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Original

# Spectrophotometric analysis of the expression of secreted aspartyl proteinases from *Candida* in leukoplakia and oral squamous cell carcinoma

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**Abstract:** ***Candida*** **species are a normal commensal of the oral cavity 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 *in vitro* level of Saps from *Candida albicans* in nonsmokers, smokers, and patients with leukoplakia and oral squamous cell carcinoma (OSCC). *Candida* cell count (CCC) at 48 h was also assessed. The Sap level was measured by spectrophotometry in 38 clinical isolates of *C. albicans* obtained from the oral cavity of the four different groups. Culturing was done in yeast carbon base-bovine serum albumin. Speciation of *Candida* was performed by using a *Candida* identification kit, and CCC was measured by hemocytometer. Sap levels and CCC were higher in individuals with leukoplakia and OSCC than in nonsmokers or smokers ($P = 0.001$); however, there was no significant difference in Sap levels or CCC between smokers and nonsmokers ($P = 0.529$). Further, an intragroup correlation between CCC and Sap level was also observed. The higher level of Saps from *C. albicans* in individuals with leukoplakia and OSCC suggests that this pathogen plays a role in disease development and could aid in identifying the pathogenic commensal. (J Oral Sci 53, 421-425, 2011)**

Keywords: *C. albicans*; sap; healthy individuals; smokers; leukoplakia; OSCC.

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## Introduction

Oral leukoplakia is one of the most frequent potentially malignant lesions of the oral cavity. Numerous studies have reported that 1-18% of premalignant oral lesions will progress to cancer. Various etiological factors have been identified, including tobacco, alcohol, nutritional deficiency, viral infection (especially human papilloma virus), and chronic irritation. *Candida* has been identified as a possible factor in the development of oral leukoplakia and its malignant transformation (1). Putative attributes contributing to candidal virulence include host tissue adherence, phenotypic switching, and release of hydrolytic enzymes (2).

*Candida* is a common inhabitant of the human oral cavity, with a prevalence of 3-48%; *C. albicans* is the most prevalent species (3). *Candida* species secrete isoforms of aspartyl proteinase (Sap), which destroy host tissue. This degraded tissue is a source of nutrition for fungi and is believed to contribute to the invasiveness and proliferation of *Candida* (4).

Sap from *C. albicans* is encoded by at least 10 genes (SAP1-10). Its molecular weight is 42-44 kDa, with

Table 1 Number of *Candida* species isolated, by group, age, and sex

| Subjects | Group | No. of *Candida* isolates | Male | Female | Mean age |
|---|---|---|---|---|---|
| Nonsmokers | Group I | 10 | 2 | 8 | 36 |
| Smokers | Group II | 10 | 8 | 2 | 38.24 |
| Leukoplakia patients | Group III | 10 | 7 | 3 | 47.25 |
| OSCC patients | Group IV | 10 | 8 | 2 | 48.90 |

OSCC: oral squamous cell carcinoma

optimal activity at pH 3.5-4.0 (5). Numerous *in vitro* studies using oral or vaginal clinical isolates of *Candida* have shown a positive correlation between the level of Sap production and its virulence, which suggests role for proteinase in pathogenesis (4). However, there has been no study of Sap levels in premalignant lesions.

The present study compared *in vitro* Sap expression in *C. albicans* isolated from healthy nonsmokers, smokers, patients with leukoplakia, and patients with oral squamous cell carcinoma (OSCC). *Candida* cell count (CCC) was estimated after an interval of 48 h.

## Materials and Methods

### Study population

The study included a total of 40 *Candida* strains isolated from 103 patients presenting to the Department of Oral Medicine and Radiology, Manipal College of Dental Sciences, Department of Radiation Oncology or the department of Surgical Oncology, Kasturba Medical Hospital, Manipal, India. The study protocol and procedure were approved by the ethical committee of a tertiary referee university hospital, and informed consent was obtained from each patients after explaining the aims and objective of the study.

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.

The 40 participants were divided into four groups of 10 participants each. Group I consisted of healthy never smokers with no pathology of the oral mucosa (2 men and 8 women). Group II comprised smokers (defined as at least 1 cigarette or *beedi* per day at least for 6 months; 8 men and 2 women). Group III consisted of adults with a diagnosis of leukoplakia (7 men and 3 women). Group IV comprised adults with a diagnosis of OSCC (8 men and 2 women) and no leukoplakic lesions. Table 1 shows the distribution of *Candida* colonies, mean age, and sex of the groups.

