Pathogenecity of *Candida* species in development of oral cancer from pre-cancer in patients of North India Population

Shaista Suhail[1],

Department of Botany ,

University of Lucknow,

Lucknow, U.P., India

Shalini Gupta[2],

Department of Oral Pathology & Microbiology,
King George's Medical University,
Lucknow, UP, India

Neeta Sharma[3]

Department of Botany ,

University of Lucknow,

Lucknow, U.P., India

ABSTRACT:

Introduction: *Candida* species, a normal commensal of the oral cavity found in healthy individuals, but can become an opportunistic pathogen when the oral ecosystem is unbalanced. Several virulence attributes have been identified in candidal infection, among which are the hydrolases, including the secreted Aspartyl proteinases (Saps).

This study evaluated and compared the level c.f.u of *Candida albicans and SAP level* among the patients of leukoplakia, eryhtroplakia, OSMF (oral sub mucous fibrosis) and oral squamous cell carcinoma (OSCC) and control samples, followed by the study of hyphae in tissue specimen of patients. *Candida* c.f.u at 48 hr was also assessed. The hyphae level was assessed by histopathology in samples of the patients undergone biopsy.

Keywords: *Candida albicans*, Pathogenecity, Oral precancer**,** Oral Squamous Cell Carcinoma (OSCC)**.**

Aims: The present study was carried out to identify the correlations between oral carriage of *Candida* in oral precancer and cancer. The aims were; 1) to assess the presence and levels of *Candida* colonization in precancer and cancer patients , 2) to assess the presence or absence of *Candidal* hyphae in biopsy specimens, 3) to correlate the number of colony forming units of *Candida* with hyphae level and sap level in specimens of precancer, cancer and control samples.

Method and Materials: Clinical isolates of *C. albicans* were obtained from the oral cavity of five different groups. Culturing of isolates was done on SDA media. Speciation of *Candida* was performed by performing germ tube test. Histopathology was done to study hyphae level. Spectrophotometric analysis of SAP level was performed.

Results: Hyphae levels and CFU were higher in individuals with leukoplakia and OSCC than in control (P = 0.001); however, there was no significant difference in hyphae levels or CFU in control (P = 0.529). Further, a correlation between CFU, hyphae level and SAP level was also observed at all the stages.

Conclusion: The increasing SAP level and hyphae of *C. albicans* in individuals biopsy tissue of patients with leukoplakia, erythroplakia to OSCC suggests that this pathogen plays a role in disease development and could aid in identifying the pathogenic commensal. This research may help us to understand the pathogenecity of oral *Candida* isolated from precancer and cancer patients in India, and would assist in getting the accurate treatment in clinical practice.

Introduction:

Candida species, a harmless eukaryotic commensal organism belongs to a members of the phylum Ascomycota which can be recovered from human and other mammalian sources. In healthy individuals *Candida* species mostly reside as a part of the normal commensal microbial flora on mucosal surfaces of the oral cavity, gastrointestinal and genitourinary tracts (Kumamoto C.A, 2011). As it is harmless it causes infection only when host immune system becomes weak. Pregnant, diabetic, elderly or immunocompromised individuals show higher percentage of *Candida* prevalence (Lockhart S.R et al 2003., Odds F.C, 1988). Small number of *Candida* species show clinical importance in humans including *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Candida dubliniensis* among which *C. albicans* is the most pathogenic of the *Candida species*, and is responsible for the majority of oral and systemic infection (Moran, G et al 2004, Thompson GR et al 2010, Zomorodian K et al 2011). *C. albicans*, is a dimorphic species which grow as a filamentous forms, and is one of the only two *Candida* species capable of forming true hyphae, the other species being *C. dubliniensis*, the closest relative of *C. albicans*. Hyphae are

considered to play important roles in processes such as adhesion and tissue invasion. Comparison of both species in both mucosal and systemic infection models have demonstrated that in spite of the ability of both species to produce hyphae, *C. albicans* is a significantly more successful pathogen (Stokes C et al 2007, Daftary DK et al 1991).

Candida albicans is the most prevalent and pathogenic among Several *Candida* species which causes opportunistic infections in humans. It is isolated from the oral cavity as a commensal as well as a pathogenic organism in healthy individuals and those with underlying disease (Odds FC et al 1988).

