fortune Journals

Research Article

ARCHIVES OF
CLINICAL AND
BIOMEDICAL RESEARCH

ISSN: 2572-5017

RAS1 is Involved in the Antifungal Resistance of *Candida Albicans* and the Inhibition of Farnesol to the Resistance of Biofilms

Lulu Zuo[1,2,3], Zhenzhen Zhang[1,2,3], Wei Duan[1,2,3], Yun Huang[1,2,3], Zheng Xu[1,2,3], Qinqin Zhang[1,2,3], Ying Lv[1,2,3], Xin Wei[1,2,3*]

Abstract

Background: Farnesol enhances the susceptibility of *Candida albicans* biofilms to antifungals; however, its molecular mechanisms are poorly understood. *RAS1* is required for the hyphal growth. This study hypothesized that *RAS1* is involved in the antifungal resistance of *C.albicans* and the inhibition of farnesol against biofilms.

Methods: The antifungal resistances of *C.albicans* biofilms formed by *RAS1* mutant and control strains were examined using spot assays and XTT reduction assays with and without farnesol, and morphological changes were observed using CLSM and SEM. Meanwhile, *RAS1* gene and RAS1 protein in biofilms exposed to farnesol were analyzed using RT-*q*PCR and Western blot.

Results: Compare with control strain, *RAS1* overexpressing strain (*RAS1*OE) was more resistant to some of the antifungal drugs, while *RAS1* deleted strain (*RAS1*Δ/Δ) was less resistant to all antifungal drugs. *RAS1* was down-regulated in the *C.albicans* biofilms exposed to farnesol. The increased resistance of *RAS1*OE to antifungals was significantly decreased by farnesol, and farnesol decreased the resistance of *RAS1*OE more obviously than wild-type and *RAS1*Δ/Δ strains ($P<0.05$). Further, farnesol reduced the expression of *RAS1* in *RAS1*OE biofilm more obviously than that in wild-type strain biofilm. In addition, morphological observation showed that *RAS1*OE increased the hyphal growth of biofilm, while *RAS1*Δ/Δ reduced that, and the inhibition of hyphal growth by farnesol was more obviously in *RAS1*OE biofilm, while that was less obvious in *RAS1*Δ/Δ biofilm.

Conclusions: *RAS1* is involved in the antifungal resistance of *C.albicans* and the inhibition of farnesol to the antifungal resistance of biofilms.

Keywords: Antifungal; *C.albicans* Biofilms; Resistance; Farnesol; *RAS1*

List of Abbreviations: ANOVA- One-Way Analysis of Variance; CLSM- Confocal Laser Scanning Microscope; *RAS1*OE- *RAS1* Overexpressing Strain; SDA- Sabourd's Dextrose Agar; SEM- Scanning Electron Microscopy; YPD- Yeast Peptone Dextrose.

Introduction

The fungus *Candida albicans* (*C. albicans*), an opportunistic human pathogen, is commonly found in the biofilms of oral cavity and gastrointestinal tract. The biofilms of *C.albicans* are significantly less susceptible to antimicrobial agents than that of planktonic cells, which seriously weakens the effectiveness of antifungals [1]. Indeed, 65~80% of *C.albicans* infections were associated with biofilms [2, 3]. The extensive use of antifungals has

Affiliation:

[1]Jiangsu Province key Laboratory of Oral Diseases, College of Stomatology, Nanjing Medical University, China.

[2]Department of Operative Dentistry and Endodontics, Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing, China.

[3]Jiangsu Province Engineering Research Center of Stomatological Translational Medicine, Nanjing, China.

***Corresponding author:**
Xin Wei, Department of Operative Dentistry and Endodontics, Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing, China

Citation: Lulu Zuo, Zhenzhen Zhang, Wei Duan, Yun Huang, Zheng Xu, Qinqin Zhang, Ying Lv, Xin Wei. *RAS1* is Involved in the Antifungal Resistance of *Candida Albicans* and the Inhibition of Farnesol to the Resistance of Biofilms. Archives of Clinical and Biomedical Research 6 (2022): 694-706.

Received: August 08, 2022
Accepted: August 18, 2022
Published: September 16, 2022

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

led to the increased resistance of biofilms to the few existing antifungals. Thus, it is crucial to explore the mechanisms of therapeutic or preventive strategies targeting biofilm-related infections.

