Original article

Indian Journal of Dental Education
Volume 5 Number 3, July - September 2012

Occurrence of Candida Albicans in Oral Leukoplakia; A Clinical & Histological Evaluation

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Abstract

Background: Leukoplakia is most common premalignant lesion of the oral cavity. Candida albicans is the most common fungal species isolated from oral leukoplakia. Malignant transformation of Leukoplakia has been linked to several factors like habits, duration of lesion, location, clinical appearance and presence of Candida albicans.

This study was done on 50 healthy individuals with oral leukoplakia to determine the prevalence of Candida in leukoplakia and to examine the relationship between presence & absence of Candida with the histological feature and to determine the possible role of Candida in its malignant transformation.

Method: The presence of Candida was determined by microscopic examination of gram stain section & by culture. Biopsy material was obtained from all lesions for histological grading.

Result: Association between occurrence of Candida & type of leukoplakic lesion clinically & histologically were observed. 82% of non-homogeneous leukoplakic lesions yielded Candida & 89% histologically grade 3 lesions were found to be associated with presence of Candida in smear.

Conclusion: Findings implicates possible role of Candida albicans in transformation of untreated leukoplakia into malignancy.

Key words: Candida albicans; Oral leukoplakic lesions; Malignant transformation.

Introduction

Oral leukoplakia occurs in 3% to 4% of the adult population and if untreated, 5% to 10% of the cases will develop into carcinoma.

Recent years have brought several reports of an associated Candida albicans infection in patients with oral leukoplakia. Some reports consider that it just acts as a secondary invader in already established lesions. At the same time it has been suggested that some clinical forms of leukoplakia are the results of invasion by hyphae of C.albicans as well as the possible role of yeasts as a factor in the malignant transformation of leukoplakia. It is well known that Candida species are keratophilic and they tend to colonize thick layers of keratin and adhesion to epithelial cells is the initial step in the process by which Candida albicans adhere to oral mucosa.

Candida possesses enzymes with the ability to destroy the protein substrates on which they reside and these include candida aspartyl proteinases, phospholipase A and lysophospholipase which are secreted extracellularly. These are complemented by a variety of intra cellular proteinases and peptidases which are released into the environment on cell death. Furthermore the hyphal extensions of Candida serve as an ideal appendages abetting colonization and invasion and they help in adherence and anchoring organisms to host surface.

This present study is an attempt to find out if Candida albicans have any role in bringing any histopathological changes in leukoplakia.

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(Received on 07.08.2012, accepted on 24.08.2012)

The aims and objectives of this study were

1. To determine the prevalence of Candida albicans in leukoplakia.
2. To compare the number of colonies obtained from the swab culture of lesion with that from apparently normal mucosa.
3. To correlate the clinical features of leukoplakia with the presence or absence of Candida albicans at that site.
4. To correlate the histological features of leukoplakia with the presence or absence of Candida albicans at that site.
5. To evaluate the role of Candida albicans in malignant transformation of leukoplakia.

Materials and Methods

Fifty healthy individuals with oral leukoplakic lesions were recruited. Individuals with dentures or with antibiotic or steroid therapy over the past 3 months, or with a history of candidosis, anemia, diabetes and any other condition or treatment known to promote oral candidosis or candidal carrier state were excluded. Any pathological condition in the oral mucosa caused exclusion from the healthy group. Patients who received or had received any treatment with antifungal agents within the latest 3 months, or had been hospitalized were also excluded.

Clinical investigation

Clinical investigation started with the examination of cases Clinical staging was done according to stages given by McCarthy,[1] as follows :

Stage-I

Represented the initial reaction of the mucous membrane to irritation, and clinically the lesion is red, granular area that gradually become slightly grey.

Stage-II

Lesions are bluish-white patches or plaques, sharply outlined and without any palpable induration.

Stage-III

Lesions are indurated plaques, white, possibly wrinkled, and sharply outlined.

