SAP3 Gene Expression as Diagnostic Marker of Oral Candidiasis In HIV/AIDS Patients

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Abstract

HIV/AIDS cases growth progressively. Oral Candidiasis frequently found in the tract of pathogenesis HIV infection, these condition due to immunocompromised condition among HIV/AIDS patient and one higher complications insidence in oral. Until now, type of cell and receptor of oral mucosa as particularly target of HIV has not been well understood. Secretory Aspartyl Proteinase (SAP) is hydrolytic enzyme secreted by C. albicans which might destruct protein in the site of infection. This enzyme coding by SAP gene and contribute to C. albicans virulency. The aim of this research is to found the expression of SAP3 as the predictor of HIV severity. 30 HIV patients divided into two groups, one group with ARV therapeutic indication (15 patients) and the other group without ARV therapeutic indication. The cotton swab has been done in buccal mucous and dorsum of tongue. The detection would be done by PCR.

The result is showed in non-ARV, revealed the presence of SAP3 gene in all samples, but SAP3 gene was not found in all HIV/AIDS samples with ARV. SAP3 could be used as indicator of HIV/AIDS severity, since it was an oral infecton marker related with the degree of host immunodeficiency and C. albicans virulence level.

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Introduction

HIV/ AIDS tends to increase from one year to another. One of the manifestation of these infection in oral cavity is the oral candidiasis. Oral candidiasis is frequently found anytime in the course of HIV infection. It occurs due immune compromised condition typically present in HIV/AIDS patients and it is one of the complications with highest incidence rate in oral cavity. The type of the cell and receptor in oral mucosa, the primary target of HIV virus, remains unclear.[1]

In the past, fungal infection in oral cavity was less taken into consideration. Today, it should be seriously taken into account since it has a potential to lead the patients into fatality. It is reported that the increase of fungal infection may enhance morbidity and mortality of immunocompromised (HIV) patients.[2] *C. albicans* is an oral commensal organism, a dimorphic fungi, an opportunistic pathogen that presents as normal flora in the oral cavity. In this site there are various *C. albicans* strains with certain phenotype characteristics, determining its nature as commensal or pathogenic. In its development to become pathogenic, *C. albicans* may take two changes, i.e. by overcolonization or switching form, from blastophore to hyphae. [3,4] *C. albicans* status alteration from commensal to pathogenic is closely related with local and systemic factors, which up to now remains uncompletely elaborated through experiments.[5,6,7]

Secretory Aspartyl Proteinase (SAP) is an hydrolitic enzyme secreted by *C. albicans* and coded by the genes SAP_1 to SAP_9.[7] SAP is a specific enzyme, as it is able to degrade various proteins in the site of infection. SAP gene-coded SAP enzyme also provides contribution to *C. albicans* virulence, due to its proteolytic nature. In addition, it also contributes to hyphal formation factor and phenotypic switching. Such condition is related to invasive capability of *C. albicans* strain biochemically, genetically, and immunochemically.[4,8] Virulence difference among

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various *C. albicans* strains influences the colonization and the production of SAP enzyme, and in *C. albicans* serotype A the SAP secretion was found to increase in vitro.[9]

The virulence of *C. albicans* in various strains are predominantly affected by 2 factors, the host immunity and the activity of SAP gene through its product, the proteatic enzyme, SAP_3. Due to these considerations, a study at molecular level is needed to reveal the role of SAP_3 in oral candidiasis in HIV/AIDS patients and could determine it as an indicator of HIV/AIDS severity. The objective of this study was to perform C. *albicans* sp identification in HIV patients and to analyze the expression of *C. albicans* SAP_3 gene in HIV patients with ARV and non-ARV.

