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Original Research Article

Screening of the *Sap1* Gene of *Candida Albicans* in Oropharyngeal Cancer Patients in Tertiary Care Unit of Kanpur

Ramesh Yadav[1], R Sujatha[2], Ajay Bhagoliwal[3], Anil Kumar[4]

[1]Ph.D. Scholar, [2]Professor and Head,
Department of Microbiology, Rama Medical College and Research Centre, Mandhana Kanpur (UP) India.
[3]Professor, Department of Community Medicine, [4]Assistant Professor, Central Research Laboratory,
Rama Medical College, Hospital and Research Centre, Mandhana, Kanpur (UP) India.

Corresponding Author: Anil Kumar

ABSTRACT

Background: In the oral cancer patients because of the immune-suppression there is high risk of *Candida albicans* infection. The aim of this study was to identify presence of *C. albicans* in the oral mucosa of 150 patients in Kanpur population.
Methods: This is a prospective study from the oral and maxillofacial OPD of Rama hospital and Research Centre, Kanpur. Swabs were collected and cultured into Sabouraud dextrose agar medium. Presence of *Candida* species was confirmed microscopically as well as biochemically according to standard procedure. The presence of *SAP1* gene was confirmed by isolating RNA and preparing cDNA followed by cDNA amplification by agarose gel electrophoresis.
Results: *C. albicans* was found the most common species in oropharyngeal cancer patients (42%). The presence of *SAP1* was confirmed in the 24 isolates (8%).
Conclusions: Detection of SAP1 gene can help in the treatment and prognosis of *C. albicans* in the immune-compromised patients especially in oropharyngeal cancer patients.

Keywords: Oropharyngeal cancer, Virulence, *Candida albicans*.

INTRODUCTION

Candida species are eukaryotic opportunistic pathogens that reside on the mucosa of the gastrointestinal tract as well as the mouth, oesophagus and vagina. [1] The frequency and prevalence of *Candida albicans* infections are common chiefly in the large population of immune-compromised patients. [2] *C. albicans* belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina, [3] and are accountable for different clinical manifestations from mucocutaneous overgrowth to bloodstream infections. [4] The *C. albicans* is commensal in healthy humans and may cause systemic infection in immune-compromised situations due to their great adaptability to different host niches. There are 17 different Candida species are common to be aetiological agents of human infection however, more than 70% of persistent infections are caused by *Candida albicans.* [5] The expanding population of immune-compromised patients that use intravenous catheters, total parenteral nutrition, invasive procedures and the increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation are factors that contribute to the increase of these infections. [6] The pathogenicity of *C. albicans* species is attributed to certain

virulence factors, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin. [7]

Critically, ill or otherwise immune-compromised patients are more prone to develop both superficial and life threatening Candida infections. [8] Candida infections also constitute the most common fungal infections in AIDS patients. [9,10] These patients predominantly develop oropharyngeal candidiasis, which can lead to malnutrition and interfere with the absorption of medication. *C. albicans* is the predominant cause of invasive fungal infections [11] and represents a serious public health challenge with increasing medical and economic importance due to the high mortality rates and increased costs of care and duration of hospitalization. [12,13] Although *C. albicans* is the most prevalent species involved in invasive fungal infections, the incidence of infections due to non-albicans species is increasing. In a study with 2000 patients at major North American medical centres, a predominance of *C. albicans* was the most frequently isolated species.

Mucosal candidiasis represents a frequent clinical problem, particularly in human immunodeficiency virus-infected patients who suffer from recurrent, severe forms of oroesophageal and vaginal infections. [14,15] The mechanisms of pathogenesis of these infections have not been totally established, but the loss of host defense mechanisms is generally a prerequisite for infection to occur. [16,17] In human immunodeficiency virus-infected subjects, in addition to the immune-depression of anti candidal T-cell-mediated immunity, [18] a selection of particularly aggressive *Candida* strains has been reported. [19,20] For *C. albicans*, the most virulent and most frequently isolated species of *Candida*, the possible virulence attributes are dimorphism, adherence, enzyme secretion, phenotypic switching, antigen variation, and possession of complement-binding receptors. [21,22] However, the actual contribution of each of these factors to the pathogenesis and severity of the disease awaits elucidation.