Previous studies have demonstrated that farnesol prevented the germination of yeast cells into mycelia, repressing hyphal growth and biofilm formation [4, 5]. Further studies showed that farnesol enhanced the microbial susceptibility to antibiotics of *Staphylococcus aureus [6], Paracoccidioides brasiliensis [7], Streptococcus mutans [8]*, and *Fusarium graminearum* [9]. Another in vitro study showed that farnesol inhibited the development of *C.albicans* biofilms formed from the resistant strains, and farnesol in combination with fluconazole, itraconazole, and 5-flurocytosine had synergistic effects against *C.albicans* biofilms [10]. Farnesol possibly enhance the susceptibility of planktonic *C. dubliniensis* and *C. albicans* to fluconazole [6] via regulation of the ergosterol biosynthesis pathway [11]. However, the exact mechanism underlying the farnesol-mediated increase in susceptibility to various antifungals is poorly understood in *C. albicans* biofilms.

C. albicans RAS proteins are small GTPases that can trigger the MAPK and cAMP signaling pathway, and involve in the morphogenesis [4, 12], stress resistance [13,14], and maintenance of hyphal state [15]. RAS1 is a highly conserved signaling protein that plays central roles in key physiological processes for both commensal and pathogenic lifestyles within the host [16], in this process *RAS1* gene might regulate a diverse array of phenotypes that are critical to the antifungal resistance of *C. albicans.* However, the role of *RAS1* in the resistance of *C. albicans* biofilms was not discussed in the previous studies, and the molecular mechanism of the *RAS1* in regulating the farnesol-relevant antifungal capacity to *C. albicans* biofilms is still unknown. In the present study, we hypothesized that *RAS1* is associated to the antifungal resistance of *C. albicans* biofilms, and also regulated the inhibition of farnesol on the antifungal resistance of *C. albicans* biofilms. To test this hypothesis, the antifungal resistance of *C. albicans* biofilms was examined in the *RAS1* mutant strains (including *RAS1* overexpressing strain (*RAS1*OE) and deletion strain (*RAS1*Δ/Δ)) with the presence or absence of farnesol.

Materials and Methods

Strains, Plasmids and Media

The wild-type clinical isolate *C. albicans SC5314* (American Type Culture Collection, MD, America) [10] and the resistant strain were used to analyze the effects of farnesol on the *RAS1* expression in *C. albicans*. The resistant strain derived from *SC5314* induced via the serial fluconazole concentration gradient method until the MIC reached or exceeded 64 μg/ml. *RAS1* overexpressing strain (*RAS1*OE) was generated from *CAI4* (William A. Fonzi, Department of Microbiology and Immunology, Georgetown University, Washington, USA) using *RAS1*-pCaEXP plasmids [10,17], and the PCaEXP-*CAI4* strain with wild-type phenotype was generated from *CAI4* using the plasmid pCaEXP (BioVector NTCC Inc., China). The transformants then grew on the plates of SD-ura-met-cys selective culture medium, and the positive clone of PCaEXP-*RAS1*-*CAI4* was verified using RT-PCR to detect the level of mRNA of *RAS1*. *RAS1* deletion strain (*RAS1*Δ/Δ) was generated from *SN152* (School of Pharmacy, The Second Military Medical University) using HIS-LEU-ARG knocking-out strategy [18]. Fusion PCR was performed to create disruption fragments of *C. albicans HIS1* and *LEU2* coding sequences, and the *HIS1* and *LEU2* disruption fragments were transformed into *SN152* to obtain *RAS1*Δ/Δ [19]. The homologous recombination transformants grew positively on selective medium (SD-his, SD-leu, and SD-his-leu). The positive clone was verified using colony PCR to detect the presence of correct knockout junctions with the designed primers. The strains and plasmids used in the study are listed in Table 1.

