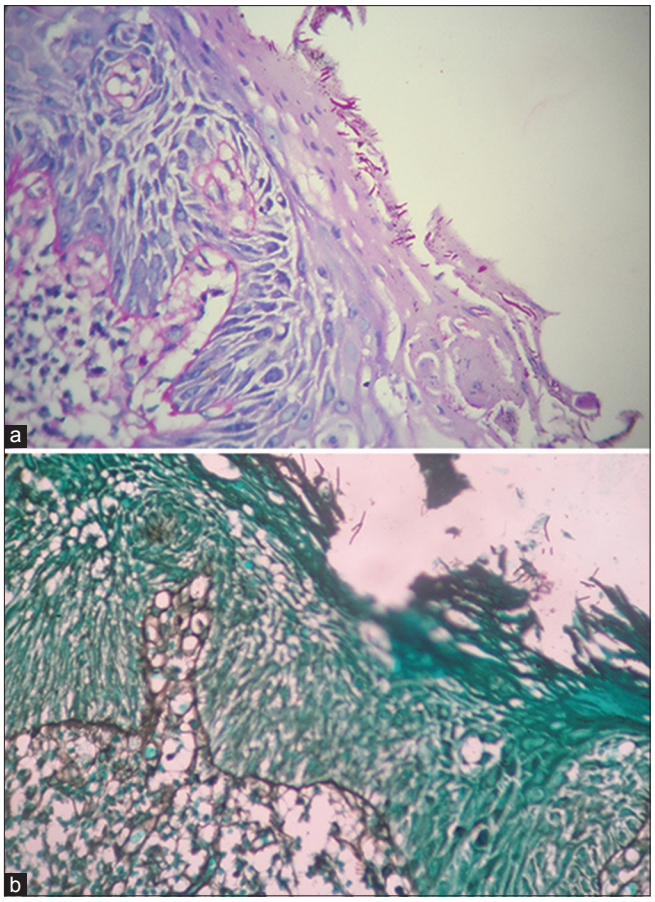


Figure 3: (a) Fungal hyphae in cornified layer in moderate dysplasia (PAS stain, x400). (b) Fungal hyphae in cornified layer in moderate dysplasia (GMS stain, x400)

termed "ichthyosis") and subsequent development of tongue carcinoma. Since then, there has been clinical, morphological and experimental evidence of a correlation between certain lesions in the oral cavity and subsequent development of oral cancer.

Conflicting evidence exists for the role of various infectious agents in the etiology of precancer and oral cancer that includes human papillomavirus (HPV) and herpes simplex virus (HSV). Among the fungi, *C. albicans* is the most common microorganism to pose a possible risk factor for the malignant transformation in premalignant lesions and conditions. Few studies have also indicated that presence of candidal infection may increase the risk of a premalignant lesion and conditions turning malignant.[3] Colonization of the mouth by candida species has a long recorded history. Oral lesions probably caused by candida have been reported since the time of Hippocrates, as early as 377 BCE. Langenbeck in 1839 is credited as a pioneer in recognizing a fungus, consistent with *C. albicans* in an oral aphtha (ulcer) of a patient with typhoid fever.[13] The association of candidal infection with leukoplakia was first reported by Cernea *et al*., and Jepsen and Winther in 1965. Fungi are eukaryotic, saprophytic microorganisms usually associated with dead cellular matter. Indeed, it is well-known that Candida species are keratophilic and they tend to colonize thick layers of keratin.[14] The role played by various Candida species in initiating oral epithelial lesions is ill understood. Cawson and Lehner (1968)[15] first suggested a possible etiological relationship between leukoplakia and candidal infection on finding mycelial elements of *C. albicans* infiltrating the cornified layer of the epithelium in some cases of human oral leukoplakia. But other authors have regarded *C.albicans* as a secondary invader rather than the cause of the leukoplakia in those cases in which it is found.[16]

The concept of carcinogenic potential of candida has received support from a study which demonstrated that certain *C. albicans* biotypes are capable of producing carcinogenic nitrosamine N-nitrosobenzyl methylamine from its precursors. They also showed that strains with high nitrosation potential were associated with lesions showing more advanced precancerous changes. Treatment with antimycotics has often resulted in partial or complete resolution of leukoplakias.[5]

