Original Article

Clinicopathologic assessment of *Candida* colonization of oral leukoplakia

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ABSTRACT

Background: Leukoplakia is the most common premalignant lesion of the oral mucosa. We studied the colonization of *Candida* in oral leukoplakia using direct microscopy, culture and histopathology to determine if there is a statistical correlation between *Candida* invasion and the clinical appearance and presence of epithelial dysplasia in leukoplakia. **Methods:** Samples were collected from 40 patients with oral leukoplakia and 21 controls. The swabs collected were used to inoculate Sabouraud's dextrose agar slant and for direct microscopy with Gram's stain. Culture growths were subjected to germ tube and corn meal agar tests to differentiate between *Candida albicans* and non-*albicans* groups. Biopsies were also done in all patients for histopathological confirmation; Gomori's methanamine silver stain was used to identify fungal invasion of lesional epithelium. **Results and Conclusions:** Nineteen cases of leukoplakia showed *Candida* on direct smears, compared to 3 controls. Eighteen cases and one control showed growth of *Candida* on culture. Non-homogenous leukoplakia showed a higher positivity rate on microscopy and culture than homogenous lesions. All these correlations were statistically significant. Forty percent of leukoplakia cases were simultaneously positive for *Candida* on direct microscopy, culture and histopathologic evaluation. No significant difference was found between non-dysplastic and distinctly dysplastic lesions with respect to *Candida* detection on microscopy or culture.

Key words: *Candida*, dysplasia, leukoplakia, malignant transformation

INTRODUCTION

The genus *Candida* is a heterogeneous collection of asporogenous yeast species categorized under fungi imperfecti.[1] Fungal colonization has been reported in about 18% of patients with clinically healthy mucosa, of which over 80% proved to be *Candida albicans*.[2] *Candida albicans* is a dimorphic fungus that occurs both in a budding yeast and a hyphal phase.

Leukoplakia is the most common potentially malignant lesion of the oral mucosa.[3,4] A range of studies report that oral leukoplakia shows a significant tendency to malignant transformation, varying from 0.13% to 6% and rising to 14% or higher when dysplasia is present.[5] Leukoplakia is defined as a predominantly white lesion or plaque of questionable behavior having excluded, clinically and histopathologically, other definable diseases. Brouns *et al*. have discussed the differentiation of hyperplastic candidiasis from *Candida*-associated leukoplakias, especially at the buccal commissures and on the dorsal surface of the tongue. Lesions that regress after antifungal treatment are those of hyperplastic candidiasis, and those that persist are termed *Candida*-associated leukoplakias.[6]

It has been proposed that tobacco carcinogens could act as initiators and *Candida* components as promoters according to classical theories of carcinogenesis.[7] Smoking and other co-factors such as denture-wearing, HIV infection, the oral environment, nutritional factors and diabetes mellitus work towards increasing

Access this article online	
Quick Response Code:	Website: www.ijdvl.com
	DOI: 10.4103/0378-6323.140299

How to cite this article: Sarkar R, Rathod GP. Clinicopathologic assessment of Candida colonization of oral leukoplakia. Indian J Dermatol Venereol Leprol 2014;80:413-8.

Received: December, 2013. **Accepted:** March, 2014. **Source of Support:** Nil. **Conflict of Interest:** None declared.

oral *Candida* carriage.[8] It was postulated that certain strains of *Candida albicans* and other yeasts might play a causal role in the development of oral cancer by means of endogenous nitrosamine production.[9] Higher levels of aspartyl proteinases from *C. albicans* in individuals with leukoplakia and oral squamous cell carcinoma may facilitate colonization by the fungus.[10]

The present study investigates the clinicopathological association between *Candida* and oral leukoplakia.

METHODS

The study comprised 40 consecutive cases of oral leukoplakia and 21 controls selected from amongst the outpatients of oral pathology department. The study was cleared by the institutional ethics committee and all subjects gave informed consent prior to inclusion.

A detailed case history was taken for every patient with special reference to habits, past dental and medical history as well as treatment taken. A detailed orofacial examination was conducted in all cases.The selection criteria for leukoplakia was a white lesion or plaque of questionable behavior having excluded, clinically and histopathologically, any other definable white disease or disorder (Brouns' criteria).[6] Investigations included complete blood counts and blood glucose as well as bleeding time and clotting time, pre-biopsy. Definable disorders such as mechanical irritation and chronic hyperplastic candidiasis were ruled out in a follow-up period of six weeks with ameliorative measures like grinding a sharp tooth or dental restoration, as well as antifungal therapy. After two weeks of completion of 15 days of topical 0.2% clotrimazole, patients were recalled for obtaining samples from the lesions. Control samples were obtained from comparable oral sites in healthy individuals of similar age, sex and tobacco habits but without clinically visible pathology. All samples were collected by the same investigator.