Biofilm Formation and Farnesol Treatment

Freshly grown yeast cells from Sabourd's Dextrose Agar (SDA) plates were incubated in yeast peptone dextrose (YPD) medium and grown overnight in an orbital shaker at 30 °C. The cells were collected and resuspended in RPMI 1640 (Gibco Ltd., Paisley, U.K.). The solution was adjusted to a cell density of 1×10^6 cells/ml for the experiments. Standardized suspensions of the strains were dispensed into flat-bottom microtiter dishes (Corning, Inc., N.Y., USA). The cells were incubated at 37 °C in a moist chamber with 5% CO_2. After 2 h of incubation, non-adherent cells were removed by thoroughly washing the biofilms three times with PBS. Then the biofilms formed depending on the selected time periods (6, 12, 24, and 36 h). For the farnesol treatment, equal volumes of farnesol (100-300 μM) or sterile water (with an equal concentration of methanol) were added to the farnesol-treated and untreated control groups, respectively. Stock solutions (100 mM) of farnesol (E, E farnesol; Sigma Chemical Co., St. Louis, Mo) were dissolved in 100% (vol/vol) methanol and frozen at -70 °C until use. The effect of methanol on the growth of *C. albicans* was evaluated by testing different methanol concentrations without antifungals, ranging from 0.05–1% (v/v) [20]. The highest methanol concentration used in the microdilution plates was 0.1% (v/v), which presented no antifungal activity.

Susceptibility Tests

The antifungals used in the tests were fluconazole (Sigma-Aldrich, St Louis, MO, USA), itraconazole (Selleckchem, Houston, TX, USA), amphotericin B (Sigma-Aldrich, St Louis, MO, USA), caspofungin (Sigma-Aldrich, St Louis, MO, USA), terbinafine (Selleckchem, Houston, TX, USA),

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

Table 1: Strains and Plasmids used in this Study.

Strains	Genotype and Description[a]	Reference
C. albicans strains		
SC5314	Wild-type clinical isolate	American Type Culture Collection, America
Resistant strain	derived from *SC5314* induced via the serial fluconazole concentration gradient method until the MIC reached or exceeded 64 μg/ml	Yu LH et al. 2011
		Xia J et al. 2017
CAI4	*ura3:: λimm434/ura3:: λimm434*	Fonzi and Irwin. 1993
	URA3 auxotrophic strain	
PCaEXP-*CAI4*	*ura3:: λimm434/ura3:: λimm434*-(pCaEXP *URA3*)	In this study
	strain transformed with pCaEXP, used as a control of overexpression experiments	
*RAS1*OE	*ura3:: λimm434/ura3:: λimm434*-(*RAS1*-pCaEXP *RAS1, URA3*)	In this study
	RAS1-overexpressing strain	
SN152	*arg4/arg4 leu2/leu2 his1/his1 URA3/ura3::imm434 IRO1/iro1::imm434*	Noble SM et al. 2005
	Arg4, Leu2, His1 auxotrophic strain	
*RAS1*Δ/Δ	*arg4/arg4 leu2/leu2 his1/his1 URA3/ura3::imm434 IRO1/iro1::imm434*	In this study
	RAS1::C.d.HIS1/RAS1::C.m.LEU2	
	RAS1-deleted strain	
Plasmids		
pCaEXP	*URA3 and MET3* promoter integrating plasmid	R. S. Care et al. 1999
RAS1-pCaEXP	Constructed by integration of *RAS1*	In this study
pSN52	With *HIS1* marker	Noble SM et al. 2005
pSN40	With *LEU2* marker	Noble SM et al. 2005

[a]C.m., *C. maltose*: the DNA fragments *LEU2* was generated from *C. m.* ; *C.d., C. dubliniensis*: the DNA fragments *HIS1* was generated from *C.d.* The DNA fragments *LEU2* and *HIS3* from *C. dublinensis and C. maltosa* have been homologous recombinated to replace the target genes *RAS1* in the *C. albicans.*