### Isolation and identification of fungi

Isolates were obtained by using oral swabs and saliva samples. Unstimulated saliva was collected in sterilized wide-mouthed universal containers, or sterile cotton swabs were wiped across mucosal sites.

The growth and identification of *Candida* species and induction of Sap from *C. albicans* were performed as described by Kuriyama et al., wherein all samples were cultured on Sabouraud's dextrose agar in an aerobic atmosphere at 37˚C for 48 h (6). Suspected yeast colonies were subcultured in a KB006 HiCandida Identification Kit (Hi-Media Laboratories, Mumbai, India) at 25˚C for 48 h for speciation, and then subcultured into yeast extract peptone dextrose broth (1% yeast extract, 2% peptone, 2% dextrose) at 37˚C for 24 h. The yeast growth was washed twice by centrifugation, after which the pellet was resuspended in 0.9% NaCl solution. The amount of yeast in the preparation was determined using a *Candida* suspension dilution (1:20) with a WBC pipette. A yeast cell suspension of $10^6$cells/ml and a hemocytometer were used for the study. A 500-µl suspension was then inoculated onto 20 ml of yeast carbon base-bovine serum albumin (BSA) medium (pH 5.6) and incubated at 37˚C for 48 hrs. The culture medium was centrifuged at 4,000-6,000 rpm and the supernatant was used for the assay.

### 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,

$\log_{10}(I_0/I) = A = elC$ or, when rearranged, $C = A/el$

Where, $I_0$ = incident radiation, I = transmitted radiation, A

Table 2 *Candida* species isolated from the four groups

| *Candida* species | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| *Candida albicans* | 10 | 9 | 9 | 10 |
| *Candida pintolepsii* | – | 1 | – | – |
| *Candida stellatoidea* | – | – | 1 | – |

Table 3 Mean *Candida* cell count (CFU/ml) and Sap levels (in micromoles) in the four groups

| Groups | Subjects | Mean *Candida* cell count | Mean Sap level |
|---|---|---|---|
| Group I | Nonsmokers | 10.29±0.32* | 3.34±1.60* |
| Group II | Smokers | 10.93±0.36* | 4.31±1.99* |
| Group III | Leukoplakia patients | 11.42±0.081 | 8.05±1.10 |
| Group IV | OSCC patients | 12.10±0412 | 9.82±1.68 |

OSCC: oral squamous cell carcinoma
One unit of Sap enzyme level was defined as the amount of tyrosine equivalent released, in micromoles, per min per ml of saliva.
Intragroup values significantly differed in all groups (Kruskal-Wallis test, $n = 10$, $P < 0.05$).
*No significant difference between groups (Mann-Whitney *U*-test, $n = 10$, $P < 0.05$).

= 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 is considered 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 (7).

### Statistical analysis

Mean CCC and Sap level were calculated in all groups. The Mann-Whitney *U* test was used for comparison of CCC and Sap level among groups. The Kruskal-Wallis test was used for intragroup comparison of CCC and Sap level. A *P* value of less than 0.05 was considered to indicate statistical significance.

## Results

A total of 40 participants (25 men and 15 women) were divided into four groups of 10 individuals. CCC and Sap level of *C. albicans* were calculated by using a hemocytometer and spectrophotometry, respectively. Sap level and CCC were significantly higher in patients with leukoplakia and OSCC than in nonsmokers and smokers ($P = 0.001$ for all tests). Sap level and CCC were significantly higher in OSCC patients than in leukoplakia patients ($P = 0.011$ and $P = 0.002$, respectively); however, there was no significant difference in Sap level or CCC between smokers and nonsmokers $P = 0.059$ and $P = 0.529$, respectively). Table 3 shows the correlation between CCC and Sap level in all groups ($P < 0.05$ for all tests).

## Discussion

The *Candida* species are indigenous to the human oral cavity and can produce a variety of oral infections. Studies show that *C. albicans* is the pathogen most commonly isolated from the oral cavity and that it is the most virulent of the *Candida* species (3). These findings are consonant with the results of the present study, in which 40 yeast colonies were isolated from saliva and swab samples of the oral cavity; 38 individuals had *C. albicans* (Table 2). This may be because *C. albicans* is able to produce several virulence factors. In addition, *in vitro* studies have shown that non-*C. albicans* species adhere less to buccal epithelial and vascular endothelial cells and secrete less Sap than does *C. albicans*, which may account for their reduced virulence (8-10). The prevalence of various yeast colonies also varies according to the site, yeast type, the age and health of the person sampled, and the sampling method used (11).

