ORIGINAL RESEARCH PAPER

Pathology

KEY WORDS: Candida Hyphae, OSCC, leukoplakia, lichen planus, SDA media. OSMF

PREVALENCE OF CANDIDA HYPHAE IN PATIENTS WITH POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

To isolate and identify candida from saliva sample and compare the colonization of oral candida in normal and patient with oral squamous cell carcinoma, leukoplakia, lichen planus, OSMF, and oral squamous cell carcinoma. The study consisted of total 40 patients. 15 individuals diagnosed with oral squamous cell carcinoma, 15 individuals diagnosed potentially malignant lesions and 10 normal individual without any obvious oral lesion. Samples were collected from saliva with sterile swabs, and processed for the detection of fungi on plates that contained Sabouraud-dextrose agar (SDA) and chloramphenicol. At the same time, a biopsy was also performed on each patient. Specimens were fixed in formalin and embedded in paraffin, and then processed. Periodic acid Schiff (PAS) stain was used to demonstrate the presence of fungal elements within tissues. The observation of hyphae and blastospores in the PAS sections was expressed as the presence or absence of Yeasts. Comparison of fungal CFU and number of hyphae as per various diagnoses (diseases) has been done using Kruskall Wallis ANOVA followed by Mann Whitney test for pair wise comparisons. When number of colony forming unit were compared among each group one by one, there was a statistically significant difference seen only between groups control and well differentiated OSCC, also between control and moderate dysplasia also moderately differentiated OSCC and OSMF with highest no of hyphae in OSCC (p value : 0.000). There was no difference between all other pairs.

Introduction:

Cancer afflicts all communities worldwide. Approximately 10 million people are diagnosed with cancer and more than 6 million die of the disease every year. Oral cavity cancer is currently the most frequent cause of cancer-related deaths among Indian men (Park's K., 2007). Certain strains of Candida albicans and of other yeasts play a causal role in the development of oral cancer, by means of endogenous nitrosamine production (Krogh P. et al, 1987). The oral cavity harbours hundreds of different microbial species and C. albicans is the most common fungal pathogen in humans. It exists as a commensal inhabitant of mucosal surfaces in most healthy individuals. However, alterations of host or environment can lead to overgrowth of fungus and infection to the host (Nagy K. et al, 2000).

Candida albicans is carried in the mouths of about 50% of the world's population as a normal component of the oral microbiota, but is kept in check by our immune system. This candidal carriage state is not considered a disease, but when *Candida* species become pathogenic and invade the host tissues, oral candidiasis can occur. This change usually constitutes an opportunistic infection of normally harmless micro-organisms because of local (i.e., mucosal) or systemic factors altering host immunity. Thus, depending on the host defense mechanisms or local oral microenvironment, *Candida* can transform from a harmless commensal to the pathogenic organism causing oral mucosal infection (Bouza E, Muńoz P., 2008 & Marol S, Yücesoy M., 2008). These opportunistic fungal pathogens may colonize, invade and induce lesions in any part of the oral cavity in immunocompromised individuals (Anila K, et al., 2011).

A multifactorial model for the pathogenesis of OSMF is postulated. Tobacco, lime, betel quid, iron and nutritional deficiencies, chronic candidiasis, genetic abnormalities, viral infections, autoimmunity etc., are considered to have either direct effect in causing OSMF or an indirect effect by intervening the immune system which is compromised in OSMF (Sudarshan R, et al., 2012). A considerable proportion of oral squamous cell carcinomas develop from preexisting potentially malignant disorders of the oral cavity (Silverman S Jr, Gorsky M, Lozada F., 1984). World Health Organization (WHO) in 2007 proposed the term potentially malignant oral disorders (PMD) for precancerous lesions and conditions (Warnakulasuriya S, Johnson NW, van der Waal I., 2007).

The aims and objective of the study are: 1. to isolate and identify Candida albicans from saliva.

2. To compare the colonization of oral Candida albicans in normal and patient with oral squamous cell carcinoma or precancerous lesions or conditions.