Materials and methods

Samples of candida species were taken from HIV/AIDS patients in Tropical Disease Outpatient Clinic, UPIPI, Dr Soetomo Hospital, Surabaya. Sampling technique was as follows: all HIV/AIDS patients who would serve as samples were informed about the objectives and benefits of this study and their willingness to participate in this study was asked by offering them to sign the informed consent for sampling collection. The samples were taken from those who met the criteria, with a consideration that the criteria have been adequately strict, which was resulting in limited number of samples. The sample size was determined based on sampling technique according to observational cross-sectional type of study. Sampling technique was carried out by selecting subject groups of HIV/AIDS patients who visited Tropical Infection internal disease outpatient clinic, UPIPI, Dr Soetomo Hospital, Surabaya, who had been treated with ARV and non-ARV. Based on sampling estimation, the valid sample size in each group was 10 individuals. Candida species were taken by instructing the patients to rinse and scrub with sterile cement spatula in the patients' oral mucosa for subsequent candida culture in Sabouraud dextrose agar (Difco).

Several stages were taken, such as: Cytological examination with Papanicolau staining to identify the presence of *C. albicans* in HIV/AIDS, *C. albicans* identification with gram staining and sugar fermentation test, *C. albicans* DNA isolation, *C. albicans* SAP3 gene expression with PCR method and the thickness of the PCR result was measured using Densitometer. To detect the expression of SAP1 and SAP3 genes, PCR method was used in order to obtain amplification of a certain DNA segment restricted by two synthetic olygonucleotides (primers). In this PCR the primer of EFB1 gene was used as internal control for *C. albicans*:

5'-ATTGAACGAATTCTTGGCTGAC–3'
and 5'-CATCTTCTTCAACAGCAGCTTG–3'.

The final volume of 25 ul PCR reaction mixture comprising 10 X Buffer Mg Free, 25 mM MgCl2, 2,5 mM dNTP mix, 25ng DNA, 20 uM primer and 100 U taq polymerase (Promega) was mixed and PCR eppendorf was put within Master Cycler machine (Gene Amp PCR System 2499, Perkin Elmer). The PCR condition was as follows: denaturation in 94°C for 1 minute, annealing in 55°C for 1 minute and extension in 72°C for 1 minute and the final step was extra extension in 72°C for 10 minutes. Total PCR cycle was 45 cycles. In PCR used to detect the presence of SAP3 genes, the following primers were used: SAP3:

5'-TGGATTGGAACATTTCTAATTC-3'
and 5'-CAATCTCCAGAGGAGTACTTCC-3'.

PCR reaction mixture comprised 10 X buffer Mg Free, 25 mM Mg Cl2, 2.5mM dNTP mix 25 ng DNA, 20uM primer SAP1 and SAP3 and 100.U Tag Polymerase (Promega). The final volume of PCR reaction mixture was 25 µl. It was gently mixed and entered into PCR tube, and then the PCR tube was put within Master Cycler machine (Gene Amp PCR System 2499, Perkin Elmer). PCR conditions: denaturation in 94°C for 1 minute, annealing in 52°C for 1 minute and extension in 72°C for 1 minute, extra-extension was in 72°C for 10 minutes and the total cycles was 45 cycles.

Results

C. albicans identification with gram staining and sugar fermentation test

In the subsequent stage, *C. albicans* indentification was performed in order to obtain biochemical data on the characteristics of *C. albicans*. The result of biochemical data indicated that Surabaya isolate *C. albicans* had specification in the examination with gram staining and sugar fermentation reaction. Cytological examination with Papanicolau staining was performed to identify the presence

of *C. albicans* in oral mucosal epithelium.

This was necessary because *C. albicans* infection is not necessarily accompanied with clinical symptoms. If cytological test revealed sporal or hyphal formation along with inflammatory cells, *C. albicans* infection is present.

PCR method to explore the expression of SAP_1 and SAP_3

To detect the expression of SAP_1 and SAP_3 genes, PCR method was used in order to obtain amplification from a certain DNA segment restricted by two synthetic olygonucleotides (primer). Both of these SAP genes may secrete the enzymes SAP_1 and SAP_3, who have important roles during the early process of *C. albicans* infection because SAP_1 and SAP_3 have important roles in the process of attachment and colonization of *C. albicans*.

. In control group of HIV/AIDS patients with ARV and non-ARV, the gene SAP_1 was apparent in almost all samples (except in sample no. 10A) (Figure 1). The result of PCR of the gene SAP_3 in Group A no. 3, 6, 7, 8, 10, 12 and in Group B no. 4, 5, 10, 11, 12, 13, 14, 15 was not apparent (Figure 2).