Oral cancer is considered to be the sixth most commonest cancer which occurs worldwide and continues to be the most prevalent cancer (Daftary DK et al 1991). It is not only alarming an increase in percentage of diseased patients but also constitutes the highest incidence parts of the globe (Shah J 2003).

Oral cancer develops in a multistep process from pre-existing potentially malignant lesions. The most common precancer is Leukoplakia which represents 85% of such lesions (Bouquot JE et al 1994) and 95% of oral cancers are squamous cell carcinomas (Chen J et al 1991, Ostman J et al 1995). In India a vast majority of oral squamous cell carcinomas arises from pre- existing Leukoplakia (Gupta PC et al 1989). Likewise, the incidence of oral submucous fibrosis (OSMF) and OSCC is also increasing like an epidemic, targeting the younger generation.

Oral leukoplakia is considered to be one of the most frequent potentially malignant lesions of the oral cavity. Several studies *Candida* have reported that 1-18% of premalignant oral lesions will develop into malignancy or cancer. *Candida* has been also identified as a possible factor in the development of oral leukoplakia and its malignant transformation (Van der Waal I et al 1997). It contributes many virulence attributes like adherence to host tissue, phenotypic switching, and release of some hydrolytic enzymes (Agabian N et al 1994).

Candida is frequently isolated from various mucosal surfaces in healthy individuals hence its presence is almost universal (Al-Abeid HM et al 2004). 3 to 48% of *Candida* prevalence has been found in clinically normal mouths of healthy adults (Scully C et al 1994, Cawson,1996) *Candida* also play a role of promoter in conversion of oral mucosal keratoses to carcinoma, which is remained a highly controversial point to date (O'Grady JF et al 1992).It is still unclear, how an increased amounts of *Candida* in the oral cavity influence the progression of epithelial dysplasia or malignancy (McCullough MJ et al 2005). Higher levels of *Candida* is present in patients with epithelial dysplasia and oral squamous cell carcinoma(Beggs KT et al 2004). More than 90% of oral-pharyngeal cancers are squamous cell carcinomas (SCC). Often, these malignancies begin as preneoplastic inflammatory lesions, such as leukoplakia, erythroplakia, and OSMF. Leukoplakia is a

common oral lesion, appearing as a highly phenotypically variable with white patches, and may be associated with candidal colonization. When these and other risk factors are present, the risk of malignant transformation to SCC may approach 17%(Silverman S Jr 2001, Gleich LL et al 1997). The leukoplakia (or other premalignant lesions) may become cancerous, especially if they demonstrate epithelial dysplasia. If epithelial dysplasia is diagnosed, the rate of cancer transformation may become as high as 42%.(Pak AS et al 1995) Alterations in host immunity, inflammation, angiogenesis, and metabolism have been noted as prominent clinical features in oral cancers.(Gleich LL et al 1997 ,Pak AS et al 1995).

Method and material:

Study population:

The study included a total of 225 samples 25-erythroplakia(I), 25-leukoplakia(II), 25-OSMF(III), 50-OSCC(IV), 100-Control(V) of patients presenting to the Department of Oral Pathology and Microbiology KGMU, Lucknow. The 225 participants were divided into five groups of participants . Group I- . Group II - Group III- Group IV -, Group V.

A detailed history of all participants was taken to ensure that they were immunocompetent. Individuals were excluded if they had received antibiotic, steroid, or antifungal therapy during the previous three months, if they had a history of underlying systemic disease, or if they were HIV-seropositive or had any other condition that could potentially decrease their immunity. We also excluded oral cancer patients who were undergoing or had undergone radiation therapy or surgical treatment for an oral lesion.

Collection of Sample:

Isolates were obtained by using oral swabs and saliva samples. Unstimulated saliva, Oropharyngeal secretions (swab from posterior pharyngeal wall) swabs were wiped across mucosal sites were collected with sterile swab in sterilized wide-mouthed universal containers, or sterile cotton to isolate *Candida* species .This specimen was sent to the laboratory for assessment of *Candida* colony-forming units. This was followed by an incision or excision biopsy of the lesion and the biopsy sample was sent for histopathologic examination for the presence or absence of hyphae and to assess the presence or absence of Candidal hyphae in sample.