Stage-IV

Lesions are indurated, leathery plaques with fissures, erosions and occasional warty proliferation of the surface.

The lesion was also classified as homogeneous and non-homogeneous according to Axell T et al in 1984.[2,3]

Homogeneous leukoplakia has been defined as a predominantly white lesion of uniform flat, thin appearance that may exhibit shallow cracks and has a smooth, wrinkled or corrugated surface with a constant texture throughout.

Non-homogeneous leukoplakia has been defined as a predominantly white or white-and-red lesions ("erythroleukoplakia") that may be irregularly flat, nodular or exophytic.

Cytological investigation

Smears were obtained from the lesional side and then heat fixed and stained by the Gram's stain. It was then evaluated for the presence or absence of Candida.

Mycological investigation

Sample from the lesional side was taken with the help of curette and from the apparently normal mucosa with a sterile cotton swab.

Both the samples were inoculated on two plates of sabouraud's Dextrose Agar (SDA). Both plates were incubated at 37^{o}C. It was observed everyday, for a week, for the appearance of growth.

Growth of the yeasts was registered as negative or positive along with quantitation of the colonies.

The tests used for species identification were germ tube test and morphology on corn meal agar.

The assimilation potential of isolated strains was studied by Modified Wickerham Medium (Tube Method).

Histological investigation

Incisional biopsy was obtained from leukoplakic side. It was fixed in 10% neutral formalin and embedded in paraffin wax. Three 5 μm sections were cut, one section was stained with haematoxylin – eosin and evaluated for histological changes and was graded histologically according to Mc Carthy et.al. [1],as follows :

Grade I - Simple hyperkeratosis

The width of the stratum corneum of the mucosal stratified squamous epithelium is increased. They may appear either as hyperkeratosis or parakeratosis. With hyperkeratosis the stratum granulosum may be accentuated. Acanthosis and some extension of rete pegs may be apparent, particularly in lesions of the alveolar mucosa and tongue. Chronic inflammatory infiltration into the underlying connective tissue is minimal or absent. This type of lesion represents a simple keratotic response to some mild irritant or stimulant.

Grade II - Hyperkeratosis and Inflammation

In this reaction there is hyperkeratosis and often notable inflammatory infiltration into the underlying connective tissue. Dilated capillaries are often in evidence. There may be extension of rete pegs, and some hydropic degeneration may be seen in the stratum spinosum. The degree of keratinization varies. It may be thick or thicker than the rest of the epithelium.

Grade III - Hyperkeratosis and Dysplasia (Dyskeratosis)

In addition to hyperkeratosis there is evidence of dysplasia or an abnormal orientation of the epithelium with cellular atypism. The chronic inflammatory cells are found in the connective tissue stroma and there is not the usual separation between epithelium and connective tissue at the basement membrane zone. The epithelium and connective tissue appear to blend into each other. However, there is no obvious invasion of the epithelium into connective tissue.

This histological findings were correlated with the Candida culture as well as the Candida found in the smear.

And finally clinical stage, histological grade and Candida's presence were correlated.

Now the other 2 sections were stained by PAS method and were evaluated for the presence of Candida, based on negative or positive finding of hyphae or pseudohyphae.

Results & Observations

A chi-square test and students't' test were used to analyse the results. Chi-square test was done to determine the extent of variations in the proportions of positive test results in following situations.

1. To examine difference in presence of Candida in smear between homogeneous and non-homogeneous subgroups (Table-VI). The result of statistical analysis shows that non-homogeneous type yielded more Candida than homogeneous type - 82% versus 7%. This difference is statistically highly significant ($p<0.001$).

2. To examine differences in candidal growth in culture between pathological mucosa and normal mucosa subgroups (Table-VII).

The results of statistical analysis showed that differences in positive test results were not statistically significant ($p<0.05$).