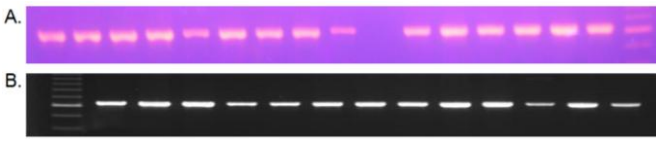

Figure 1. PCR results of genes SAP1.
Note: Lane 17 A: 100 bp DNA Marker, Lane 1 B:100 bp DNA Marker.

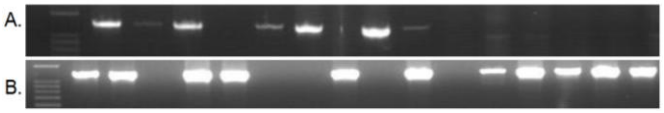

Figure 2. PCR results of gene SAP3.
Note: Lane 1 A: 100 bp DNA Marker, Lane 1 B: 100 bp DNA Marker.

Discussion

The result of examination revealed the presence of *C. albicans*, either in the form of spora or hypha, as well as the presence of inflammatory cells. Such condition indicated the presence of *C. albicans* infection in oral mucosa of HIV/AIDS patients and control group. *C. albicans* infection is not necessarily accompanied with clinical manifestations. Therefore, cytological examination is imperative to find whether there is infection or simply the presence of commensal *C. albicans*. Furthermore, the observation was focused to observe or evaluate the presence of *C. albicans* through culture media. In *C. albicans* identification, we used gram staining and sugar fermentation test to differentiate various candida species colonizing the oral mucosa.

The result of identification of *C. albicans* using gram staining and sugar fermentation test revealed 12 *C. albicans* from HIV/AIDS with ARV, 12 from HIV/AIDS non-ARV who were *C. albicans* positive and from 10 samples of control group visiting Dentistry Clinic, Airlangga University, five patients were found to have been infected with *C. albicans*. The remaining were positively infected with other candida species in distributing numbers, such as C. galbrata, C. stelatoidea, C. krusei, C. parapsilosis and C. tropicalis. Based on biochemical analysis in *C. albicans*, there was specification of *C. albicans* in examination with gram staining and sugar fermentation. Data on gram staining indicated that candida isolate was actually *C. albicans*. The result of sugar fermentation test also revealed positive result with a change in glucose, maltose, and sucrose, while the lactose provided negative result.

Oral candidiasis can be found anytime during the course of HIV infection. This is due to immunocompromized condition generally accompanying HIV/AIDS patients, one of the complications with highest rate of incidence in oral cavity. Immunodeficiency condition in host cells also results in disordered production of cytokines, such as IL-1beta and TNF-alpha, resulting in disordered phagocytotic function of Polymorphonuclear (PMN) and macrophage.[10,11] The disorder of immunocompetent cells function also reduces the tissue's defence against excessive growth of *C. albicans*, facilitating the binding of *C. albicans* to mucosal epithelium.

This study was focused on samples containing positive culture from oral mucosal scrubing. The result of the culture revealed the presence of *C. albicans* infection. This finding confirmed that of Willis[6] who suggested that 40% of *C. albicans* carrier HIV/AIDS patients do not show clinical symptoms. Erythematous symptom does not necessarily manifest, confirming a study by Phan[12] who found that *C. albicans*, either as yeast, blastophore, or hyphae, can interact with host cells. Such interaction takes place through the attachment on mucosal epithelium, and this process actually depends on protein composition

at *C. albicans* cell wall, which is functioning as adhesin molecules. Up to the moment, *C. albicans* infection in oral mucosa of HIV/AIDS patients remain a persisting problem that cannot be overcome completely in dentistry. This is because the pathogenesis underlying the infectious process is very complex, encompassing disorders in immune system and molecular interactions, which has not been completely elaborated until this day. According to Elahi[13], *C. albicans* infection in oral mucosal epithelium or HIV/AIDS patients are recurrent and persistent, and data revealed that oral candidiasis is found in about 90% of HIV/AIDS patients. Today various resistance cases in candidiasis treatment are beginning to be found. This is a clinical problem that has not been satisfactorily addressed. Although the species candida is a normal flora, and there are various candida species in different strains, but one does have opportunistic pathogenic character, is the predominant one, *C. albicans*.