Isolation of Candida sp.:

The cotton end of each swab was inserted into 0.5 ml of sterile water in a microcentrifuge tube, the tube was rigorously mixed for 30s with a laboratory tabletop vortex mixer and 0.15 ml of the samples were inoculated on SDA media with antibiotics and incubated at 35^0 C in BOD incubator for one week. SDA plates were observed daily after 24 hr for growth and plates which did not yield any colonial growth after one week were considered as negative.

SDA plates with growth were processed. The growth that showed Gram positive budding yeast cells on Gram's staining was further processed by germ tube test, inoculation in SDA broth, for speciation of *Candida*.

Method of Identification:

The swabs were inoculated immediately on the Sabouraud's slope and incubated at 35 °C for 48 hrs. (Figure 1). From those samples demonstrating positive fungal growth, single colonies were isolated, transferred to Sabouraud agar and incubated at 35 °C hrs. The pure white colonies were tested for germ tube production. For the control group , smears were obtained from the posterior dorsal surface of the tongue and buccal mucosa of normal healthy individuals and for the study group , smears were prepared from the lesional site.The smears were Gram stained and observed under a light microscope where *Candida* was confirmed as gram-positive , dark blue colored hyphae and yeasts.

Germ tube test:

The germ tube test provides a simple, reliable and economical procedure for the presumptive identification of *Candida albicans*. About 95% of the clinical isolates produce germ tubes when incubated in serum at 35 ^{0}C for 2.5-3 hours. A germ tube represent the initiation of a hypha directly from the yeast cell. They have parallel walls at their point of origin. Germ tube formation is influenced by the medium, inoculum size and temperature of incubation. Fresh normal pooled human sera or a commercially available germ tube solution (Remel Lenexa kansa) are to be used as the medium for the test. The inoculums should result in a very faintly turbid serum suspension. Over-inoculation will inhibit the development of germ tubes. Incubate in at 35^0 C-37^0 C for 2.5-3 hours.

Histopathology:

Fragments of the plaque material smeared on a microscopic slide, macerated with 20%potassium hydroxide and examined for the typical hyphae. In addition, the organisms may be cultured in a variety of media, including blood agar, cornmeal agar and Sabouraud's broth, to aid in establishing the diagnosis.

Histologic sections of a biopsy from a lesion of oral Candidiasis show the presence of yeast cells and hyphae or mycelia in the superficial and deeper layers of involved epithelium.These are more easily visualized with the sections are stained with PAS since the organisms are positive in both instances. Chlamydospores are seldom seen on oral smears or histologic sections.

Assessment of Sap level by spectrophotometer

A spectrophotometer was used to determine absorption spectra of compounds and the concentration of organic and inorganic analytes in solution. The principle of spectrophotometry is based on the Beer-Lambert law, which states that when a sample is placed in the beam of a spectrophotometer, there is a direct linear relationship between the amount (concentration) of its constituent(s) and the amount of energy it absorbs. This may be stated mathematically as,

$\log10 (I_0/I) = A = elC$ or, when rearranged, $C = A/el$

Where, I_0 = incident radiation, I = transmitted radiation, A
= absorbance, e = extinction coefficient at a given wavelength,
l = length in cm, and C = molar concentration (M).

When the molar absorptivity constant is known for a substance at a specific wavelength, the Beer equation is used to determine concentration directly.

A 0.1-ml volume of culture supernatant was mixed with 0.4 ml of 0.1 M citrate buffer containing 1% BSA at pH 3.2 and incubated for 15 min at 37˚C. The reaction was stopped with 0.5 ml of 5% trichloroacetic acid (TCA) on ice for 15 min, and the mixture was centrifuged at 8-12,000 rpm for 10 min. Then, the absorbance was read at 280 nm against distilled water, which was considered as neutral. The controls consisted of the same ingredients and 0.1 ml of pepstatin A solution added along with 0.1 ml of 0.2 M citrate buffer containing 2% BSA. Pepstatin A solution (20 µg/0.1ml) was added as a proteinase inhibitor in controls. One unit of Sap enzyme level was expressed as the amount of tyrosine equivalent, in micromoles, released per min per ml of saliva. A spectrophotometric assay was used to evaluate Sap level, using the technique described by Wu and Samaranayake (Wu T, Samaranayake LP 1999).