5-flucytosine (Selleckchem, Houston, TX, USA), and nystatin (Selleckchem, Houston, TX, USA). The susceptibility of *RAS1* mutant strains in planktonic form to antifungals was determined using a spot assay [21]. The strains were incubated in the mediums and grown for 16 h in an orbital shaker at 30 °C. The yeast cells were harvested during the logarithmic growth phase, washed twice with PBS and suspended in fresh medium to an OD_{600nm} of 1.0. A 5 μl of tenfold serial dilution of the suspension was spotted onto plates in the presence or absence of antifungals with serial concentration gradients. Antifungals were added to the medium at a concentration of 4 μg/ml for fluconazole, 0.5 μg/ml for itraconazole, 2 μg/ml for amphotericin B, 0.5 μg/ml for caspofungin, 15 μg/ml for terbinafine, 8 μg/ml for 5-fluorocytosine, and 2 μg/ml for nystatin. Growth differences were measured after incubation at 30 °C for 48 h. All experiments were performed in thrice on three separated occasions. The susceptibility of *RAS1* mutant strains in biofilms to antifungals was determined using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]-menadione (0.5 mg/ml XTT, 1 μM menadione) reduction assay (Sigma-Aldrich, St Louis, MO, USA) [22]. Colorimetric changes were analyzed in a microtiter plate reader (BioTek Instruments, Inc., VT, USA) at a wavelength of 490 nm, which measured the changes in the metabolic activity of the biofilms [23]. The biofilms were incubated as described above. A series of antifungal agent-free wells and biofilm-free wells were also included to serve as the positive and negative controls. Drugs were prepared in serial 2-fold dilutions, and their final concentrations ranged from 4 to 1024 μg/ml for fluconazole, 1 to 256 μg/ml for itraconazole, 0.25 to 64 μg/ml for amphotericin B, caspofungin, and nystatin, and 2 to 512 μg/ml for terbinafine and 5-flucytosine. Antifungals were added to the biofilms and incubated for an additional 24 h at 37 °C. The value of the background OD measurement, obtained from biofilm-free wells processed in the same manner as the inoculated wells, was subtracted from the OD measurement of each well. After this subtraction, the percentage of growth in each well was calculated as the OD of each well /OD of the drug-free well. The lowest drug concentration that inhibited biofilms growth by 50% was considered the sessile MIC50 (SMIC50) of this antifungal. Each concentration was tested in five wells and the average value from the five wells was used as the SMIC50. All experiments were performed in thrice on three separated occasions.

The Morphological Observations using CLSM and SEM

Biofilms from mutant, control, and farnesol-treated mutant and control strains were formed on the glass bottom of cell culture dishes for morphological observation. For confocal laser scanning electron microscopy (CLSM) observation,

the formed biofilms were fixed with 4% paraformaldehyde overnight, washed with PBS and stained with 500 µl calcofluor white stain (0.0025 g/ml; Sigma Chemical Co., St. Louis, MO) [24] for 30 min at 37 °C in the dark. The biofilms were observed using a Zeiss LSM700 microscope with a video capture system, automatic camera, image analysis hardware and software (Carl Zeiss, Inc., Oberkochen, Germany), and a 488 nm argon ion laser. For scanning electron microscopy (SEM) observation, the formed biofilms were fixed with 2.5% glutaraldehyde solution overnight. Biofilms were subsequently washed twice with distilled water, dehydrated in ethanol series (70% for 10 min, 95% for 10 min, and 100% for 20 min), and air-dried overnight in a desiccator. The biofilms were then metalized by gold sputtering for 45 s in a High Vacuum Evaporator, followed by bonding to carbon double-side tape and processing for SEM (FEI Inc., Hillsboro, USA).

RT-*q*PCR and Western Blot Analysis

RNA samples of the farnesol-treated and untreated biofilms were purified using a modified hot phenol method [25]. The cDNA was synthesized using the reverse transcription system (TaKaRa, Bio Co., Ltd., Dalian, China) and performed on an ABI 7300 Fast real-time PCR machine (Applied Biosystems, Rotkreuz, Switzerland) using *q*PCR SYBR Green Mix (Thermo Scientific, Waltham, MA, USA). The PCR program used was as follows: activation at 95 °C for 10 min; 40 cycles of amplification (95 °C for 15 s, 55 °C for 1 min); 70 °C for 20 s; cooling at 4 °C. After amplification, a melting curve analysis was performed to ensure the absence of primer dimers. The expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method [11]. ACT1 was used as a reference gene. Primers were all designed by Shanghai Generay Bio-Tech Co., Ltd (Shanghai, China). (Table 2).