Sap level and CCC were lowest in nonsmokers, possibly due to exhaustion of available nutrients caused by the presence of commensal bacterial flora or antifungal substances in saliva—which could be intrinsic to saliva or produced by bacteria—as human saliva contains numerous antimicrobial proteins (7). Lysozyme is resistant to proteolytic activity and has effects on the yeast cell membrane that impede Sap secretion, which leads in turn to intracellular accumulation of Sap or Sap precursor proteins (12, 13). However, the effects of lysozyme on *Candida* are controversial (7).

Sap level and CCC were higher in smokers than in nonsmokers, but not significantly so (Table 3). The effect of smoking on mycotic flora is not clear. Gregely and Uri (14) reported that smoking neither increased nor decreased oral mycotic flora. Similar observations were made by Beasely (15), Olert and Shillote (16), and Rasool (17). However, Arendorf and Walker found that smoking significantly increased the carrier rate of *C. albicans* and might cause mild or clinically undetectable alterations of the oral mucosa that facilitate colonization by *Candida*. *In vitro* findings have also suggested that tobacco smoke may contain a saliva-soluble candidacidal factor, the effect of which was time- and concentration-dependent (11).

Leukoplakia belongs to a large group of potentially malignant oral mucosal lesions. Among the various etiological agents, *Candida* has long been a subject of debate. *Candida* can colonize the superficial layers of the oral mucosa, producing thick granular plaque with a mixed white and red clinical appearance. *Candida*-associated leukoplakia is associated with a higher risk of malignant transformation as compared with non-candidal leukoplakia (18). It has been suggested that *Candida* species play a role in oral carcinogenesis by triggering nitrosamine compounds to activate specified oncogenes, thereby initiating oral neoplasia (19). It has also been noted that leukoplakia invaded by *Candida* shows evidence of cellular atypia (20). McCullough et al. found that fungal infection was more common in potentially malignant lesions than in lesions without malignant potential, which suggests interaction between oral yeast infection and oral epithelial dysplasia (21).

In this study, a higher Sap level of *C. albicans* and CCC were observed in patients with premalignant lesions than in nonsmokers and smokers. Data from *in vitro* studies suggest that the main proteinase secreted by *C. albicans* is Sap-2, which is capable of degrading a number of substrates in the oral cavity, including extracellular matrix proteins, mucin, secretory immunoglobulin A (sIgA), and host proteins (22). Furthermore, digestion of these *in vivo* nutrients aids in the acquisition of essential nitrogen for growth and attachment to or penetration of the oral mucosa, and sIgA digestion might allow *C. albicans* to evade the host immune response (23). The expression of proteolytic activity is governed by the SAP gene family, i.e., the SAP1-10 proteins.

In the present study, the mean Sap level of *C. albicans* and CCC were significantly higher in patients with OSCC as compared with the other groups. The host immune response—particularly the integrity of cell-mediated immunity—is the principal factor in determining whether *C. albicans* behaves as a commensal or as a pathogen. In OSCC, reduced salivary flow, epithelial cell surface changes, and alterations in oral microflora might influence the candidal microenvironment. These changes, along with impaired humoral or cell-mediated mucosal immunity and/or impaired nonspecific host defenses, degrade molecules such as salivary lactoferrin, lactoperoxidase, cathepsin D, and complement. Sap is also known to degrade sIgA by providing essential nitrogen for growth and by enhancing attachment, colonization, and penetration of host tissue (24). Moreover, *C. albicans* is a polymorphic pathogen that can exist in yeast or hyphal state and undergo phenotypic switching (25). Therefore, it seems logical to assume that, in the present OSCC patients, the increase in Sap was associated with an increase in the level of cell-surface associated *Candida* and secreted Sap in saliva. Our study indicated that *Candida* species secrete Sap and that the rate of release is related to yeast growth. Recent studies have shown that, in addition to their primary enzymatic role, various Saps may act singly or synergistically to enhance proliferation and adhesion of yeasts. Here, it should be emphasized that the use of BSA alone as a substrate for Sap determination may have some limitations. Further, substrate specificity has not yet been defined for all Sap proteins and the possibility thus cannot be excluded that non-Sap proteins that hydrolyze BSA might contribute to Sap levels (26).

We found that *C. albicans* secreted more Sap and that yeast growth was greater in leukoplakia and OSCC patients than in nonsmokers with no apparent clinical abnormalities. Thus, increased Sap might facilitate *C. albicans* colonization. However, the precise regulatory factors that control or influence these expression profiles *in vivo* remain to be elucidated. Further studies of the association between proteinase secretion and the selective genetic lineage of fungi are required. Such studies would be helpful in understanding the interaction of *C. albicans* with the human host and, consequently, its role in the progression to neoplasia.

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