Statistical analysis

Mean CFU and Sap level were calculated for all the groups. The Chi-square test was done for comparison of positive results among all the groups and ANOVA test was used for comparison of CFU and Sap level among all the groups. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results:

A total of 225 participants were divided into five groups. Candida albicans was the most predominantly isolated species. Mean of CFU and Sap level of *C. albicans* were calculated.

A highly significant association of *Candida* was seen both in precancer and cancer samples. Statistical comparisons showed significant correlation of presence of *Candida* between oral precancer and cancer. The difference between the SDA positive and negative groups was statistically significant in the present study ($P < 0.001$). The correlation between CFU mean and SAP level was also statistically significant in the present study ($P < 0.001$). Sap level and CCC were significantly higher in OSCC patients than in leukoplakia patients ($P < 0.001$ and $P < 0.001$, respectively); however, there was no significant difference in Sap level or CCC in control ($P = 0.059$ and $P = 0.529$, respectively). Table 4 shows the correlation between CCC and Sap level in all groups ($P < 0.001$ for all tests). Sap level and CFU were significantly higher in patients with OSCC> Leukoplakia > Erythroplakia > OSMF > Control. Sap level and CFU along with hyphae penentration were higher in OSCC patients than in leukoplakia patients and in Leukoplakia it is higher than other pre cancers and control patients.

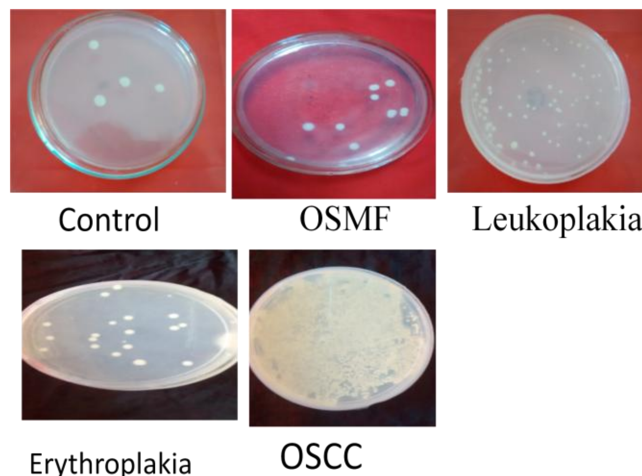
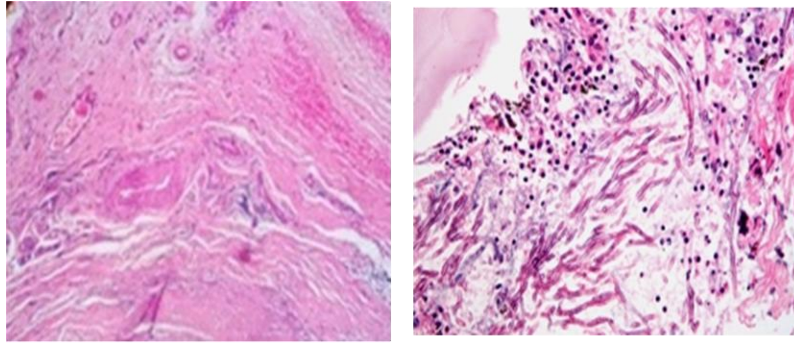


Fig : 1 Colonies of *Candida albicans* showing positive results.



Tissue control Tissue (OSCC)

Fig: 2 Histologic sections of a biopsy from a tissue of control and oral cancer samples show the presence of hyphae or mycelia in the superficial and deeper layers of involved epithelium.

Table: 1 *Candida* species isolated from the five groups

Subjects	Group	Total sample(n)	Positive	Negative
Erythroplakia	I	25	16	9
Leukoplakia	II	25	17	8
OSMF	III	25	11	14
OSCC	IV	50	43	7
Control	V	100	33	67

[(chi square test) χ^2=42.2 (df=4); p<0.001]