Secreted aspartyl proteinases (SAP appear to be a virulence-associated attribute of *Candida* species). These enzymes can cleave several proteins which are important in host defenses, such as antibodies of both immunoglobulin G and A isotypes. [23] Also, SAP may promote the colonization, penetration, and invasion by *C. albicans.* [24] Since the expression of these enzymes in systemic candidiasis may be less important than when the organism colonizes mucosal surfaces [25,26] in mouth of the patients. Following this observation, we have now studied the expression of two aspartyl proteinase genes (*SAP1*) in oral cancer patients. We accomplished this objective using several strains of *C. albicans* which differ in virulence, according to previously published reports. [27-29]

MATERIALS AND METHODS

Total of 150 samples from patients were included the study from January 2016 to December 2017. Samples were collected from 75 males and 75 females patients suffering from oropharyngeal cancer in the study group. The study was approved by the Ethical Committee of Rama medical college and research centre, Rama University, Kanpur (India). Total RNA was extracted from fugal colonies using Trizol (Qiagen, Germany) following protocol according to manufacturer's guidelines. The primers for *SAP1* gene were synthesized by Chromous biotech. Pvt. Ltd. (Bangaluru). The obtained primers were dissolved in TE buffer (1mM, pH-8.0) and further diluted with addition of nuclease free water and made them 10 pm/µl. cDNA were prepared by using Fermentas cDNA synthesis kit with following manufacturer's guidelines. The total volume of synthesized cDNA was 20 µl. PCR was conducted in 20 µl reaction volume containing 10µl master mix

(Takara), 5µl nuclease free water, 1 µl forward and reveres primer each and 3µl cDNA template. Conditions for PCR was initial denaturation 94 ˚C for 5 min, and then 34 cycle at 94 ˚C for 30 sec for denaturation, 51 ˚C for 45 for annealing for *SAP1* gene then after extension was performed at 72 ˚C for 1min and final extension performed at 72 ˚C for 7 min. 1% agarose gel was used for electrophoresis with using 1X TAE buffer.

DNA fragments of target sequences of *SAP1* genes were amplified using polymerase chain reaction (PCR) on BIORAD T100 Thermal Cycler, Singapore. Primers of PCR are listed in Table 1.

Table 1, List of primers and their Tm

Forward primer	5'-CAATAATTACAATAGAAAAATGTGGC-3 Tm-51
Reverse primer	5'- CCAGTAGCATTAACAGGAGTTTTAATGACA -3' Tm-56

The amplified DNA fragment containing *SAP1* gene was obtained and electrophoreses on 1.2% agarose gel and stained with ethidium bromide. Standard strain ATCC 10261 strains of *C. albicans* was used during the study. Their sources, morphologies, and virulence characteristics were brought from vender. [30,31]

Statistics:

Sample size calculation

Sample size was calculated in order to control type I & type II error. Assuming a minimum power 80% and 95% significance level using formula:

We assume the incidence of oral cancer 0.84 in India.

We accepted the allowable error to be 10% using the formula:

$$Z_{power} = \frac{p1 - p2}{2\ S.E\ of\ difference} - Z_{\alpha/2}$$

Formula for sample size calculation

$$\text{sample size } n = \frac{2pq(Z_\beta + Z_{\alpha/2})^2}{d^2}$$

$$p\ (\text{incidence of disease}) = 0.43$$

$q = 1 - p$

$d = p1 - p2$- is the difference which we want to detect at a specified power & level of confidence. Z_β – power of statistical test we want to be minimum 80% for which is Z_β is 0.84.

$Z_{\alpha/2}$ -is the level of confidence we have chosen 95% confidence in this $Z\alpha/_2$=1.96. Solving the above equation the sample size for oral cancer comes out 308 rounding off; we can safely assume that sample size of 300.

RESULT AND DISCUSSION

C*andida albicans* were identified in 135 cases in total of 150 samples (Table-2).

