行政院國家科學委員會補助專題研究計畫成果報告

以分子基因方法探討*ALSI*基因在白色念珠菌吸附到血管內皮細胞所扮演的角色

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計畫主持人：陳宜君
共同主持人：陸坤泰
李芳仁
張上淳

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中文摘要

爲了探討念珠菌的黏附因子，之前 Fu et al(1998)將白色念珠菌(*Candida albicans*)的 genomic library 在酵母菌表現，以功能篩選找到 *ALS1* (agglutinin-like sequence1)基因。爲了探討這個可能是黏附基因在念珠菌致病機轉中所扮演的角色，本研究主要是由分子生物學方法來探討，利用 target mutagenesis 將白色念珠菌的 2 alleles of *ALS1* 基因破壞，基於其他基因背景相同下，比較母株與變異株(homozygous mutants of *ALS1* gene)對人類內皮細胞，上皮細胞以及細胞外 matrix 黏附能力的差別。反覆實驗顯示 *ALS1* gene knock-out 變異株對人類內皮細胞的黏附能力降低，且有 gene dose effect。此結果相當令人振奮，後續研究將會在適當的動物模式中探討母株及變異株所造成死亡率的差別。此外，將 *ALS1* 基因可調控地表現在白色念珠菌以及酵母菌，一方面探討其 phenotype, 一方面可製備 Als 蛋白，進一步製備抗血清。利用 Als1 蛋白及抗血清，可在 in vitro model 探討念珠菌 Als1 蛋白或抗血清對內皮細胞是否有阻斷作用，或者對內皮細胞有保護作用。並進一步在適當的動物模式中測試其預防或治療的角色。若上述研究成果是肯定的，最終目標希望發展疫苗來保護高危險群宿主，以減少相關的死亡率，以及減緩抗藥性的發生及散播。

Summary

The opportunistic pathogen *Candida albicans* cause disseminated infections in susceptible hosts and attribute a high mortality and morbidity. The adherence of *C. albicans* to the vascular endothelium is likely a pivotal step in the initiation of a hematogenously spread and establishment of metastatic foci. Among three *C. albicans* adherence genes identified, expression of *the C. albicans ALS1* gene (for agglutinin-like sequence 1) in *S. cerevisiae* uniquely induces adherence to endothelial cells as well as epithelial cells. In this study, we constructed *C. albicans als1* null mutants using targeted mutagenesis. Our finding that disruption of *ALS1* gene result in decreased adherence to endothelial cells confirm previous hypothesis. Besides, comparing adherence of *als1* heterozygous and homozygous mutants to human endothelial cells, there was a dose effect. This limited evidence also support the hypothesis that *ALS1* gene function is dominant, or at least important, as compared to other members of *ALS* gene family in the interaction of *C. albicans* and host cells. This finding is very encouraging because it is difficult to handle the experiments and interpretate the results when dealing with one member of a gene family. The result is essential to elucidate the function of *ALS1* gene and its contribution to *C. albicans* pathogenesis

Keywords: *C. albicans*, adherence, *ALS1* gene, endothelial cells, extra-cellular matrix

Introduction

The opportunistic pathogen *Candida albicans* cause disseminated infections in susceptible hosts and attribute a high mortality and morbidity. The adherence of *C. albicans* to the vascular endothelium is likely a pivotal step in the initiation of a hematogenously spread and establishment of metastatic foci. In recent years the advance of genetic and molecular biology approaches have facilitated the investigation of adherence gene in the *C. albicans* pathogenesis. Among three *C. albicans* adherence genes identified, expression of *the C. albicans ALS1* gene