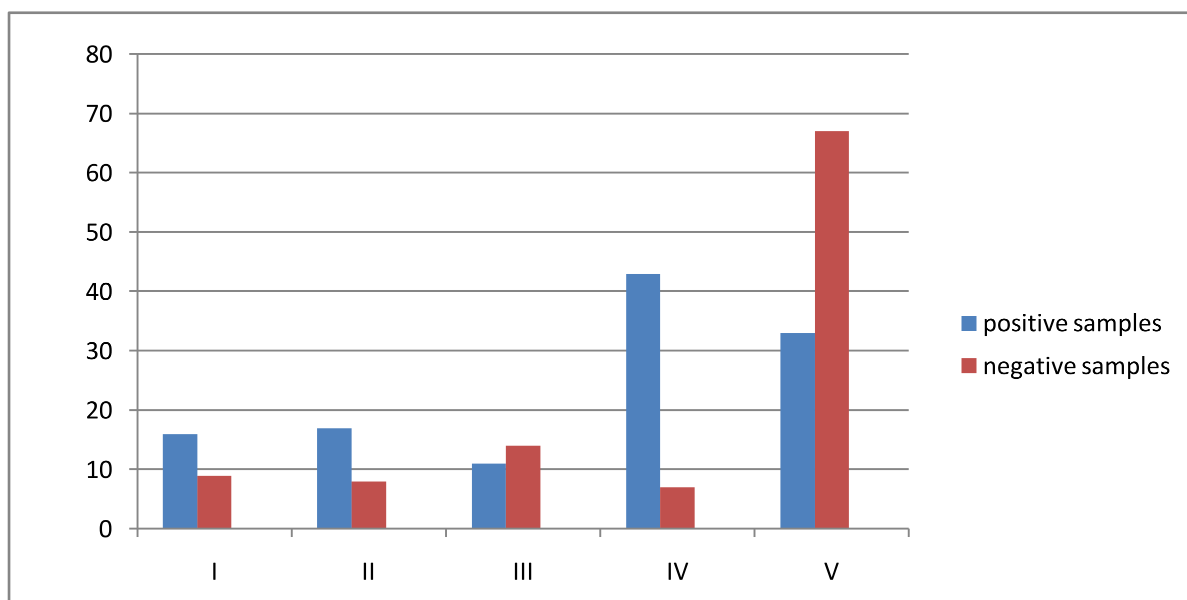


Fig: 3 *Candida* species isolated from the five groups

Table : 2 Mean *Candida* Cell Count or CFU (CFU/ml) (in micromoles) of all the groups and its correlation with hyphae level.

Group	Total sample(n)	Positive	Mean c.f.u/ml	Hyphae in sample
I	25	16	08±0.33	++
II	25	19	12±0.51	++
III	25	11	08±0.48	++
IV	50	33	15±0.58	++
V	100	43	06±0.21	--

Table: 3 Candida positive samples releasing sap enzyme

Group	Total	Positive(sap)	Negative	Sap level(in micromoles)
I	16/25	11	5	++
II	17/25	13	04	++
III	11/25	04	07	++
IV	43/50	37	06	++
V	33/100	0	33	--