Materials and Method:

The study consisted of total 40 patients. All the patients selected were those coming to the outpatient department. The selected patients were divided into three groups, each containing A (15), B (15) and C (10) patients.

Study groups

Group A: 15 individuals diagnosed with oral squamous cell carcinoma.

Group B: 15 individuals diagnosed potentially malignant lesions.
Group C: 10 normal individual without any obvious oral lesion.

Preparation of Culture media

Samples were collected from saliva with sterile swabs, and processed for the detection of fungi on plates that contained Sabouraud-dextrose agar (SDA) and chloramphenicol. Colony forming units resembling yeast growth were removed from the plates and processed further for identification using Gram staining, a germ tube test, chlamydospore formation and sugar assimilation tests. After 48 hrs of incubation at 37°C, growth was assessed by enumeration of colonies and expressed as candidal colony forming units per mL (cfu mL–1) of rinse (Anila K. et al, 2011).

Identification of Candida species

Very small inoculum from an isolated candidal colony was picked up with a sterile inoculating loop and was suspended in a test tube containing normal human serum (0.3-0.5 ml) by rubbing the inoculated loop against the wall of the test tube. This helps in diluting the pasty colonies by giving the serum turbid appearance. The mixture was incubated at 42°C for 2-3 hours. A drop of mixture was placed in a clean glass slide and covered with a clean cover slip. This was first examined under a low power objective to locate the group of cells and later, the presence of germ tube was confirmed under high power objective of the microscope.

Histopathological diagnosis

At the same time, a biopsy was also performed on each patient. Specimens were fixed in formalin and embedded in paraffin, and then processed. Periodic acid Schiff (PAS) stain was used to demonstrate the presence of fungal elements within tissues. The observation of hyphae and/or blastospores in the PAS sections was expressed as the presence or absence of Yeasts.

Statistical analysis

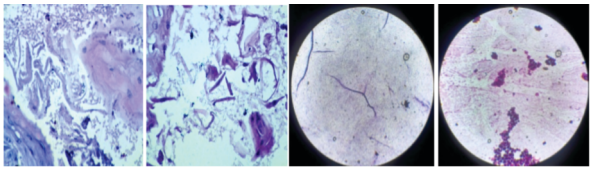
Statistical Package for Social Sciences (SPSS 16.0 version) was used for analysis. One way ANOVA (Post hoc) followed by Dunnet t test was applied to find statistical significance among the groups. P<0.05 considered statistically significant at 95% confidence interval.

Result and Discussion:

Out of total 40 cases 75% [30] cases were male and 25% [10] cases were female (Figure: ABCD). 20% cases were well differentiated carcinoma, 17.5% were moderately differentiated data obtained was compiled on a MS Office Excel Sheet (v 2010). Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 22.0, IBM).

FIGURES: A B C D

A. CLINICAL PHOTOGRAPH SHOWING SQUAMOUS CELL CARCINOMA INVOLVING LEFT BUCCAL VESTIBULE AND GINGIVA
B. CLINICAL PHOTOGRAPH SHOWING LICHEN PLANUS INVOLVING DORSAL SURFACE OF TONGUE
C. CLINICAL PHOTOGRAPH SHOWING LEUKOPLAKIA INVOLVING RIGHT BUCCAL
D. CLINICAL PHOTOGRAPH SHOWING ORAL SUBMUCOUS FIBROSIS



A B C D

A. PAS STAINED HISTOPATHOLOGICAL SECTION SHOWING FUNGAL HYPHAE (x400)
B. PAS STAINED HISTOPATHOLOGICAL SECTION SHOWING FUNGAL HYPHAE (x400)
C. PAS STAINED CULTURE SMEAR SHOWING CANDIDAL HYPHAE (x400)
D. PAS STAINED CULTURE SMEAR SHOWING CANDIDAL SPORE(x400)