Presence of SAP_1 dan SAP_3 Genes

SAP enzymes, particularly SAP_1 and SAP_3, are proteatic hydrolytic enzyme coded by the genes SAP_1 and SAP_3.[14] Both SAPs are predominantly secreted during the early process of *C. albicans* infection especially in *C. albicans* attachment and colonization, so that the presence of both genes should be identified in *C. albicans*-infected in HIV/AIDS patients with ARV, HIV/AIDS patients with non-ARV, and non-HIV/AIDS (control) group since the SAP_1 and SAP_3. In addition to its correlation with *C. albicans* colonization, the presence of SAP_3 also plays a role in phenotypic switching, the change of *C. albicans* formation from blastophore to hyphae, which is correlated with *C. albicans* virulence level. The formation change from blastophore to hyphae is a change from normal to pathogenic.

The result of data analysis on the presence of *C. albicans* SAP_1 and SAP_3 genes in HIV/AIDS patients and in control group indicates that SAP1 gene has the same predisposition of existence. This was based on the mean of SAP1 gene existence rate in control group (39104.19 ± 4156.22), HIV/AIDS with ARV, (25259.79 ± 7429.71) and HIV/AIDS non-ARV (26281.29 ± 4312.39). This shows that all samples had SAP1, assuming that *C. albicans* does exist in oral mucosal epithelium and able to attach.

The subsequent result in the analysis of *C. albicans* SAP3 gene in HIV/AIDS patients showed that the mean of SAP3 presence value in control group was 11512.86 ± 1348.26, lower than that in HIV/AIDS patients with ARV (13106.33 ± 9028.21) as well as that in HIV/AIDS with non-ARV (14793.86 ± 8851.28). HIV/AIDS patients with non-ARV had predisposition of mean SAP3 presence value higher than that in HIV/AIDS patients with ARV and control group. The presence of SAP_3 gene in non-ARV HIV/AIDS patients in this study demonstrated that, in addition to its capability in attaching to oral mucosal epithelial cells, C. albicans is also capable to colonize.

SAP enzyme is a pathogenic indicator of C. albicans has been proved in the results of various studies on experimental animals, which were based on the followings: First, mutation in SAP gene results in inhibited candidiasis distribution, indicated by the reduction of *C. albicans* virulence. Second, tissue biopsy from *C. albicans* infected rats showed that SAP was produced in vivo, as seen from the result of Indirect Fluorescent-antibody staining. Wu et al[15] presented in their study that SAP is a target for future's research in regard with the development of drug resistance and the virulence factor of C. albicans.

The result of PCR of SAP3 gene reveals that not all samples in control group and HIV/AIDS with ARV presented SAP_3. This indicated that, although *C. albicans* are able to attach to oral mucosal epithelial cells, not all of them are able to colonize. However, the result of PCR in non-ARV HIV/AIDS patients, the SAP_3 gene was apparent in all samples, indicating difference in the attachment to oral mucosal epithelial cells and in the capability to colonize. In non-ARV HIV/AIDS patients, all *C. albicans*, which are able to attach to oral mucosal epithelial cells, are also able to colonize, while those in HIV/AIDS with ARV in in control group, not all are able to colonize. The success of *C. albicans* in attachment and colonization in oral mucosa is the beginning of the infection. This is a critical stage of the incidence of C. albicans infection in oral mucosa.