(for agglutinin-like sequence 1) in *S. cerevisiae* uniquely induces adherence to endothelial cells as well as epithelial cells. Besides, the primary structure of *ALS1* protein suggested a cell surface localization, which will be confirmed by indirect immunofluorescence with an anti-Als antiserum. All these features provide a rational target for antifungal design or vaccine strategy, which prevent the progression of *Candida* colonization or super-infection into deep tissue invasion and/or haematogenous dissemination, but not interfere the ecology of microbes on mucocutaneous surfaces. So far, at least 5 members of *ALS* gene family were isolated. Among them, *ALS1, ALS2, ALS3, ALS4* were isolated from a fosmid library by PCR screening, *ALS1* gene was also identified independently in our previous study by functional screening. It assumed that *ALS1* gene function is dominant, or at least important, as compared to other members of *ALS* gene family. The goal of the present study was to construct a null mutant for *ALS1* gene using targeted mutagenesis, and to evaluate its phenotype. The result is essential to elucidate the function of *ALS1* gene and its contribution to *C. albicans* pathogenesis.

Materials and Methods

Strains. The Ura$^-$ strain CAI4 of *C. albicans* was used for disruption of *ALS1* gene. The growth rates and adherence were compared with those of the parental Ura$^+$ clinical isolate SC5314 as well as Ura$^+$ revertant CAI12. Other strains and their genotypes are described in table1. *Escherichia coli* XL1-blue (Promega) was used for propagation of plasmids constructed in this study.

Media and growth conditions. *Candida* strains were routinely maintained and grown in YPD medium consisting of 1% yeast extract (Difco), 2% peptone (Difco), and 2% glucose, unless otherwise mentioned. *Candida* transformants generated during the Ura-blaster protocol were grown on synthetic dextrose (SD) agar plates, which contained 0.17% with yeast nitrogen base without amino acids (Difco) and 2% of glucose. Media were supplemented with uridine (25ug/ml) as required. All cultures were incubated at 30°C.

Ura$^-$ auxotrophy was selected on SD agar plate containing 5-fluoroorotic acid (5-FOA) (1mg/ml) and uridine (25 ug/ml) (Boeke, 1984; Fonzi, 1993). Prior to selection, strains were plated on YPD medium supplemented with uridine and incubated 48 hr at 30°C. Individual colonies were restreaked onto 5FOA plate and incubate at 30°C for 2 to 4 days.

PCR. Full length (4.9 kb) of *ALS1* gene was amplified from plasmid pYF-5 (Fu, 1997) using primers 5'-CCG CTC GAG ATG CTT CAA CAA TTT ACA TTG TTA-3' (sense), 5'-CCG CTC GAG TCA CTA AAT GAA CAA GGA CAA TA-3' (antisense), which introduce *XhoI* site at each ends. A 1.3 kb C-terminal fragment of *ALS1* gene was amplified from pYF-5 using primers 5'-CTA CAT CCT CCA ATG ATA TAA C-3' (sense) and 5'-ATA ACA GGA ACA AGT AAA TCA CT-3' (antisense). This fragment was considered to be specific for *ALS1* gene among *ALS* gene family (Hoyer, 1995; Hoyer, 1998a). A 1.3 kb fragment of *URA3* gene was generated with pMB-7 was template using primers 5'-AGC TCT AGA AGG ACC AC-3' (sense) and 5'- GCT CTA GAT AAG CTA CTA ATA GGA AT-3' (antisense).

Plasmid Construction: Plasmid pMB-7 was kindly provided by Fonzi (Fonzi and Irwin, 1993), which consists *C. albicans URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* sequences and contains I-*Sce*I recognition sequence within each of the *hisG* repeats . Two different *ALS1*-deletion cassettes were constructed with different lengths of residual *ALS1* gene flanking *hisG-CaURA3-hisG* cassette. The *ALS1* PCR product was cloned into pGEM-T vector

(Promega) and 4.5 kb *KpnI-HindIII* internal fragment of coding region of *ALS1* was replaced by 4.1 kb *KpnI-HindIII* fragment containing *hisG-CaURA3-hisG* cassette isolated from pMB-7 (Fonzi and Irwin, 1993) to generate pYC1. To construct second cassette, *HindIII-BglII* fragment containing *hisG-CaURA3-hisG* cassette was isolated from pMB-7 and cloned into pUC19. The resulting plasmid was digested with *HindIII* and *SmaI* to release *hisG-CaURA3-hisG*, which was in turn used to replace *HpaI-XbaI* internal fragment of coding region of *ALS1* gene to generate pYC3.