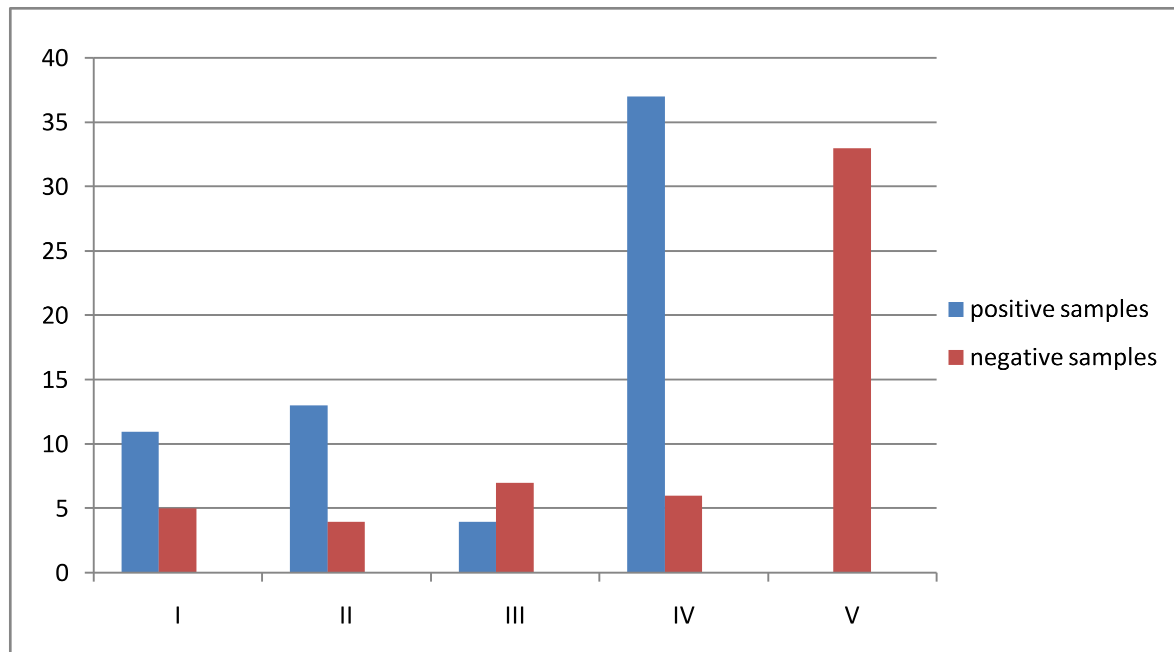


Fig: 4 *Candida* positive samples releasing sap enzyme

Table: 4 Mean *Candida* Sap levels (in micromoles) of all the groups and its Correlation with c.f.u

Group	Total	Positive	c.f.u/ml (mean)	Sap level(mean)
I	25	22-17	08±0.33	04±0.32
II	25	19-14	12±0.51	09±1.53
III	25	11-04	08±0.48	04±0.81
IV	50	43-37	15±0.58	11±1.22
V	100	33-00	06±0.21	1±.50
			F=4639; p<0.001	F=1,245;p<0.001

[ANOVA= F=4639; p<0.001, F=1,245; p<0.001]

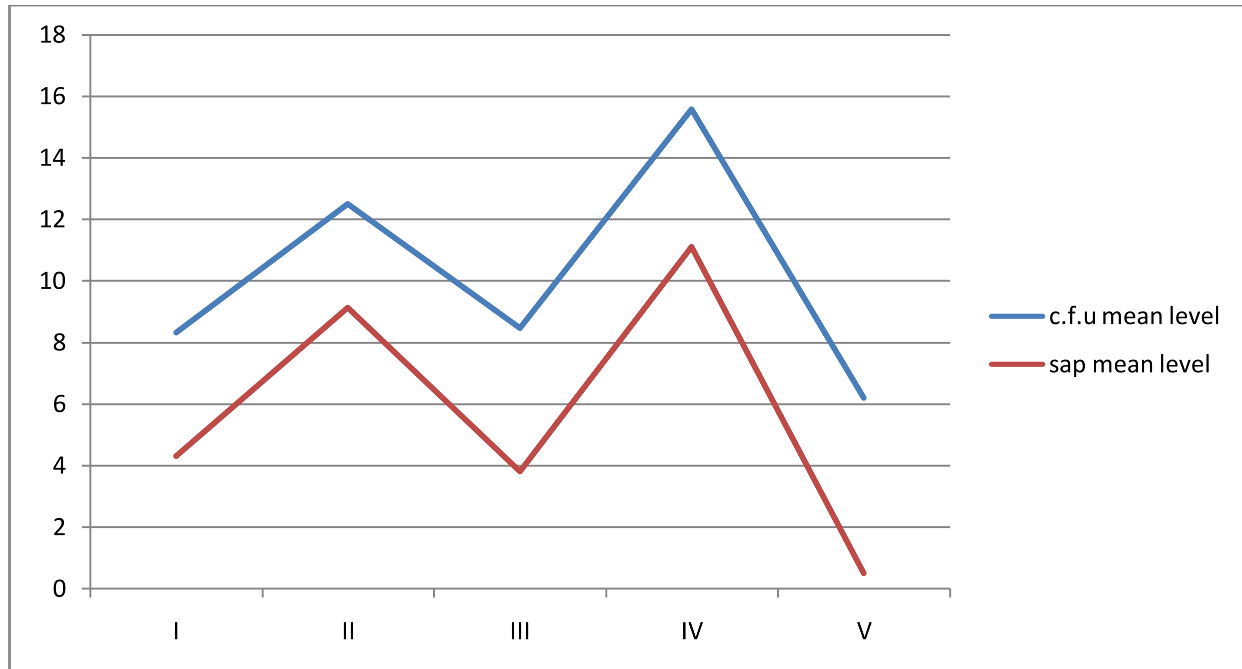


Fig: 5 Mean *Candida* Sap levels (in micromoles) of all the groups and its Correlation with c.f.u level.