3. To examine differences in presence of Candida in smear between grade-I, grade-II and grade-III subgroups (Table-XII).

Results showed that grade-III leukoplakia yielded more Candida than grade-II – 89% versus 40%. No cases in grade-I showed Candida in smear. This difference is statistically highly significant ($p<0.001$).

Students't' test was done to determine the significance in difference of mean colony count between pathological and normal mucosa subgroups (Table-VIII).

Result showed that mean colony count were higher in pathological mucosa subgroup than normal mucosa – 22 versus 9.5. This difference is statistically significant ($p<0.001$).

Table I: Distribution of examined individuals by age and sex

Age Range in years	Number of Individuals (n)	Female	Male
15 – 25	11	0	11
26 – 35	21	3	18
36 – 45	10	1	9
46 – 55	3	0	3
56 – 65	3	2	1
66 – 75	2	0	2
Total	**50**	**6 (12%)**	**44 (88%)**

Table II : Distribution by Habits

Sr. No.	Habits	No. of Individuals	Percentage
1	Tobacco with lime only	43	86.00
2	Bidi only	3	6.00
3	Tobacco with lime & Arecanut	1	2.00
4	Tobacco with lime & Gutkha	1	2.00
5	Pan with lime only	1	2.00
Total		**50**	**100.00**

Table III: Topographic Distribution of Lesions

Sr. No.	Site	No. of Individuals	Percentage
1	Buccal Mucosa	34	68.00
2	Lower vestibule	8	16.00
3	Gingiva	5	10.00
4	Labial mucosa	3	6.00
Total		**50**	**100.00**

Table IV: Types of Leukoplakic Lesion

	Number of Individuals	Percentage
Homogeneous	28	56.00
Non-homogeneous	22	44.00
Total	**50**	**100.00**

Table V: Distribution of Cases According To Clinical Staging of Lesion

Clinical stage	Number of Individuals (n)	Homogeneous	Non-homogeneous
Stage-I	10 (20%)	10 (100%)	0
Stage-II	27 (54%)	18 (66%)	9 (33%)
Stage-III	13 (26%)	0	13 (100%)
Stage-IV	0	0	0

Table VI: Candida Albicans Observed in Smear from the Leukoplakic Lesion

	No. of patients showing Candida	^{2}	Significance
Leukoplakic lesions (n=50)	20 (40%)		
Homogeneous (n = 28)	2 (7%)		
Non-homogeneous (n = 22)	18 (82%)	28.6	p<0.001 (HS)

Table VII: Growth of Candida – Isolated and Demonstrated In Culture from Pathological and Clinically Normal Mucosa in Patients with Leukoplakic Lesions

	No. of cases showing Candida	^{2}	Significance
Pathological Mucosa (n=50)	30 (60%)	0.36	p > 0.05 (NS)
Normal Mucosa (n=50)	27 (54%)		

Table VIII: Mean Colony Count of Isolated Candida in Culture from Pathological and Clinically Normal Mucosa in Patients with Leukoplakic Lesions

	Mean Colony count	't'	Significance
Pathological Mucosa (n=30)	22 (Min = 10; Max.=34)	4.9	p<0.001 (HS)
Normal Mucosa (n=27)	9.5 (Min = 4; Max.=20)		

Table IX: Histopathological Grading of Leukoplakic Lesions

	Grade-I	Grade-II	Grade-III
Homogeneous lesion (n=28)	11 (39%)	16 (57%)	1 (3%)
Non-Homogeneous lesion (n=22)	0	14 (64%)	8 (36%)
Total	11 (22%)	30 (60%)	9 (18%)

Table X: Presence of Candida in Smear and Dysplasia in the Leukoplakic Lesions

	No. of cases showing presence of Candida	No. of cases showing Epithelial dysplasia
Leukoplakic lesion (n=50)	20 (40%)	9 (18%)
Homogeneous (n=28)	2 (7%)	1 (3%)
Non-homogeneous (n=22)	18 (82%)	8 (36%)