***Candida* transformation and gene disruption.** Transformation of *C. albicans* with linear DNA fragments containing the gene disruption constructs was performed by a high efficiency lithium acetate transformation methods described by Gietz et al. (1995). Disruption of the *ALS1* gene followed the conventional "Ura-blaster" protocol described by Fonzi et al. (1993), and the *ura3* auxotroph was regenerated by selection for 5-FOA-insensitive segregants (Boeke, 1984; Fonzi, 1993).

Strain Construction: Heterozygous mutant of *ALS1* gene (CAYC1) was generated by transformation of CAI4 (Fonzi, 1993) using 4.5 kb *Xho*I fragment of *ALS1*-deletion cassette released from pYC1. The ura3 auxotroph (CAYC2) was selected on 5FOA agar plate as a Ura- spontaneous mitotic recombinant of strain CAYC1. Homozygous mutant (CAYC3) was generated by transforming CAYC2 using 6.0 kb *XhoI* fragment of *ALS1*-deletion cassette released from pYC3. The deletion of *ALS1* gene and integration of the *hisG-URA3-hisG* fragment at the *ALS1* locus of CAYC1 and CAYC3 was verified by Southern blot analysis. The 5FOA-resistant colonies (CAYC2, CAYC4) were confirmed by Southern blot analysis that the *URA3* fragment has been popped-out.

Southern blotting and DNA probe hybridization. *Candida* genomic DNA was isolated according the method described by Chen and Fonzi (1992). Genomic DNA digested by *EcoRI* and *HindIII* was size fractionated by agarose gel electrophoresis and capillary transfer onto inherently charged nylon membrane (MAGNACHARGE membrane; MSI). Prehybridization and hybridization of the membrane with labeled DNA probe were performed at 42°C in a solution containing 50% formamide, 5xSSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate, and 100 ug of denatured salmon sperm DNA per ml. A 1 kb C-terminal fragment of *ALS1* gene generated by PCR amplification using primers G611-3 and G611-2, and a 1.3 kb *Xba*I fragment of *URA3* gene were used as hybridization probes in this study. DNA fragments were labeled with [α-^{32}P] dCTP (Amersham Pharmacia Biotech, Inc. N.J.) by means of a random-primed DNA labeling kit (New England BioLab). The membrane was washed in high stringency and moderate stringency conditions, respectively and exposed at –70°C on a Kodak X-ray film with an intensifying screen.

Determination of growth rates. Freshly streaked colonies of *C. albicans* CAI12, the heterozygous and homozygous mutants of *ALS1* gene were grown in YPD at 30°C for 24h on a rotary shaker (200 rpm). The cells were washed in PBS and counted with a hemacytometer. They were adjusted to a cell density of 10^4/ml and used to inoculate 100 ml YPD to a final optical density of 0.015 to 0.020 at 600 nm. The optical density was measured every hour until the stationary phase of the growth curve was reached. The doubling time during the log phase was determined by the formula: $In_2xt/(In_b - In_a)$ (t-time period in hours; a = optical density at the beginning of time period; b = optical density at the end of time period).

Germ tube formation. The germination of CAI12 and mutants was determined as descreibed previously. Briefly, cell suspension in the density of $5x10^6$/ml was incubated at 37°C in 24-well tissue culture plates containing prewarmed 50% fetal bovine serum. At selected intervals, samples were fixed with equal volume of 2% glutaraldehyde (vol/vol in distilled water). The number of yeast cells with germ tubes, at least one blastospore diameter in length was counted, and the lengths of germ tubes were measured with a micrometer under inverted microscope. In these experiments, 100 organisms per low-power field were examined. Each strain was tested in triplicate.