The present study was carried out in patients with potentially malignant lesions and conditions such as leukoplakia, lichen planus and submucous fibrosis with the aim to compare the prevalence of presence of fungal hyphae; to find out

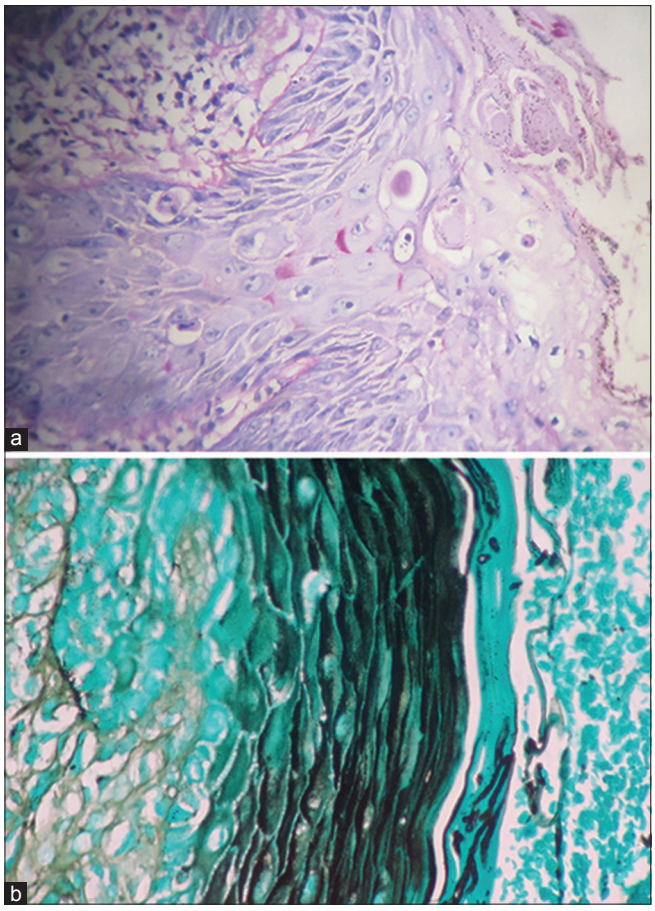


Figure 4: (a) Hyperkeratosis and hyperplasia, superficial colonization of fungal hyphae in severe dysplasia (PAS stain, x400). (b) Fungal hyphae in cornified layer in severe dysplasia (GMS stain, x400)

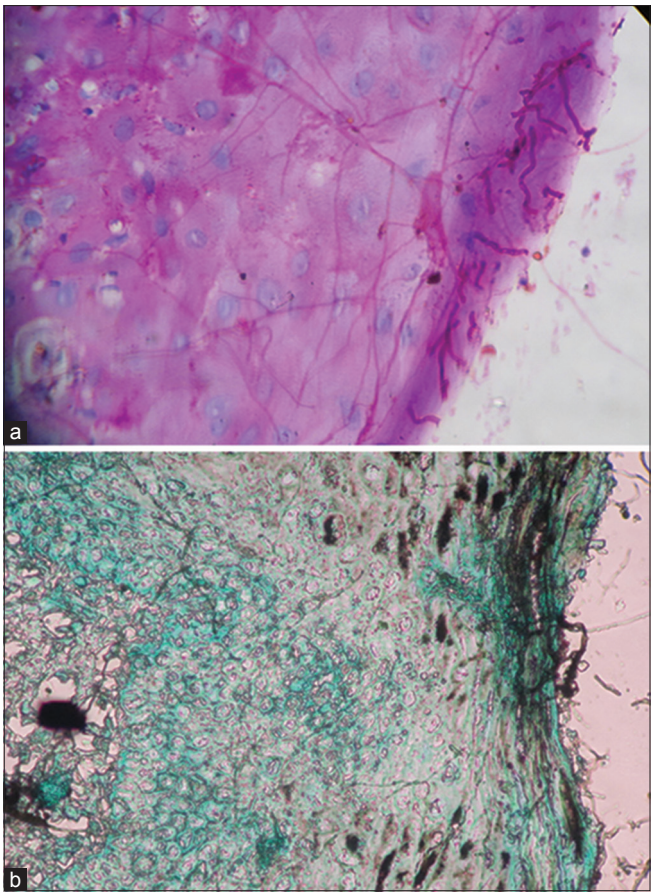


Figure 5: (a) Invading fungal hyphae in cornified layer in lichen planus a (PAS stain, x100). (b) Invading fungal hyphae in cornified layer in lichen planus (GMS stain, x100)