Total protein extracts were prepared as described for *C. albicans* biofilms using an immunoprecipitation protocol [26]. Protein concentrations were determined using a BCA Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). A total of 15 µg protein diluted in 6× loading buffer was separated on a 10% SDS-PAGE gel and blotted onto PVDF membranes (Millipore, MA, USA). Membranes were blocked in 0.01 M PBS containing 5% skim milk and 0.1% Tween-20 at room temperature, followed by incubation of membranes overnight with the primary antibodies (RAS1, 1:1000 dilution; GAPDH, 1:8000 dilution; Bioworld, MN, USA) at 4 °C. Membranes were then rinsed with PBST (0.01 M PBS plus 0.1% Tween-20) and incubated with secondary antibodies (1:10000 dilution, Bioworld, MN, USA) at room temperature for 1 h. Membranes were visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and exposed to Kodak X-ray films. GAPDH was used as an internal control in the experiments.

Table 2: Primer Sequences in Identifying *RAS1* Expression Strains.

Gene	Sequences
RAS1	**F:** GGTAAATCCGCTTTAACCATTC
	R: GCCAGATATTCTTCTTGTCCAG
ACTIN	**F:** GCCGGTGACGACGCTCCAAGAGCTG
	R: CCGTGTTCAATTGGGTATCTCAAGGTC

Statistical Analysis

All quantitative experiments were performed in thrice for statistical analyses. Data were analyzed using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the differences between the groups, while a paired t-test was performed for intragroup comparisons. The rank sum test was used to analyze ranked data. Comparisons resulting in *P* values of less than 0.05 were considered statistically significant.

Results

RAS1 is Involved in the Resistance of *C. albicans* to Antifungals

For the spot assay to analyze the resistance of planktonic *C. albicans* to antifungals, *RAS1*OE was more resistant to fluconazole (4 µg/ml) and itraconazole (0.5 µg/ml) than control strain (pCaEXP-*CAI4* with wild-type phenotype) (Fig. 1). Moreover, *RAS1*Δ/Δ was more susceptible to fluconazole (4 µg/ml), itraconazole (0.5 µg/ml), amphotericin B (2 µg/ml), nystatin (2 µg/ml), caspofungin (0.5 µg/ml), terbinafine (15 µg/ml), and 5-fluroctosine than wild-type strain (SN152) (Figure 1).

For the XTT-reduction assay to analyze the resistance of *C. albicans* biofilms to antifungal drugs, the biofilms of *RAS1*OE strain had higher SMIC50 values for fluconazole (at 12 and 24 h biofilms), 5-flurocytosine (at 6 and 12 h biofilms), nystatin (at 6, 12, and 24 h biofilms), itraconazole, amphotericin B, caspofungin, and terbinafine (at 6, 12, 24, and 36 h biofilms) than the biofilms of control strain with wild-type phenotype (Table 3-1, Part A). Furthermore, biofilms of *RAS1*Δ/Δ had lower SMIC50 values for itraconazole (at 6, 12 and 24 h biofilms), amphotericin B (at 12 and 24 h biofilms), nystatin (at 6 h biofilm), fluconazole, caspofungin, terbinafine, and 5-flurocytosine (at the 6 and 12 h biofilms), than the biofilms of control strain (*SN152*) (Table 3-1, Part A). On the other hand, the SMIC50 of the antifungal drugs were increased with the mature of biofilms of all the studied strains.

RAS1 of the *C. albicans* Biofilms Regulated by Farnesol

When the biofilms exposed to farnesol, compare with the farnesol-untreated control, *RAS1* expression and RAS1 level in the biofilms of *SC5314* and resistant strain (formed from *SC5314*) were all down-regulated at four growth phases (6, 12, 24, and 36 h), respectively ($P<0.01$) (Figure 2).

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

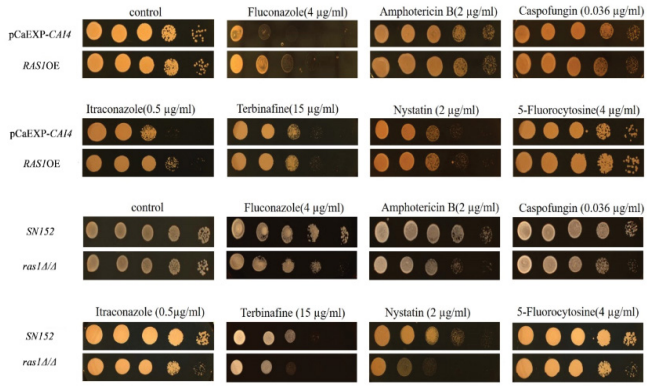

Figure 1: Susceptibility Tests of the *RAS1* Mutant Strains of Planktonic *C. albicans*.