Preparation of endothelial cells. Endothelial cells were obtained from human umbilical cord veins by our prior modification of the methods of Jaffe et al (1973). Briefly, the cells were grown in M-199 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Intergen, Purchase, NY), 10% defined bovine calf serum (Hycloine, Logan, Utah), 2 mM L-glutamine, and penicillin and streptomycin as previously described (Ghannoum, 1992). For use in the adherence assay (see below), second-passage cells were grown to confluence in specially treated six-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) coated with 0.2% gelatin.

Endothelial cell adherence assay. The adherence of *C. albicans* to endothelial cells was determined by methods described previously (Ghannoum, 1992) with modification. Briefly, stationary-phase *C. albicans* were harvested by centrifugation, washed twice in PBS, and resuspended in PBS-CM (Irvine Scientific, Santa Ana, Calif.). After the organisms were sonicated for 10 s, the singlet blastospores were counted with a hemacytometer and adjusted to the desired concentration in PBS-CM. Next, 350 cell suspended in PBS-CM were added to confluent endothelial cells in sex-well tissue culture plates. The inocula were confirmed by quantitative culture on YPD agar. Following incubation for 30 min at 37°C in the presence of 5% CO_2, the non-adherent organisms were aspirated and washed twice with 10 ml of PBS-CM. The intact of confluent endothelial cell monolayer was checked under inverted microscope immediately. Then, the wells were overlaid with YPD agar, and the number of adherent organisms was quantified by colony counting. Adherence was expressed as a percentage of the original inoculum. All assays were performed in triplicate and were repeated at least three times with endothelial cells from different umbilical cords.

Adherence of heterozygous and homozygous mutants to extra-cellular matrix. The procedure was as above except incubation of *Candida* in 0.2% gelatin-coated plates without endothelial monolayer.

Results

Disruption of *ALS1* gene. Evidence for the function of the your-interest-gene in vivo can be resolved by the disruption of target gene and comparison of the resulting mutants with the parental strain. Consequently, we disrupted the *ALS1* gene in the Ura$^-$ strain CAI4, using the Ura-blaster protocol. The strategy of the Ura-blaster protocol is as followed: the majority of the open reading frame of the cloned target gene is deleted and replaced by the *hisG-URA3-hisG* cassette (Fig. 1,2). The resulting *ALS1*-deleted-*hisG-URA3-hisG* cassettes was released from the plasmid and used to disrupt the wild allele of Ura3$^-$ strains (either CAI4, or ura auxotroph of *ALS1* heterozygous mutants) by integrative transformation (Table 2).

Ura$^+$ transformants and 5-FOA-resistnat segregants of these transformants were analyzed by PCR and Southern analysis. For PCR screening, primers which amplified the full-length of the ORF were used (Table 1). For Southern analysis, genomic DNA was digested with *EcoRI* and *HindIII*, and hybridized with C-terminal fragment of *ALS1* gene, which is specific to *ALS1* gene among *ALS* gene family (Fig. 3). For all phenotype study, Ura$^+$ strains of heterozygous and homozygous mutants were used.

Growth rates in YPD medium. Various growth rates of heterozygous mutants in YPD medium incubated at 30°C were observed. Two strains of *ALS1* heterozygous mutants were selected for further genetic manipulation (reasons seen discussion). One strain had disrupted larger allele of *ALS1* gene, another strain had disrupted smaller allele. Heterogenicity of growth rate was also observed among different strains of *ALS1* homozygous mutants.

Germ tube formation. Several strains of *ALS1* heterozygous mutants had impaired germ tube formation either induced by RPMI or serum for 2-3 hr. Others had comparable germ tube formation. For *ALS1* null mutants, germ tube formation was delayed initially, but nearly approach to that of CAI12 after 4 hr of incubation with RPMI or serum.