the association if any, with presence or absence of fungal hyphae and various grades of dysplasia; and to test the prognostic significance of this information. Biopsy-based studies have reported candidal hyphae to constitute 7–50% in leukoplakia.[17] In our study, we found 41.44% prevalence of presence of fungal hyphae in cases of leukoplakia with or without dysplasia [Table 3 and Graph 1]. This finding was in close approximation with the findings of the other studies, such as studies by Krogh *et al*. 1987 (50%)[8] Roed-Pertersen *et al*., 1970 (31%);[18] Rindum *et al*., 1994 (47%);[7] Dorko *et al*., 2001 (35.9%);[19] and Kumar *et al*., 2009 (46.66%).[6] In contrast to our findings, few studies showed much lower prevalence of fungal hyphae in tissue sections; they are, Berret *et al*., 1998 (7.1%);[20] Banoczy and Csiba 1976 (13.5%),[21] Silverman *et al*., 1984 (23%).[22] In our study among 29 cases of leukoplakia, 12 (44.44%) cases showed presence of fungal hyphae and all the 12 (100%) cases showed dysplasia in various grades [Table 4].

The prevalence of fungal hyphae in cases of lichen planus in our study was 36% with or without dysplasia [Table 3 and Graph 1]. In biopsy-based studies, the reported candidal colonization rates ranged from 0to 17.7%, without preference for a particular clinical presentation.[6] Our study showed substantially greater prevalence of fungal hyphae in lichen planus than that suggested by previous biopsy-based studies by Vuekovic *et al*., 2004 (22.22%);[23] Lundstrom *et al*., 1984 (7%);[24] Holmstrup and Dabelsreen, 1974 (2.3%);[25] and Hatchuel *et al*., 1990 (17.4%).[26]

In contrast to our findings, study conducted by Krogh *et al*. 1987[8] and Kumar *et al*., 2009;[6] none of them showed hyphae in tissue sections. However, the lower prevalence in a study conducted by Lundstrom *et al*., 1984[24] could have been caused by prior treatment with amphotercin B before biopsy in six of 39 patients. In a study by Krogh *et al*., 1987;[8] only reticular lichen planus was included and possibly this restriction excluded infected cases that could have had an altered clinical presentation, further possibility might be that in study by Holmstrup *P* and Dabelsreen 1974[25] lichen planus cases with candidal infection could have been classified as candidiasis and hence would not have been accessible in retrospective studies.

Comparatively higher prevalence of candidal hyphae in lichen planus biopsies in our study suggests that lichen planus could be predisposing condition to candidal infection. In lichen

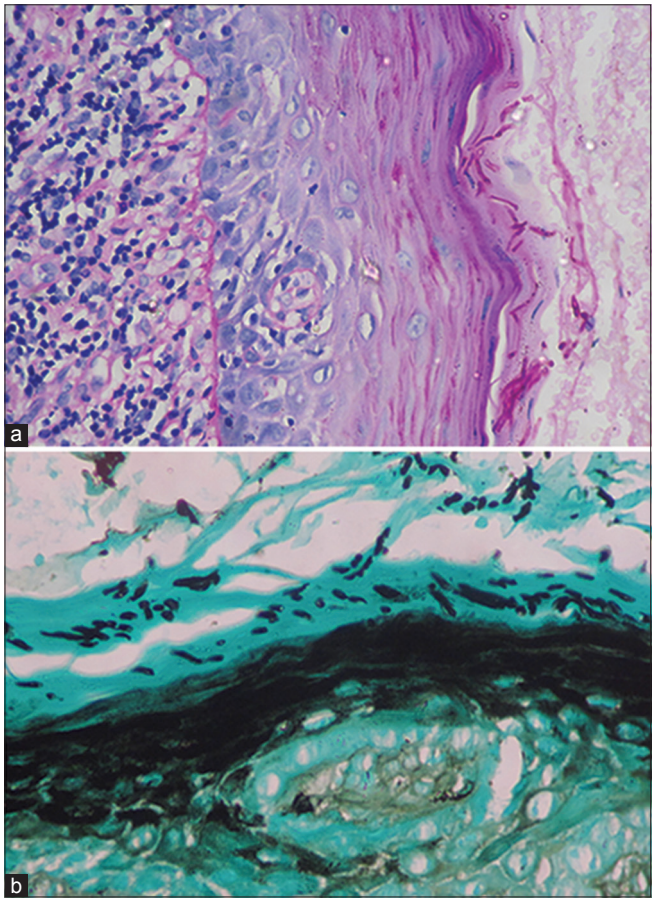


Figure 6: (a) Fungal hyphae in cornified layer in oral submucous fibrosis (PAS stain, x 400). (b) Fungal hyphae in cornified layer in oral submucous fibrosis (GMS stain, x400)