Mean age of the participants, number and percentage of males and females participating in the study have been mentioned (Table No.: 2). To rule out any effect of age/gender of the participants on fungal CFU and number of hyphae, correlation was calculated (Photographs: ABCD). Comparison of fungal CFU and number of hyphae as per various diagnoses (diseases) has been done using Kruskall Wallis ANOVA followed by Mann Whitney test for pair wise comparisons. For all the statistical tests, p<0.05 was considered to be statistically significant, keeping error at 5% and error at 20%, thus giving a power to the study as 80%. The age range of selected cases (Total 40) was 22 to 68, with mean age 42.18 and standard deviation being 14.261 (Table No.: 1). Carcinoma, 2.5% were severe epithelial dysplasia, 2.5% moderate epithelial dysplasia, 7.5% were mild epithelial dysplasia, 12.5 % were lichen planus, 12.5% were Oral sub mucous fibrosis, 25% were control (Table No.: 3 and Graph No.: 1). The co-relation coefficient Spearman's rho was found to be weaker hence suggesting no relationship between age and number of colony forming unit and hyphae (Table No.: 4). There was no significant difference was found gender wise on number of fungal colony forming units and hyphae thus was nullified, i.e. gender has no effect on both of these variables (p value of Mann Whitney test for colony forming unit was 0.095 # and for number of hyphae was 0.318 # in terms of gender) (Table No.: 5). There was an overall statistically high significant difference seen with type of diagnosis and No of CFU and Hyphae. With highest no of CFU in Moderate OSCC (p value of Kruskall Wallis ANOVA: 0.000) and highest no of hyphae in Moderate OSCC (p value of Kruskall Wallis ANOVA: 0.001) (Table no: 6 and Graph No.: 2).

TABLE NO: 1 MEAN AGE OF THE STUDY PARTICIPANTS

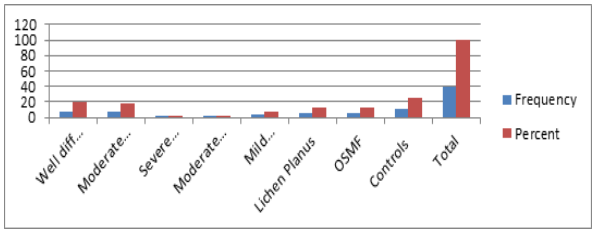
	N	Minimum	Maximum	Mean	Std. Deviation
Age	40	22	68	42.18	14.261

TABLE NO: 2 GENDER DISTRIBUTION OF CASES

S. No.	Sex	Frequency	Percentage
1	Male	30	30
2	Female	10	10
Total		40	40

TABLE NO: 3 DISTRIBUTIONS AS PER FINAL DIAGNOSIS

S. No.	Final Diagnosis	Frequency	Percentage
1	Well diff OSCC	8	20.0
2	Moderate OSCC	7	17.5
3	Severe epithelial dysplasia	1	2.5
4	Moderate epithelial dysplasia	1	2.5
5	Mild epithelial dysplasia	3	7.5
6	Lichen Planus	5	12.5
7	OSMF	5	12.5
8	Control	10	25.0
Total	40	100.0	



GRAPH NO. : 1 DISTRIBUTIONS OF CASES AND CONTROLS AS PER HISTOPATHOLOGICAL DIAGNOSIS

TABLE NO: 4 CORRELATION COEFFICIENT

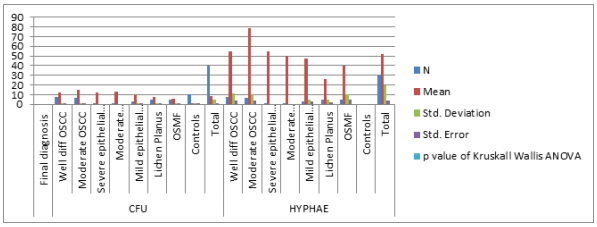
	No. of Fungal colony forming unit (CFU)	Hyphae
Correlation coefficient (r)	0.374	-0.063
Spearman's rho age sig. (-tailed)	0.017	0.741
N	40	30