Adherence of heterozygous and homozygous mutants to extracellular matrix and human umbilical endothelial cells (Fig. 4-6)

Discussion

Disruption of *ALS1* gene resulting lower adherence to endothelial cells. The predicted *ALS1* protein has several motifs which are characteristic of a protein expressed on the cell surface. In addition, the N termini of both the *ALS1* and AGα1 gene products have homology with the immunoglobulin superfamily. This superfamily contains adhesins such as intercellular adhesion molecules 1, 2, and 3, which mediate the cell-to-cell attachment of mammalian cells. This similarity to *S. cerevisiae* and mammalian adhesins is consistent with the hypothesis that *ALS1* encodes a *Candida*l adhesin. Our finding that disruption of *ALS1* gene resulted in decreased adherence to endothelial cells confirm this hypothesis. Besides, comparing adherence of *ALS1* heterozygous and homozygous mutants to human endothelial cells, there was a dose effect. This limited evidence also confirm the hypothesis that *ALS1* gene function is dominant, or at least important, as compared to other members of *ALS* gene family. This finding is very encouraging because it is difficult to handle the experiments and interpretate the results when dealing with one member of a gene family.

The significance of the tandem repeats. Recently, Gaur and Klotz described another member of the *ALS* gene family, *ALA*1. This gene was identified by its ability to cause *S. cerevisiae* to bind to extracellular matrix proteins. *S. cerevisiae* expressing *ALA1 (ALS5)* exhibited enhanced adherence to human buccal epithelial cells. The presence of tandem repeats in *ALA1* and *ALS1* but not in AGα1 suggest that they may have a unique function in *C. albicans*. Although the *ALS1* and *ALA1* tandem repeats are similar, they are no identical. Both contain consensus N glycosylation sites (N-X-S/T) which may be modified by N-glycosylation and several threonine residues which may be modified by O-glycosylation. If the threonine-rich region and the tandem repeats play a role in *C. albicans* adherence, the initial recognition of the target may occur through sugar moieties. In this regard it is significant to note that many pathogens utilize protein repeats for adherence to ECM proteins or cell surfaces. As two alleles of *ALS1* gene of *C. albicans* SC5314 have different length due to different copy numbers of tandem repeats, we

were interested to find out the impact of different length of tandem repeat on the adherence of *C. albicans* to endothelial cells. However, comparing several colonies of *ALS1* heterozygous mutants, there were no significant difference between disruption of different alleles of *ALS1* gene.

Heterogenous phenotypes of *ALS1* heterozygous mutants. Before processing further to select ura3 auxotroph of *ALS1* heterozygous mutants, and then construct *ALS1* homozygous mutants, we evaluate the phenotypes of all *ALS1* heterozygous mutants, including growth rate, germ tube formation and adherence to endothelial cells and extra-cellular matrix. We found that there were difference in the growth rate as well as the adherence to extra-cellular matrix, and lesser extent, difference in the germ tube formation and adherence to endothelial cells. Because growth rate and germ tube formation have great impact for *C. albicans* to establish infection in host, it is anticipated that using mutant strains with slow growth rate and impaired germ tube formation will result in lower mortality rate and longer survival time in study animals. It will confound the investigation of the effect of your-interest-gene on the pathogenesis of candidiasis. Similar phenomenon has been found in previous study regarding disruption of *C. albicans CAD1* gene (Gunter, submitted). Therefore, we select *ALS1* heterozygous mutants with growth rate comparable to *C. albicans* SC5314 (parent strain) and CAI12 (URA3 revertant) to avoid the undesirable mutation during transformation. It is conceded that there is an unlikely but definite possibility that targeted gene disruptions generated by the Ura-blaster protocol could generate unforeseen changes due to mitotic recombination and gene conversion, etc., and strain isogenicity may not always be sustained. In order to control whether the Ura-blaster procedure and lithium acetate transformation caused unforeseen alterations in the genetic background of the host strain, there is a tendency to re-introduce a copy of wild type allele to the null mutants to evaluate whether it can rescue the gene effect or not.