Table-2, Number of samples that have fungal growth

Total number of samples estimated	No of samples having fungal growth	Type of fungus
150	135	*C. albicans*

The presence of a *SAP1* gene in *C. albicans* provides an efficient proteolytic system that may causes severe infection in oropharyngeal cancer patients. Additionally, *Sap* production is a highly regulated and controlled process, which play a central role in many processes of *C. albicans* such as its virulence and is investigative of the multiple functions. Sap production degrades host tissues by distorting host cell membranes and degradation of host surface molecules ultimately it digests cells host immune system to make resist antimicrobial attack. [32]

The results of our study are quite similar with the findings available in literature [33] in which it has mentioned that *C. albicans* is accountable for the occurrence of 40% of the oropharyngeal cancer in patients. This study results also correlate with the results of other findings, [34] who described out that the *C. albicans* is dependable for the events of 43.2% of the oropharyngeal cancer in patients. In our study it has found that 24 (8%) of *C. albicans* isolates consists of *SAP1* gene. This was the virulence factor in *C. albicans* oropharyngeal cancer patients. Results of

this study are co-related with the findings [35] in which it has mentioned that 10% of *C. albicans* isolates, had shown *SAP1* gene in the clinical isolates. Remaining 92 % clinical isolates may have some other species of *Candida* or some other pathogens. [36] In the severely immune-compromised patient, *C. albicans* may also cause deep seated systemic infections. [37]

The total RNA was isolated (Fig. 2) and prepared cDNA. cDNA was amplified and found 24 (Fig. 2 and 3) *SAP1*positive samples in the oropharyngeal cancer patients out of 150 cases. In the controls we have obtained 2 samples of positively expressed with *SAP1* gene. The amplified DNA band size was obtained around 900bp.

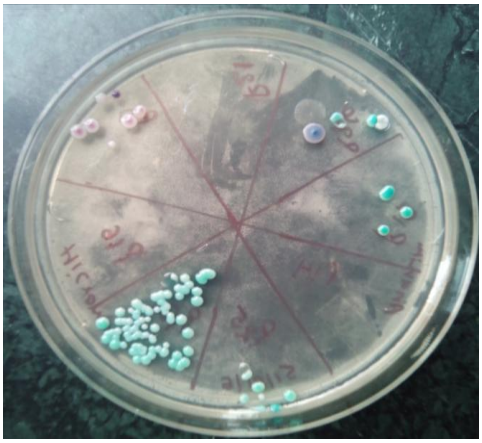

Fig. 1, *C. albicans* colonies on HiCrome agar

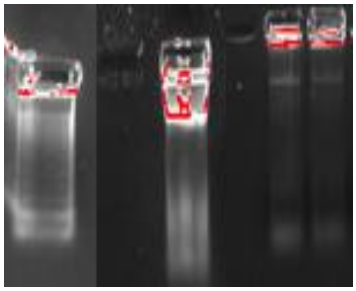

Fig. 2, Isolated total RNA from of the *C. albicans.*

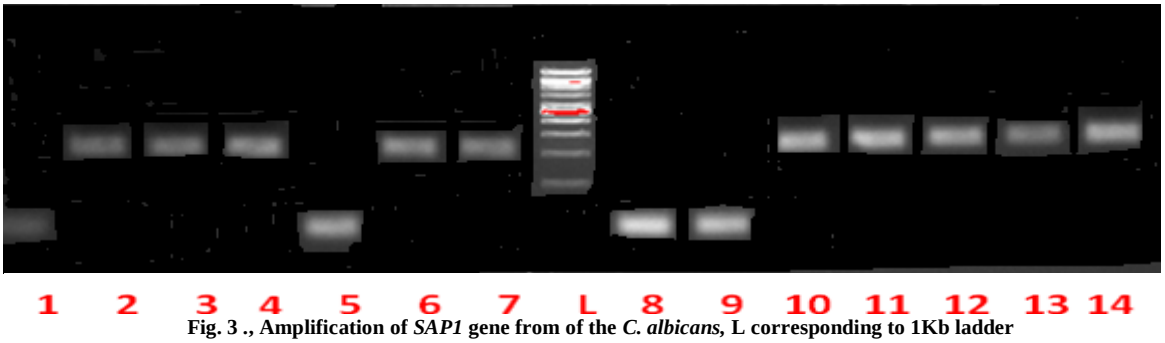

Fig. 3 ., Amplification of *SAP1* gene from of the *C. albicans,* L corresponding to 1Kb ladder