Discussion:

The *Candida* species are indigenous to the human oral cavity and can produce a variety of oral infections. These findings are consonant with the results of the present study, in which 44/75 (precancer) and 43/50 (cancer) samples gave positive colonies from saliva and swab samples of the oral cavity. Studies show that *C. albicans* is the most commonly isolated pathogen from the oral cavity and that it is the most virulent of the *Candida* species. This may be because *C. albicans* is also able to produce various virulence factors. 10 genes (SAP1-10) of *C. albicans* is responsible for encoding SAP enzyme of molecular weight 42-44 kDa, whose optimal activity is at pH 3.5-4.0 (Yamamoto T et al 1992). Numerous *in vitro* studies using of *Candida* clinical isolates have shown a positive correlation between the level of Sap production and its virulence, which indicates its pathogenetic role (Naglik JR et al 2003). However, there has been no study of Sap levels in premalignant lesions. At precancer stage the epithelial dysplasia could improve after elimination of *Candida* spp., and in precancer patients. Other studies reported that *C. albicans* comprises 78% of isolated yeasts, which makes it the most frequent species in cancerous and precancerous lesions. It was previously suggested that, certain strains of *C. albicans* probably have properties that are important in the development of pathological conditions and premalignant changes (McCullough M et al 2002). The histological methods discloses the hyphae and blastospores in tissue specimens,

which may indicate that the yeast have invaded the tissue. Our results support the frequent presence of *Candida* spp. in the precancerous and cancerous lesions of the oral cavity (McCullough M et al 2002) and increasing CFU and SAP level indicates the pathogenic effects at both the stages.

Conclusion:

The best way to help improve survival rates of oral cancers is early detection and treatment.
Candida, overcome two main obstacles to be a successful pathogen, host mechanisms to interfere the adhesion of *Candida* to human tissues and the production of hydrolytic enzymes. The first step in the initiation of and invasive process in oral cavity and other human mucosa is the microbial adherence to mucosal surfaces. *C. albicans*, the most adherent and pathogenic species of *Candida*, uses a diversity of mechanisms to adhere to human surfaces. The increasing SAP level and hyphae of *C. albicans* in individuals biopsy tissue with leukoplakia, erythroplakia to OSCC suggests that this pathogen plays a role in disease development and could aid in identifying the pathogenic commensal. This research may help us to understand the pathogenecity of oral *Candida* isolated from precancer and cancer patients in India, and would assist in getting the accurate treatment in clinical practice.

Acknowledgement: We all like to thanks Dr. Ritu Srivasta, Dr. Madhu and Dr. Fahad M Samadi for their valuable guidance in this research work and patients who have visited the Dept. Of Oral Pathology and Microbiology, KGMU, Lucknow.

References:

Agabian N, Odds FC, Poulain D, Soll DR, White TC, 1994. Pathogenesis of invasive candidiasis. J Med Vet Mycol; 32: 229-237.

Al-Abeid HM, Abu-Elteen KH, Elkarmi AZ, Hamad MA, 2004. Isolation and characterization of Candida Spp. in Jordanian cancer patients: prevalence, pathogenic determinants and antifungal sensitivity. Jpn J Infect Dis 57, 279-284.

Asmundsdo, L.R., Erlendsdo, H., Agnarsson, B.A., Gottfredsson, M., 2009. The importance of strain variation in virulence of Candida dubliniensis and Candida albicans: results of a blinded histopathological study of invasive candidiasis. Clin. Microbiol. Infect. 15, 576–585.

Beggs KT, Holmes AR, Cannon RD, Rich AM, 2004. Detection of Candida albicans mRNA in archival histopathology samples by reverse transcription-PCR. J Clin Microbiol 42, 2275-2278.

Bouquot JE, Whitaker SB,1994. Oral Leukoplakia rationale for diagnosis and prognosis of its clinical subtypes or "phases". Quintessence Int, 25:133-140.

Chen J, Eisenberg E,1991. Changing trends in oral cancer in United States 1935–1985. A Connecticut Study. J Oral Maxillofac Surg, 49:1152-1158.