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References

Bradford MM. A rapid and sensitive assay for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

Benting J, Mattei D, Lingelbach K (1994) Brefeldin A inhibits transport of the glycophorin-binding protein from *Plasmodium falciparum* into the host erythrocyte. Biochem J 300, 821-6

Donaldson JG, Finazzi D, Klausner RD (1992) Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. Nature 360, 350-2.

Fu Y, Rieg G, Fonzi WA, Belanger PH, Edwards JE Jr, Filler SG. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. Infect Immun 1998;66:1783-6.

Fu Y, Filler SG, Spellberg BF, Fonzi W, Ibrahim AS, Kanbee T, Ghannoum MA, Edwards JE, Jr. Cloning and characterization of *CAD1/AAF*1, a gene from *Candida albicans* that induces adherence to endothelial cells after expression in *Saccharomyces cerevisiae*. Infect Immun 1998;66:2078-84.

Gale C, Finkel D, Tao N, Meinke M, McClellan M, Olson J, Kendrick K, Hostetter M. Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*. Proc Ntl Aca Sci USA 1996;93:357-61.

Gaur ND, Klotz SA. Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. Infect Immun 1997;65;5289-94.

Ghannoum MA, Filler SG, Ibrahim AS, Fu Y, Edwards JE, Jr. Modulations of interaction of *Candida albicans* and endothelial cells by fluconazole and amphotericin B. Antimicrob Agent Chemother 1992;36:2239-44.

Helms JB, Rtothman JE (1992) Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature 360, 352-4.

Hube B, Sanglard D, Odds FC, Hess D, Monod M, Schafer W, Brown AJP, Gow NAR (1997) Disruption of each of the secreted aspartyl proteinase genes *SAP1, SAP2,* and *SAP3* of *Candida albicans* attenuates virulence. Infect Immun 65, 3529-38.

Jaffe EA, Nachman RL, Becker CG, Ninick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745-56

Klausner RD, et al. (1992) J Cell Biol 116, 1071

Lay J, Henry K, Clifford J, Koltin Y, Bulawa CE, Becker JE. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. Infect Immun 1998;66:5301-6.

Myers KK, Sypherd PS, Fonzi WA. Use of *URA3* as a reported of gene expression in *C. albicans*. Curr Genet 1995;27:243-8.

Pelham HRB (1991) Cell 67, 449.

Randazzo PA, Yang YC, Rulka C, Kahn RA (1993) Activation of ADP-ribosylation factor by Golgi membranes: evidence for a brefeldin A and protease sensitive activating factor on Golgi membranes. J Biol Chem 268, 9555-63.

Rothman JE, Orci L (1992) Nature 355, 409

Sanglard D, Hube B, Monod M, Odds FC, Gow NAR (1997) A triple deletion of the secreted aspartyl proteinase genes *SAP4, SAP5,* and *SAP6* of *Candida albicans* causes attenuated virulence. Infect Immun 65,3539-46.

Tamura G, et al. (1968) J Antibiotics 21, 160.

Yoshimoto A, Umezu K, Kobayashi K, Tomita K. Orotidylate decarboxylase (yeast). Methods Enzymol. 1978;54:74-9

Table 1. Lists of strains used in this study

Strain	Genotype	Source/reference
SC5314	*URA ALS1/URA ALS1*	Gillum, 1984
CAF2-1	*ALS1Δura3::imm434/ALS1URA3*	Fonzi, 1993
CAI4	*Δura ALS1/ Δura ALS1*	Fonzi, 1993
CAI12	*ALS1Δura3::imm434/ALS1Δura3::imm434 + URA3*	Becker, 1995
CAYC1	*Δals1::hisG-URA3-hisG/ALS1*	This study
CAYC2	*Δals1::hisG/ALS1*	This study
CAYC3	*Δals1::hisG/Δals1::hisG-URA3-hisG*	This study