PCR results in several C. albicans samples colonizin oral mucosal epithelium in control group and HIV/AIDS patients with ARV showed the presence of SAP_3. This may result from two possibilities, CD4 reduction due to HIV infection, which is reducing response capability

against immunogenic stimulation, and candida carrier in control group and HIV/AIDS with ARV patients. The reduction of immune response in immunogene recognition by T-helper (CD4) lymphocyte also results in the reduction of immunoglobulin production, a product of humoral immunity, so that the capability of the antibody, particularly the SIgA, cannot prevent the attachment of *C. albicans* to oral mucosa. This proved that there is strong correlation between humoral immunity, particularly salivary SIgA, with the presence of *C. albicans* in HIV/AIDS with ARV. HIV-resulted chang in oral epithelial mucosal cells brings about changes in CD4 T-cell in the mucosa and salivary Th1 cytokine in chronic HIV patients. This triggers the occurrence of opportunistic infection. Based on such condition, HIV/AIDS patients should be alert to various factors that are able to trigger complications, particularly the local factor in oral cavity, since the candida colonizing the oral mucosa is the pathogenic one. Although not suffering from HIV/AIDS, if the environmental factor enables, the patients in control group have the opportunity to have oral candidosis as well.

The result of this study indicated that not all Surabaya-isolate C*. albicans* had SAP_3 gene, but SAP1 gene was found in all samples of *C. albicans*. This confirmed the finding of Naglik JL et al.[16] that SAP_1 and SAP_3, to SAP7, are only produced by certain strains of *C. albicans*, while SAP_2 is produced by all strains of *C. albicans*. In non-ARV HIV/AIDS cases, the result of PCR examination revealed the presence of SAP_3 gene in all samples, but SAP_3 gene was not found in all HIV/AIDS samples with ARV or in control group, so that it can be concluded that SAP3 gene does not always emerge in every *C. albicans*. This demonstrates that SAP_3 gene has correlation with the host virulence and immunity. In HIV/AIDS patients with ARV, not all samples had SAP_3 gene. This proved that ARV therapy is able to improve the immunity of HIV/AIDS patients, reducing the susceptibility against infection in oral cavity, particularly the infection by C. albicans fungi.

In non-ARV HIV/AIDS it is not uncommon to find severe oral mucosal tissue damage due to *C. albicans* infection. This is because the activated-SAP gene is secreting SAP enzyme, which is proteolytic and able to degrade various components of natural defense in oral mucosa, such as lactoferrin, mucine and lactoperoxidase. It also has capability to degrade albumin, hemoglobin, collagen, and sIgA. Various literatures reported that these enzymes have primary role in the occurrence of *C. albicans* infection, which is in the attachment and colonization process in oral mucosa. *C. albicans* colonization process is supported by various factors, such as the immunodeficient condition of the host, the role of glycomannoprotein (which is an adhesin agent) and the presense of SAP enzyme.[17]

In the early stage of binding between C. albicans and mucosal epithelium, the SAP gene will be activated and secrete the proteatic enzymes SAP_1 and SAP_3.[5] Despite its proteatic nature, SAP_1 and SAP_3 gene products have not induced tissue damage, because it is expected that their function is only to affect or lyse proteins synthesized in the attachment process, enabling the *C. albicans* to undergo colonization.

In advanced process after colonization, the subsequent SAP enzymes, SAP_2, SAP_4 and SAP_6, will be secreted. These enzymes are also proteatic and have function to degrade Extra Cellular Matrix Protein components, such as laminin, fibronectin, integrin, and serotype IV collagen.[5,14] In the subsequent stage, supported by the reduction of tissue survival due to host immunodeficiency, *C. albicans* will develop easily, penetrating basal membrane and invading deeper into the tissue through its binding with vascular endothelium[12], resulting in the increase of systemic inflammatory process.

Conclusions

The presence of SAP1 gene were found in all HIV/AIDS samples and control groups. It was because *C. albicans* attached to the mucosa as a normal flora of the oral cavity. However, the presence of SAP_3 gene were not found in all HIV/AIDS samples with ARV and in control groups, but it were found in all samples of HIV/AIDS non-ARV patients. These indicated that not all *C. albicans* attached to oral mucosa were able to colonize. This showed that high prevalences of oral candidiasis in HIV/AIDS patients, the presence of *C. albicans* SAP_3 gene can be used as an indicator of HIV/AIDS severity, since it was an oral infecton marker related with the degree of host immunodeficiency and *C. albicans* virulence level.

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Declaration of Interest

The author reports of conflict of interest..