planus, the oral mucosal pathology alters the integrity of the oral epithelium and predisposes the lesion to fungal infection (Lundstrom *et al*., 1984).[24]

Similarly, we found 25% prevalence of fungal hyphae in cases of submucous fibrosis with or without dysplasia [Table 3 and Graph 1]. Our results are compatible with results obtained by Kumar *et al*., 2009;[6] where they found positive hyphae in eight (33.33%) out of 24 cases. Lack of published data on the occurrence of fungal hyphae in biopsy-based studies of submucous fibrosis makes comparison of our data with other studies difficult.

The prevalence of fungal hyphae was studied in cases with signs of dysplasia and we found significantly higher proportion of fungal hyphae in dysplasia cases (leukoplakia 44.44%, lichen planus 66.66% and submucous fibrosis 100%) [Table 4 and Graph 2]. We obtained statistically significant association between histopathologically determined fungal hyphae and various grades of epithelial dysplasia in these individual lesions [Tables 5-8 and Graph 3]. The results of our study were compatible with results obtained by Zhang *et al*., (1994);[27] Berret *et al*., (1998);[20] McCullough *et al*., (2002);[28] Spolidorio *et al*., (2003).[29]

The discrepancy in percentage prevalence of fungal hyphae in tissue sections in our study when compared to various studies can be explained as follows. It is possible that overall detection rate of 5% represents an underestimate and there is abundant evidence that values of fungal infection as assessed by PAS staining are lower than those obtained by culture and using the PAS stain there is 13% chance of missing fungal infection, particularly if hyphae are scarce or only one section is analyzed (Roed-Petersen *et al*., 1970).[18]

On the other hand, histopathology is the quickest means of identifying those cases in which microorganisms have invaded the epithelium where they are more likely to be pathogenic. Non-invasive hyphae and yeasts may be detected in smears or grown in culture, but lost during histological processing (Cawson, 1968),[15] producing a negative result on staining the section with PAS. Despite this possible underestimate, our study has observed a significant association of fungal hyphae with various grades of dysplasia and only in few cases without dysplasia. Our study confirms an increased frequency of fungal hyphae in some potentially malignant lesions and conditions and these results are in agreement with Field *et al*., 1989.[30]

Though the staining pattern of fungal hyphae by PAS and GMS was similar, we found that the staining intensity was better with GMS than PAS.

Our study suggests that, routine PAS or GMS staining should be advised whenever signs of candidal infection such as superficial abscess formation, chronic inflammation in lamina propria, epithelial hyperkeratosis and hyperplasia and presence of weak hematoxyphilic hyphae are observed in H and E stained sections of oral mucosal biopsies. This technique will frequently detect fungal hyphae where lesions are not clinically visible. It also emphasizes the important role histopathology plays in the diagnosis of fungal infection. On histopathological confirmation of epithelial dysplasia, antifungal therapy should be considered in the management of these lesions. A histopathological report of unexpected fungal infection is likely to be acted on clinically and may affect the patient's management and prognosis (Berret *et al*., 1998).[20]

Our study did not include species specification of Candida. So we suggest future studies involving larger sample size and species isolation in potentially malignant lesions and conditions to bring about a revolution in the early detection, prevention, management and prognosis of these lesions.

CONCLUSION

Epithelial dysplasia and prevalence of fungal hyphae in leukoplakia was significantly higher as compared to lichen planus and submucous fibrosis.

The degree of epithelial dysplasia was significantly associated with presence or absence of fungal hyphae in all cases. Higher

grades of dysplasia were associated with increased prevalence of fungal hyphae. This indicates a significant association between presence of fungal hyphae and various grades of epithelial dysplasia. Future research should include studies with large sample size and species isolation of Candida.

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