After wiping the area clean of saliva and debris, lesions were gently rubbed with sterile swabs, avoiding contamination from the rest of the oral cavity. Two swabs were collected per patient, one for direct microscopy and the other for culture. One of the swabs was gently rotated on a clean slide to make a smear which was air-dried, heat-fixed and stained using Hucker's modification of Gram's stain. The stained smear was examined under oil immersion for gram positive yeast cells as well as budding and pseudohyphae.[11]

The other swab was immediately streaked across the surface of a Sabouraud's dextrose agar (SDA) slant with chloramphenicol. Following incubation at 37°C for 2-3 days, the slant was inspected for growth.[12] The types of colonies formed, the number of each type and any confluent growth were noted. Colonies showing confluent growth were sub-cultured onto fresh Sabouraud's dextrose agar plates for isolation of discrete colonies.

All the different types of colonies were studied by a gram-stained smear[12,13] and only those showing yeasts were followed. Colony morphology was studied with respect to color, size, topography and texture. *Candida albicans* was confirmed by the germ tube test (on serum) and corn meal agar test.[14-16]

Incisional or excisional biopsies obtained from the patches of leukoplakia were processed and stained with hematoxylin and eosin. Epithelial dysplasia was assessed as 'no or mild dysplasia' (P0) or 'distinct dysplasia' (P1). Two independent investigators looked at each of the slides for dysplasia and a consensus decision was adopted. Gomori's methanamine silver stain was used for demonstration of fungi. Fungal elements were sharply delineated in black with the inner parts of hyphae stained an old rose color, on a pale green background. A Gomori-stained slide from a known case of candidiasis was used as a positive control.

Statistical analysis was carried out using the Chi Square test.

RESULTS

Patients' demographics and habit profiles are as in Table 1. Barring one, all patients with leukoplakia were smokers or tobacco users. Homogenous and non-homogenous leukoplakia were seen respectively in 23 and 17 cases. Most (60%) of the lesions were located on the buccal mucosa. The buccal commissure and the lower anterior vestibule were less often affected (12.5% each), followed by the palate, tongue and retromolar areas. The lesions varied in size from 2 to 6 cms. Histologically, there were 11 cases of P0 (no dysplasia) and 29 cases of P1 (distinct dysplasia).

Nineteen cases of leukoplakia showed pseudohyphae and/or budding yeast cells on direct gram stained smears compared to 3 controls who showed single or occasional hyphae ($P < 0.001$) (Figure 1a and b). Eighteen cases of leukoplakia and only one control showed growth on Sabouraud's agar slant ($P < 0.001$) (Figure 2a and b). Further, non-homogenous leukoplakia showed a significantly higher positivity rate on microscopy (64.7%) than the homogenous type (34.8%, $P < 0.01$). Non-homogenous lesions also yielded more positive cultures (64.7%) than homogenous leukoplakia (30.4%, $P < 0.001$). There was however no significant difference between non-dysplastic and distinctly dysplastic lesions with respect to both microscopy and culture results ($P > 0.05$) [Table 2].

Of the 18 culture-positive patients, 14 were positive on both germ tube and corn meal agar tests i.e. 77.8% of the isolated fungal species (35% of all cases) were *Candida albicans* [Table 2, Figures 3a and b, 4].

On tissue sections, hyphal invasion was seen in 42.5% of all cases, with comparable results in homogenous and non-homogenous groups. Small intraepithelial abscesses were also identified alongside the hyphal structures. Dysplastic and non-dysplastic lesions showed concomitant *Candida* invasion in 41.4% and 45.5% respectively ($P > 0.05$). (Figures 5a and b, 6a and b).

Table 1: Tobacco and alcohol use in cases and controls

	Cases	Controls
Age (years, mean±SD)	43.5±12.4	42.1±12.4
Sex distribution (M:F)	7:1	6:1
Substance use (%)		
Smoking	5 (12.5)	0.3 (14.3)
Tobacco chewing	20 (50)	11 (52.3)
Alcohol	0	0
Smoking+tobacco chewing	5 (12.5)	2 (9.5)
Smoking+alcohol	7 (17.5)	4 (19)
Tobacco+alcohol	2 (5)	0
No tobacco/alcohol use	1 (2.5)	1 (4.7)
Total	40 (100)	21 (100)

SD: Standard deviation

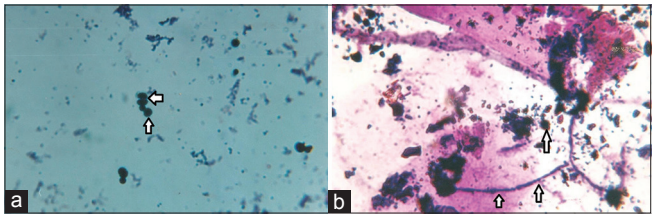

Figure 1: (a and b) Budding yeast cells and hyphal forms in direct microscopic smears (Gram- stained oil immersion, ×1000)

Overall, 16 (40%) of our leukoplakia cases gave positive results on direct microscopy, mycology as well as on histopathology.