References

1. Greenspan D and Greenspan J S. Oral manifestation of HIV Infection. http://www.hivdent.org/oralm/, last updated October 12,2005; accessed December. 2, 2006.
2. Nasronudin.HIV dan AIDS. Pendekatan Biologi Molekuler, Klinis dan Sosial. 1st edition, Surabaya. Airlangga University Press. 2007: 203-213.
3. Gale C A, Bendel C M, McClellan M and Hauser M. Linkage of Adhesion, Filamentous Growth and Virulence in Candida Albicans to a single gene 1NT1. Science. 1998;279:1355-8.
4. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S and Nichterlein T, 2002. Candida albicans Hyphal Formation and the Expression of the Efg1-Regulated Proteinases SAP4 to SAP6 are Required for the Invasion opf Parenchymal Organs. Infection and Immunity. 2002; 70 : 3689-3700.
5. Staib P, Kretscmar M, Nichterlein T; Hof H and Morschhauser J, 2000. Differential Activation of a Candida albicans Virulence Gene Family During Infection. PNAS. 2000 ; 97: 6102-7.
6. Willis A M, Coulter W A, Fulton C R, Hayes J R and Bell P M, 2001. The Influence of Antifungal Drugs on Virulence Properties of Candida Albicans in patients with Diabetes Mellitus. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001; 91:317-321.
7. Schaller M, Januschke E, Caroline S, Woerle B and Korting H C. Different Isoforms of Secreted Aspartyl Proteinases (SAP) are Expressed by Candida albicans during Oral and Cutaneous Candidosis in vivo. J Med Microbiol. 2001;50:743-7.
8. Koelsch G, Tang J,Loy J A, Monod M, Jackson K and Founding S I.Enzymic Characteristics of Secreted Aspartic Proteinases of Candida albicans. Biochimica et Biophysica Acta. 2000; 1480 : 117-131.
9. Santos Andre L S, Carvalho I M, Da Silva B A, Portela M Band Soares R M. Secretion of Serin peptidase by Clinical strain of Candida albicans : influence of Growth Conditions and cleavage of Human Serum proteins and Extracellular matrix Components. FEMS Immunol Med Microbiol. 2006;46:09-220.
10. Jean-Se´bastien Ripeau, Marie Fiorillo, Francine Aumont, Pierre Belhumeur, Louis de Repentigny. Evidence for Differential Expression of Candida albicans Virulence Genes during Oral Infection in Intact and Human Immunodeficiency Virus Type 1–Transgenic Mice. The Journal of Infectious Diseases. 2002; 185: 1094–102.
11. Herring A C and Huffnagle G B. Innate Immunity and Fungal Infections, In Immunology of Infectious Diseases**.** Washington : ASM Press American Society for Microbiology. 2002:127 – 134.
12. Phan Q T, Belanger P H and Filler S G. Role of Hyphal Formation in Interactions of Candida albicans with Endothelial cells. Infection and Immunity. 2000**;** 68:3485-3490.
13. Elahi S, Clancy R and Pang G. A Therapeutic Vaccine for Mucosal Candidiasis. Vaccine. 2001; 19(17-19) : 2516-2521.
14. Pichova I, Pavlickova L, Dostal J and Dolejsi E. Secreted Aspartic Proteases of Candida albicans, Candida tropicalis, Candida parapsilosis and Candida lusitaniae. Inhibition with peptidomimetic inhibitors. Eur J Biochem. 2001;268:2669-2677.
15. Wu T, Wright K, Hurst S and Morrison C. Enhanced Extracellular Production of Aspartyl Proteinase, a Virulence Factor by Candida albicans Isolate following Growth in Subinhibitory Concentrations of Fluconazole. Antimicrobial Agents and Chemotherapy. 2000; 44: 1200-8.
16. Julian R. Naglik, Stephen J. Challacombe, Bernhard Hube. Candida albicans Secreted Aspartyl Proteinases in Virulence and Pathogenesis. Microbiology and Molecular Biology. Reviews. 2003; Vol. 67, No. 3: 400–428.
17. Santos Andre L S and Soares R M. Candida Goilliermondii Isolated from HIV-Infected Human Secretes a 50 kDa Serine Proteinase that Cleaves a Broad Spectrum of Proteinaceous Substrates. FEMS Immunol Med Microbiol. 2005; 43: 13-20.