Table XI: Co-Relation between Presence of Candida in Smear and Histological Grading of Lesion

Total No. of patients showing presence of Candida in smear	Grade-I	Grade-II	Grade-III
20	00	12 (60%)	8 (40%)

Table XII: Co-Relation between Histological Grade of Lesion and Presence of Candida in Smear

Histological grade	Total No. of patients	No. of patients showing presence of Candida in Smear	^{2}	Significance
I	11	0	16.3	p<0.001 (HS)
II	30	12 (40%)		
III	9	8 (89%)		

Discussion

The importance of the protocol established for this study was based on the idea that Candida albicans is associated with malignant changes at the site of leukoplakia.

In this study, out of 50 patients there were 44 (88%) males and 6 (12%) females with an average age of 35 years. The maximum number of patients was in the age group of 26-35 years (Table I). In a similar study by Pindborg et al (1980) [4] all patients were males with an average age of 45 years. Krogh et al (1987) [5] reported 84% males and 18% females with an average age of 57.5%.

In this study 43 (86%) patients were smokeless tobacco users in the form of tobacco with lime. 3 (6%) had the habit of bidi smoking (Table-II). Silverman and Richard (1968) [6]reported that 95% of men in their study used tobacco in various forms; 63% were cigarette smokers. 75% of the women smoked cigarette and in addition, three female patients used snuff. The duration of the habit ranged from 1 year to 25 years. Maximum of 34 (68%) cases had duration of habit for 5 years.

In the present study, the location of the lesion was prevalently buccal mucosa (68%) followed by lower vestibule (16%) and gingiva

(10%) respectively (Table-III). Nagai et al (1992) [7] reported, among 18 cases 9 were from the tongue, 5 from the buccal mucosa, 5 from the gingiva, 1 from the palatal mucosa, 1 from the floor of the mouth and 1 from the labial mucosa. Silverman and Richard (1968) [6] showed that most common oral site was buccal mucosa, followed by floor of the mouth, tongue, alveolar mucosa, labial mucosa and gingiva.

On the basis of clinical appearance lesions were grouped into homogeneous and non-homogeneous type. In this study 28 (56%) cases had homogeneous leukoplakia and 22 (44%) had non-homogeneous leukoplakia (Table-IV). In a study of Rindum et al (1994) [8] out of 32 cases, 11 (34%) cases had homogeneous leukoplakia and 21 (65%) cases had non-homogeneous leukoplakia.

Clinical staging of the lesion on the basis of clinical findings were also done which was proposed by McCarthy.[1] 10 (20%) were in stage-I, 27 (54%) were in stage II and 13 (26%) were in stage-III. No cases were found in stage-IV. All lesions in clinical stage-I were of homogeneous type while all lesions in stage-III were of non-homogeneous type. Lesions in Stage-II exhibited both homogeneous and non-homogeneous type (Table-V).

Cytological Examination

Cawson and Lehner (1967) [9] demonstrated that the microscopic examination of PAS stained smears were the most helpful single investigation in candidal infection. They opined that hyphae in direct smears are of greater significance as it is in this phase that organisms actively invade the tissues. When a plaque is firmly scraped hyphae can often be seen embedded in detached fragments of epithelium. And they finally concluded that the most helpful single investigation is the direct smear, the discovery of hyphae which would raise strong suspicion that Candida albicans is the cause of the lesion. However, the diagnosis must be confirmed by biopsy.

In the present study 20 (40%) patients demonstrated candidal hyphae in the smear, in which 12 (60%) cases in histological grade-II and 8 (40%) cases were in grade-III, while no case in grade-I demonstrated candidal hyphae in smear (Table-VI, XI). In homogeneous leukoplakia subgroup 2 (7%) cases showed hyphae in smear whereas 18 (82%) cases showed hyphae in smear in non-homogeneous leukoplakia subgroup (Table-VI). Rindum et al (1994) [8] reported the presence of hyphae in 63% of cases. Cawson and Lenher (1967) [9] regarded hyphal form as a reliable indicator of active infection whereas Rindum et al (1994) [8] demonstrated that 4-47% of smears contained hyphae or pseudohyphae like structures in case of healthy dentate individuals with the same frequency in women as in men and most commonly from the tongue. According to them these structures are not an unequivocal sign of candidal infection.