DISCUSSION

Dysplastic lesions have been called pre-cancers,[17] but the term "potentially malignant disorder" is now considered more appropriate than 'premalignant lesion' or 'precancerous lesion'. There is no doubt that some oral white lesions especially leukoplakia are potentially malignant.[3,4,18,19] Studies report that 1 to 18% of oral potentially malignant disorders will transform into cancer.[20]

The role of *C. albicans* in erythroleukoplakia is at present unclear.[21] The term '*Candida*-associated leukoplakia' has been recommended to indicate a preliminary diagnosis of leukoplakia as well to point to a possible etiologic role for *Candida*. A higher rate of malignant transformation has been reported in *Candida*-infected leukoplakias. *Candida* therefore figures amongst the risk factors to look for in addition to other prognostic markers such as a non-homogenous morphology and epithelial dysplasia.[22] Diagnosis and treatment of *Candida* infection should be instituted in a lesion suspected to be leukoplakia.

Histologically, *Candida* leukoplakia has been defined by the presence of hyphae in the superficial epithelium and a neutrophilic infiltrate.[23] *Candida* is keratinolytic and hence observed more on parakeratotic epithelium. All histological sections in leukoplakia should hence be checked for *Candida* using special stains, particularly if they show a non-homogenous appearance and parakeratosis.

A rare *Candida albicans* biotype having a high nitrosation potential has been isolated from non-

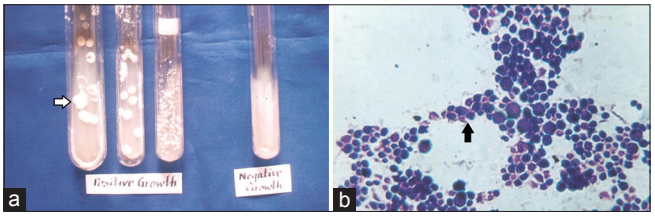

Figure 2: (a) Positive growth showing colonies of *Candida* on Sabouraud's dextrose agar slant, compared to a slant without growth (extreme right) (b) Colony characterization and confirmation of growth of colonies with Gram-stained smear. (×1000 oil immersion)

Table 2: Test results in clinical and histological variants of leukoplakia

	Total	Smear positive (%)	*P* value	Culture positive (%)	*P* value	Germ tube/corn meal agar positive (%)	*P* value	Gomori stain positive (%)	*P* value
Controls	21	3 (14.3)	<0.001	1 (4.8)	<0.001	-	-	-	-
Cases	40	19 (47.5)		18 (45)		14 (35)		17 (42.5)	
Clinical variants									
Homogenous	23	8 (34.8)	<0.01	7 (30.4)	<0.001	5 (21.7)	<0.001	8 (34.8)	>0.05
Non-homogenous	17	11 (64.7)		11 (64.7)		9 (52.9)		9 (52.9)	
Histological variants									
P_0 (no or mild dysplasia)	11	4 (36.4)	>0.05	4 (36.4)	>0.05	4 (36.4)	>0.05	5 (45.5)	>0.05
P_1 (distinct dysplasia)	29	15 (51.7)		14 (48.3)		10 (34.5)		12 (41.4)	

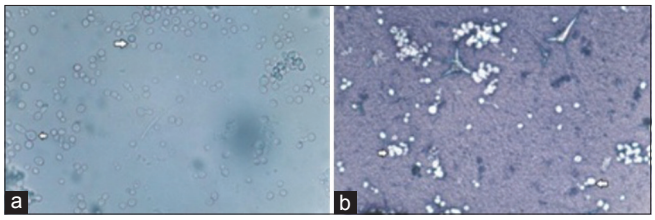

Figure 3: (a and b) Wet mount of positive germ tube test (×450) and India ink negative staining of positive germ tube test (×450)

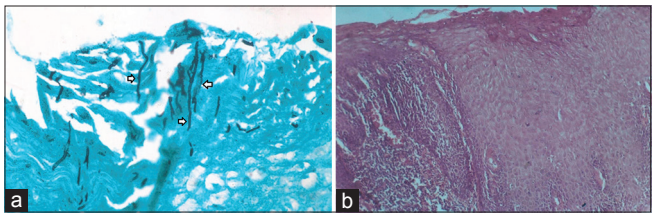

Figure 5: (a and b) Distinct dysplasia (H and E, ×100) and hyphal invasion in tissue (Gomori'smethenamine silverstain, ×450)