TABLE NO. : 5 STANTARD DEVIATION

	Sex	N	Mean	Std. Deviation	Std. Error Mean	P value of Mean Whitney test
CFU	Male	30	7.57	5.412	0.988	0.095#
	Female	10	10.70	3.433	1.086	
Hyphae	Male	20	54.80	19.870	4.443	0.318#
	Female	10	47.00	19.686	6.225	

TABLE NO: 6 COMPARISON OF NO OF CFU AND HYPHAE WITH FINAL DIAGNOSIS

	Fungal diagnosis	N	Mean	Std. Deviation	Std. Error	P value of Kruskall Wallis ANOVA
CFU	Well diff OSCC	8	12.25	1.488	0.526	0.000**
	Moderate OSCC	7	14.71	1.496	0.565	
	Severe epithelial dysplasia	1	12.00	-	-	
	Moderate epithelial dysplasia	1	13.00	-	-	
	Mild epithelial dysplasia	3	9.67	1.528	0.882	
	Lichen Planus	5	7.20	1.304	0.583	
	OSMF	5	5.60	1.517	0.678	
	Controls	10	1.50	1.509	0.477	
	Total	40	8.35	5.137	0.812	
HYPAE	Well diff OSCC	8	54.38	11.513	4.071	0.001**
	Moderate OSCC	7	78.86	10.621	4.014	
	Severe epithelial dysplasia	1	55.00	-	-	
	Moderate epithelial dysplasia	1	50.00	-	-	
	Mild epithelial dysplasia	3	47.67	5.033	2.906	
	Lichen Planus	5	26.00	5.244	2.345	
	OSMF	5	40.20	9.884	4.420	
	Controls	0	-	-	-	
	Total	30	52.20	19.822	3.619	



GRAPH : 2 COMPARISON OF NUMBER OF COLONY FORMING UNIT AND HYPHAE WITH FINAL DIAGNOSIS

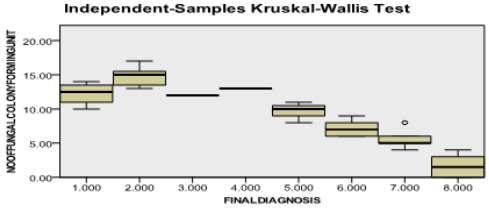
The distribution of number of colony forming unit and distribution of hyphae across all categories reject the null hypothesis (Table No.: 7). When number of colony forming unit were compared among each group one by one, there was a statistically significant difference seen only between groups control and well differentiated OSCC, also between control and moderate dysplasia also moderately differentiated OSCC and OSMF. There was no difference between all other pairs (Table No.: 8).

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of NOOFFUNGALCOLONYFORMINGUNIT is the same across categories of FINALDIAGNOSIS.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
2	The distribution of HYPHAE is the same across categories of FINALDIAGNOSIS.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Table No.: 7 Hypothesis Test Summary

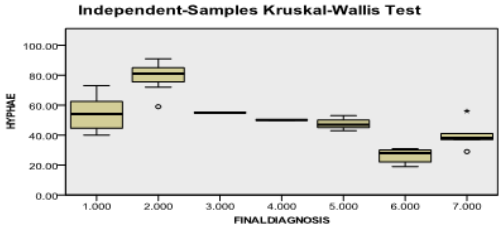


Total N	40
Test Statistic	36.013
Degrees of Freedom	7
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

Table No. : 8 Independent samples Kruskal Wallis Test

When number of hyphae were compared among each group one by one, there was a statistically significant difference seen only between groups moderately differentiated OSCC and Lichen planus, also moderately difference OSCC and OSMF. There was no difference seen between all other pairs (Table No.: 9).