As results indicate, non-homogeneous leukoplakia yielded Candida more frequently than homogeneous type – 82% versus 7%. These results are in accordance with those of Rindum et al(1994) [8] where 81% of non-homogeneous leukoplakia yielded candida. Krogh P et al (1987) [5,10] suggested that malignant development of oral precancer may be elicited by particular biotypes of C.albicans. It is also evident from the result that in cases with histological grade-III lesions, 8 (89%) cases has demonstrated presence of Candida in smear, in cases of histological grade-II lesions 12 (40%) cases has demonstrated presence of Candida in smear, while no case with histological grade-I lesion has demonstrated Candida in smear. It indicates that cases with histological grade-III lesions yielded Candida more frequently than grade-II lesions (Table-XII). As non-homogeneous leukoplakic lesions and histological grade-III lesions yielded Candida more frequently, presence of Candida can be associated with leukoplakias which has the potential to undergo malignant changes. Banoczy and Sugar (1972) [11] also found a 61% incidence of Candida in their cases of speckled leukoplakia undergoing malignant transformation.

Mycological Examination

In the present study, candidal colony was found in culture of 60% of samples taken from lesional mucosa and in 54% of samples taken from apparently normal mucosa (Table-VII). Rindum et al (1994) [8] reported candidal colony in culture of 95% of samples taken from lesional mucosa and in 89% of samples taken from apparently normal mucosa.

The colony count varied from 10 to 34 (Mean 22) in the culture from the lesional side and 4 to 20 (Mean 9.5) in the culture from apparently normal mucosa. Increased colony count in lesional side may be due to keratophilic nature of the Candida.

Histological Examination

In this study 11 (22%) cases were in grade-I, 30 (60%) cases were in grade-II and 9 (18%) cases were in grade-III (Table IX). None of the cases showed hyphae in PAS stained section. The reason might be as described by Cawson (1966) [12] that non-invasive hyphae and yeasts may be detected in smears or grown in culture but lost during histological processing, producing a negative result on staining the section with PAS. Again Roed Peterson (1970) [13] revealed that 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. Negative results in PAS may also be due to scraping of the mucosa for isolation of Candida in smear and culture. Scraping may lead to removal of hyphae from the superficial keratinized portion of the lesion.

Histopathological changes as studied in H & E stained section were correlated with the Candida found in smear and culture. No case with histological grade-I has shown the presence of Candida in the smear, 12 (40%) cases of histological grade-II have shown presence of Candida in smear, whereas 8 (89%) cases with histological grade-III have shown presence of Candida (Table-XII).

Presence of Candida in almost all cases of histological grade-III but one implicates its possible role in transformation of untreated leukoplakia into malignancy, whether the Candida are causally involved in the development of leukoplakia and in the transformation of leukoplakia into carcinoma are matters still being debated.

Krogh et al (1990) [10] postulated possible mycological etiology of oral mucosal cancers. They found that certain strains of Candida albicans and other yeasts may play a causal role in the development of oral cancer by means of endogeneous nitrosamine production. This findings were supported by Rindum et al(1994) [8] also.

Birman, Kignel, Silveriar, Paula (1997) [14] added that the patients should be drinkers and smokers since these agents as well as yeast like Candida albicans, are prone to favour the production of nitrosamines, which could be an adjuvant of neoplasia development.

Barrett et al (1998) [15] demonstrated that Candida species have been identified in as many as 31% of biopsied leukoplakias, but it was not clear which changes are due to candidal infection and which are due to other factors in studies using human material.