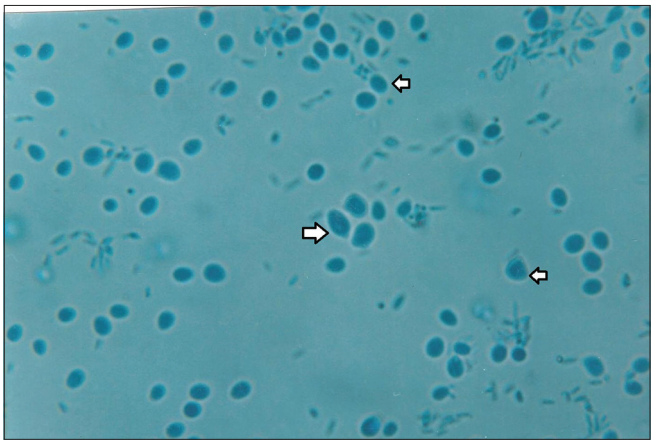

Figure 4: Chlamydospore formation by *Candida albicans* on cornmeal agar. Lactophenolcotton blue mount (×1000 oil immersion)

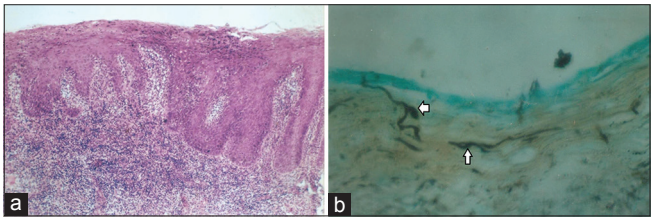

Figure 6: (a and b) Distinct dysplasia (H and E, x100) and dimorphic presence of Candida albicans GMS, ×450

homogeneous leukoplakias, suggesting endogenous production of carcinogenic nitrosamines.[20] *Candida albicans* isolated from potentially malignant disorders may also produce mutagenic amounts of acetaldehyde.[24] Further, a biological injection phenomenon has been proposed by which hyphae may serve as tracts through which carcinogens travel deep into the epithelium even though *Candida* affects only the superficial layers initially.[25]

Barring one patient, all the patients in our study smoked, chewed tobacco and/or used alcohol. It can be speculated that tobacco and alcohol prime the oral mucosa in such a way that *Candida* invasion is facilitated. Higher levels of aspartyl proteinases from *C.albicans* in individuals with leukoplakia and oral squamous cell carcinoma may facilitate colonization by the fungus.[10] Phospholipase enzyme activity of *Candida* also may enable membrane penetration and epithelial invasion.[26]

We found *Candida* on direct smears in 47.5% of leukoplakia patients and 14.3% of controls, while other studies have reported smear positivity rates ranging from 11% to as high as 63%.[25,27,28] *Candida* was isolated on culture in 45% of all leukoplakia lesions and in 64.7% of the non-homogenous lesions in our study; the respective figures in some previous studies were 59% and 76-91%.[26,28] Yet another study found a 30% positivity for *Candida* diagnosed by any one diagnostic procedure in a sample of 30 cases.[23] The relatively low incidence of positive mycological findings could be attributed to the fact that *Candida* species invade the superficial epithelium, and the use of swabs limits

sampling to tissue surfaces.[29] Additionally, once infection by *Candida* is established, there is a strong tendency for the surface layer to separate. This could lead to hyphae being lost by natural desquamation or during handling and processing of the biopsy specimen.

Candida albicans was more prominent in our patients than non-albicans species, in line with previous findings.[28,29] However some workers have reported that *Candida stellatoidea* was more prominent in their patients.[26,28,30]

Culturing of samples in oral leukoplakia cases has been recommended.[31] However, the rarer *Candida albicans* biotypes 051,147,151,153,157 and 353, exhibit the highest nitrosation potential,[28] and species identification may be more relevant than mere isolation on culture.

A positive correlation between dysplasia and *Candida* colonization has been reported previously,[32] but a statistically significant result evaded us in the histological determination of *Candida* amongst both clinical and histopathological variants of leukoplakia. A larger sample size especially concentrating on nonhomogenous leukoplakia may address this issue.

Dysplasia may conceivably also be a change subsequent to *Candida* invasion.[33] *Candida* infection secondary to leukoplakia may lead to keratinolysis and increased submucosal inflammation, thereby affecting the epithelial architecture leading to dysplastic changes; longer follow-up would reveal if the dysplastic changes are permanent.

In conclusion, we found a positive clinical association between leukoplakia and colonization by *Candida*. Further, the incidence of *Candida* using direct microscopic and culture techniques was significantly higher in the more ominous non-homogenous leukoplakia than in homogenous leukoplakia.

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