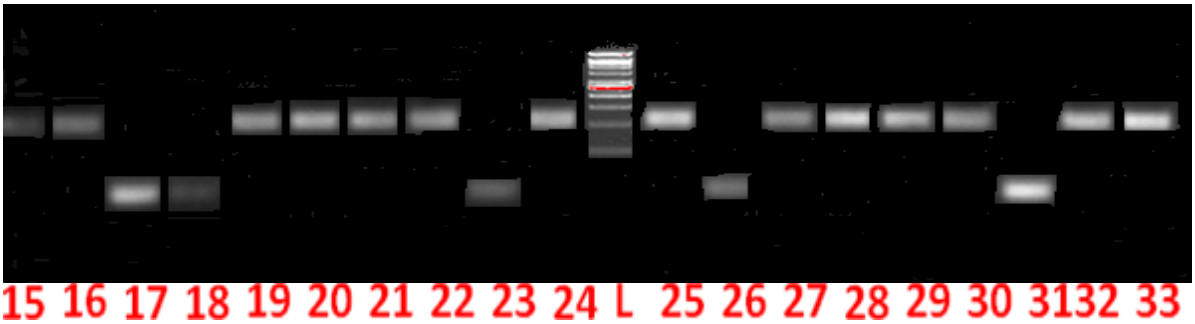

Fig. 4, Amplification of *SAP1* gene from of the *C. albicans,* L corresponding to 1Kb ladder

CAATAATTACAATAGAAAAATGTGGCTTTCAAAAATAGAAACAGCAATTTTAAACAAACGTTTTCTGTGTATGTCAATAAGT
ACAACTATAATAGCTAAATGTTCATTATACTATAATATTTCATGATGATAACAATAGAAGGTTTGGAACATTGATGAACAAA
GTACTATTTTTAGTCGTGTATTTTTGCGCGCATTTTCAAATCTTGATTCTGTTGCATTCAATGTTACATTGGAAATCTTTATCTC
CATTTTCTGTTCCATACTTCTCTTGAAGAACTTGTCGCTAAAAGATTTGATTCAAAGATATACCTTTAATTTCGATCACATATA
ACTACAGATTGTATTTAAGTAACTACTGTCCTCAAAAGATGGATTATCAAATAATGGTAGTTCCTATTTTTAGTTTTGGTTTTA
GTACTGTGGACATGATTGAAATGGAATTATACTGATAAAAAGTGGTTCTTGACACAAACTGAATGAAAGCAATATTCAATAA
TTTCACATCATAACCATTATCAACAACTATACGGCACCATGTTATTCAAAAAAACTAACCAGTTATCGCGTTATAACTGGGAG
GGGAGAATGTAAAATTAATCAAATTGTGCTATCTTTTAAAAATGGTACACCTCCTTCCCCCCCCCTATAGCTTTTGCTGGTTG
ATGCCATACTCAAATGGATAATATTCTGATGGATAATGTTATATCTTGAAAAACATATAAATATGGGAGTTGGATCTATAACT
TTATTGAAATAAATCATATTTAATCCAACAATCAATCAATTCACTCTTCCATTTCTAACAAACAATGTTTTTAAAGAATATTTT
CATTGCTCTTGCTATTGCTTTATTAGTTGATGCTTCTCCAGCTAAAAGATCCCCAGGTTTTGTCACTTTAGACTTTGATGTCATT
AAAACTCCTGTTAATGCTACTGG

Fig. 5, Obtained gene sequences of *SAP1* gene in *C. albicans*

CONCLUSION

Oropharyngeal cancer caused by *C. albicans* having *SAP1* gene is believed as virulence factors and resistant to antifungal agents and potentially deadly disease that affects patients with both intact and reduced immune systems. Early diagnosis and treatment is important for cure. Patients with oropharyngeal cancer have multisystem disturbances and require a well systematic and executed plan of treatment. The preliminary way to help progress survival rates of oral cancers is early detection and treatment. Candida defeats two main obstacles to be a successful pathogen, host mechanisms to interfere the adhesion of Candida to human tissues and the generation of hydrolytic enzymes. The first step in the initiation n 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 gene level and hyphae of *C. albicans* in individuals biopsy tissue with leukoplakia, erythroplakia suggests that this pathogen acting a role in disease formation and could aid in identifying the pathogenic commensal. This research may help us to know the pathogenicity of oral Candidiasis from cancer patients in India.

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