Daftary DK, Murti PR, Bhonsle RR, Gupta PC, Mehta FS, Pindborg JJ, 1991. Risk factors and risk areas of the world. In Oral cancer : the detection of patients and lesions at risk Edited by: Johnson NW. Cambridge University Press; 29-63.

Gupta PC, 1989. Leukoplakia and the incidence of oral cancer. J Oral Pathol Med , 18:11.

Gleich LL, Biddinger PW, Duperier FD, et al, 1997. Tumor angiogenesis as a prognostic indicator in T2-T4 oral cavity squamous cell carcinoma: a clinical-pathologic correlation. Head Neck.;19(4):276-280.

Kumamoto C.A., 2011. Inflammation and gastrointestinal Candida colonization. Curr. Opin. Microbiol, 14, 386–391.

Lockhart S.R, Daniels K.J, Zhao R, Wessels D, Soll D.R, 2003. Cell biology of mating in Candida albicans Eukaryot, Cell 2, 49–61.

Moran G, Stokes C, Thewes S, Hube B, Coleman D.C, Sullivan D, 2004. Comparative genomics using Candida albicans DNA microarrays reveals absence and divergence of virulence-associated genes in Candida dubliniensis. Microbiology 150, 3363–3382.

McCullough M, Jaber M, Barrett AW, Bain L, Speight PM, Porter SR, 2002. Oral yeast carriage correlates with presence of oral epithelial dysplasia. Oral Oncol 38, 391-393.

McCullough MJ, Savage NW, 2005. Oral candidosis and the therapeutic use of antifungal agents in dentistry. Aust Dent J 50, Suppl 2, s36-39.

Naglik JR, Challacombe SJ, Hube B, 2003. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67, 400-428.

Ostman J, Anneroth E: Malignant oral tumours in Sweden 1962–1989. An Epidemiological Study – Eu. J Cancer & Oral Oncology 1995, 8:106-112.

Odds F.C, 1988. Ecology of Candida and epidemiology of candidosis. In: Odds, F.C. (Ed.), Candida and Candidosis. Bailiere Tindall, London, pp. 68–92.

Odds FC, 1988. Candida and candidosis, a review and bibliography. 2nd ed, Baillière Tindall, London, 42-59.

OGrady JF, Reade PC, 1992. Candida albicans as a promoter of oral mucosal neoplasia. Carcinogenesis 13, 783-786.

Pak AS, Wright MA, Matthews JP, et al, 1995. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34+ cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res*; 1(1):95-103.

Scully C, El-Kabir M, Samaranayake LP, 1994. Candida and oral candidosis: a review. Crit Rev Oral Biol Med 5, 125-157.

Shah J, Johsnon N, Batasakis J, 2003. Global epidemiology, oral cancer, Martin Duntiz Group, 3.

Silverman S Jr, 2001. Demographics and occurrence of oral and pharyngeal cancers: the outcomes, the trends, the challenge. J Am Dent Assoc; 132(suppl):7S-11S.

Stokes C, Moran G.P, Spiering M.J, Cole G.T, Coleman D.C, Sullivan,D.J, 2007. Lower filamentation rates of Candida dubliniensis contribute to its lower virulence in comparison with Candida albicans. Fungal Genet. Biol. 44, 920–931.

Thompson G.R., Patel, P.K, Kirkpatrick W.R, Westbrook S.D, Berg D, Erlandsen J, Redding S.W, Patterson T.F, 2010. Oropharyngeal candidiasis in the era of antiretroviral therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109, 488–495.

Van der Waal I , Schepman KP, van der Meij EH, Smeele LE, 1997. Oral leukoplakia: a clinicopathological review. Oral Oncol 33, 291-301.

Wu T, Samaranayake LP, 1999. The expression of secreted aspartyl proteinases of Candida species in human whole saliva. J Med Microbiol 48, 711-720.

Yamamoto T, Nohara K, Uchida K, Yamaguchi H, 1992. Purification and characterization of secretory proteinase of Candida albicans. Microbiol Immunol 36, 637-641.

Zomorodian K, Haghighi N.N, Rajaee N, Pakshir K, Tarazooie B, Vojdani M, Sedaghat F, Vosoghi M, 2011. Assessment of Candida species colonization and denture-related stomatitis in complete denture wearers. Med. Mycol. 49, 208–211.