Total N	30
Test Statistic	23.129
Degrees of Freedom	6
Asymptotic Sig. (2-sided test)	.001

1. The test statistic is adjusted for ties.

Table No. : 9 Independent samples Kruskal Wallis Test (compared)

Candida species within humans either as commensals or pathogens has been a subject of interest, among physicians. It is reported that the oral yeast carriage in patient with potentially malignant lesions and oral squamous cell carcinoma may be different from those who are healthy; therefore the coexistence of. It is hypothesized also the association of Candida with various potentially malignant and malignant lesions, as a causative agent. Studies in this field are fraught with difficulty as candida organisms are commensals in the oral cavity; thus, establishing their role in carcinogenesis is challenging. This study is done to evaluate the presence of Candidal hyphae in oral squamous cell carcinoma and potentially malignant lesion.

The similar study was done by (Saigal S. et al, 2013) to evaluate the association of Candida albicans with normal control group, potentially malignant and malignant lesions of oral cavity by using two different liquid culture media. In normal control groups no fungus growth was found; however, potentially malignant and malignant cases showed fungus growth. In our study in normal control groups fungal growth was found but comparatively very less than lesional specimen. Similar study was done by (Galle F. et al) to assess the presence of Candida spp. in a sample of patients with precancer or cancer of the mouth and evaluate the limitations and advantages of microbiological and histological methods.

In the study done by (Galle Francesca et al) all the samples which were negative to culture method was also negative to histological techniques. The similar result was obtained in our study. In the study done by (Galle Francesca et al) site of the lesion was included as one of the criteria and found that the most frequent cancer site was the floor of the mouth (14%), while for precancerous lesions it was the cheek (31%) and predisposing factors considered for the two groups studies were alcohol, tobacco, prosthesis, and poor oral hygiene. In contrast to this study there are no such criteria included in our study.

The similar study by (Hebbar B. P. et al, 2013) aimed to assess the presence and level of colonization of Candida in patients with oral mucosal lesions, to determine the presence or absence of candidal hyphae in biopsy specimens and to correlate the degree of epithelial dysplasia with the number of colony- forming units of Candida. In contrast to this our study excluded other infectious condition in oral cavity or in the body, poorly differentiated carcinoma, Oral submucous fibrosis and Lichen planus with dysplasia. The results of (Hebbar et al) study revealed a correlation between higher colonization of Candida and increasing severity of dysplasia, which is similar to our study.

Similar study was done by (Vuckovic N. et al, 2004) to detect the presence of Candida albicans in potentially malignant oral mucosal lesions. The study done by (Nishanthi L. et al, 2015) to assess the presence of candidal species in Periodic Acid Schiff stained histopathologic sections of intraoral lesions. Out of 45 biopsy tissues stained for presence of candida using PAS stain, 17 tissues showed fungal forms. In oral squamous cell carcinoma, C. albicans count was more accounting for 53% (8 out of 15 tissues) whereas it was 40% (4 out of 10) in oral submucous fibrosis, 30% (3 out of 10) in oral leukoplakia and 20% (2 out of 10) in normal controls. Malignant lesions showed more candidal load compared to premalignant although statistically no significant differences (p-value =0.085) were observed. In contrast to this in our study there was an overall statistically highly significant difference seen with type of diagnosis and no. of colony forming unit and hyphae (p-value < 0.01). With highest number of colony forming unit and no. of hyphae in moderate OSCC.

Conclusions:

Today laboratory diagnosis of oral Candidiasis is not always performed and a presumptive diagnosis is often the only one made, based on patient's history, clinical presentation, and response to antifungal treatment rather than on cultural and histopathologic method. This study is done to evaluate candida by both culture and Histopathological method. There is a statistically significant association between fungal infection and oral squamous cell carcinoma, epithelial dysplasia, Lichen Planus, and Oral submucous fibrosis. The candidal loads were seen more in oral squamous cell carcinoma than other premalignant lesions and conditions. However the culture method is limited because it may highlight occasional fungi in the oral cavity that are not responsible for infection. Histopathological methods, by contrast may disclose hyphae and blastospores in tissue specimen which may indicate that the yeast have invaded the tissue, although they are not as sensitive as culture methods. Our findings suggest that association of candidal load where possible should be more extensively studied by using both culture and Histopathological method, that could be considered complementary.

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