In the present study absence of Candida from one histological grade III case, from few grade-II and from all grade-I cases were observed. It may be due to the salivation which has a constant flushing action which keeps the mucosa moist but may probably remove the unattached or loosely attached Candida from the oral cavity.

In order to establish an infective process Candida must adhere to the host surface and subsequently proliferate and penetrate the host defense that is, the mucosal barrier. One more reason which can be suggested for absence of Candida in the mucosa in most of the cases might be that the proteins within the keratinized mucosal cells may act as antifungal and retard candidal invasion. If the integrity of the epithelium is breached, for example in the presence of prosthesis causing maceration of the oral mucosa or epithelial atrophy due to any reason may result in microscopic breaches of the epithelium and invasion of Candida may occur. This probably explains the presence of candidal hyphae in non-homogeneous leukoplakia where the epithelium has undergone alterations which may affect the efficacy of the barrier mechanisms of the mucosa.

In homogeneous leukoplakia the superficial layers consists of a thick band of keratinized squames which could be either para or orthokeratinized. Probably the invasion of candidal hyphae is prevented as keratin acts as an endogeneous barrier.

As the surface keratin is formed, which is made up of the filaggrin and loricrin, there is dehydration of the cells and cells become packed with filaments surrounded by filaggrin facilitating their dense packing. Thus the cells of the keratinized layers become dehydrated and extremely flattened which become more resistant to mechanical damage and chemical solvents, hence maintaining the cell viability. In non-homogeneous leukoplakia the surface keratinized layers are not always uniform, this which facilitate candidal invasion.

For the colonization of the candidal organisms not only the mucosal changes but also other factors play an important role. As mentioned in various studies iron, folate and vitamin deficiencies may increase the individual susceptibility via systemic pathway and facilitate epithelial invasion.

Tobacco consumption may be a predisposing factor for candidal infection. When compared with the frequency and years of tobacco habit, patients who had the habit for more than 6 years and who chewed tobacco continuously were found to have dysplastic changes.

The other local factor may play a direct role on candidal colonization. For example, the flushing action of the salivary flow, the secretary IgA component of the saliva, the antifungal factors such as lysozyme, lactoperoxidase, lactoferrin, histidine rich polypeptides all of which may keep the oral candidal population at bay. The salivary pH and dietary carbohydrate content may influence the candidal growth.

Summary & Conclusion

1. Leukoplakia was more prevalent in 26-35 years of age range (42%).

2. Leukoplakia was found to be more prevalent in males.

3. In 86% of cases leukoplakia was associated with smokeless tobacco and lime consumption habit.

4. The most common site of lesion found was buccal mucosa (68%).

5. Presence of Candia in smear was noted in 40% of cases.

6. Non-homogeneous leukoplakia yielded more Candida than homogeneous leukoplakia- 82% versus 7% which is statistically highly significant ($p<0.001$).

7. In culture growth, Candida was noted in almost equal number of samples from pathological and normal mucosa whereas the mean colony counts from pathological mucosa were significantly higher than those from normal mucosa.

8. Most of the homogeneous leukoplakia histologically were in grade-I (39%) and grade-II (57%), only 3% were in grade-III. In non-homogeneous type 64% were in grade-II and 36% were in grade-III, none were in grade-I.

9. Overall 9 (18%) cases showed epithelial dysplasia in which 8 (89%) were found to be associated with presence of Candida in smear and were of non-homogeneous type.

Presence of Candida in almost all cases of histological grade-III lesions implicates its possible role in transformation of untreated leukoplakia into malignancy. Growth of Candida in almost equal number of cultures from normal and pathological mucosa implicates possible role of some other predisposing factor for malignant transformation and supports the view that tobacco consumption may be a predisposing factor for candidal infection and premalignant changes.

The exact role of Candida as etiological factor in malignant transformation of leukoplakia still remains a question. Hence, further studies are required to find the cause and effect relationship of Candida in malignant transformation of leukoplakic lesion.

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