Yellow spot: represents resistance of planktonic *C. albicans* to antifungal drugs under serial concentration gradients.

Table 3-1: The SMIC Values of Antifungals in *C. albicans* Biofilms of Mutant Strains.

Part A	SMIC50 of antifungals(μg/ml) with 0 μM farnesol						
	6 h biofilms						
	Flu	Itr	AmB	Terb	5-Fc	Cas	Nys
pCaEXP-*CAI4*	>1024	>16	1	>256	2	0.125	2
*RAS1*OE	>1024	64	4	512	512	1	4
SN152	128	16	8	64	>512	16	4
RAS1Δ/Δ	4	4	8	16	256	4	2
	12 h biofilms						
	Flu	Itr	AmB	Terb	5-Fc	Cas	Nys
pCaEXP-*CAI4*	512	>16	0.5	>256	256	>8	4
*RAS1*OE	>1024	128	64	>512	512	32	8
SN152	1024	>256	8	512	>512	32	4
RAS1Δ/Δ	>1024	4	4	64	128	4	4
	24 h biofilms						
	Flu	Itr	AmB	Terb	5-Fc	Cas	Nys
pCaEXP-*CAI4*	1024	>16	1	>256	512	>8	4
*RAS1*OE	>1024	256	64	>512	512	32	8
SN152	1024	>256	32	>512	>512	64	4
RAS1Δ/Δ	1024	>64	16	>512	>512	64	4
	36 h biofilms						
	Flu	Itr	AmB	Terb	5-Fc	Cas	Nys
pCaEXP-*CAI4*	>1024	>16	0.25	>256	512	>8	8
*RAS1*OE	>1024	256	64	>512	512	32	8
SN152	1024	>256	32	>512	>512	>64	4
RAS1Δ/Δ	1024	>256	32	>512	>512	>64	4

* "Flu" is an acronym for Fluconazole; "Itr" is an acronym for Itraconazole; "AmB" is an acronym for Amphotericin B; "Cas" is an acronym for Caspofungin; "Terb" is an acronym for Terbinafine; "5-Fc" is an acronym for 5-Flucytosine; "Nys" is an acronym for Nystatin. *Part A: SMIC50 of antifungals (μg/ml) to *C. albicans* biofilms without farnesol (0 μM farnesol).

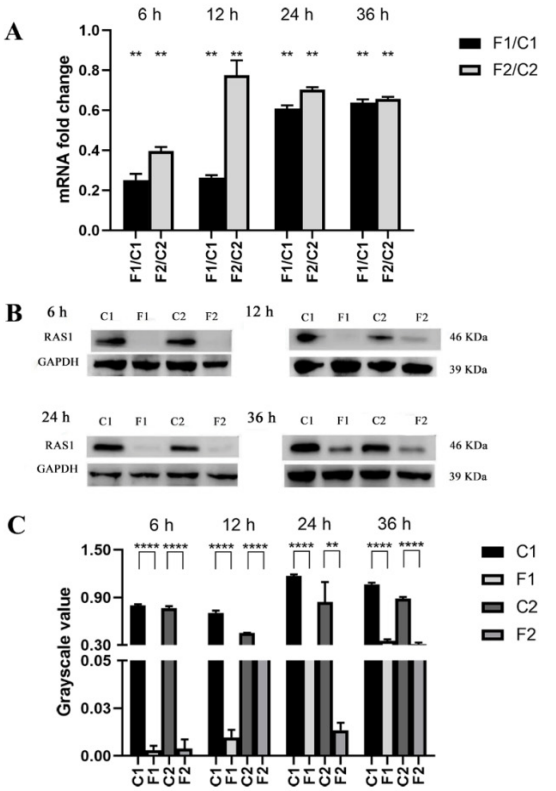

Figure 2: *RAS1* and RAS1 Regulated by Farnesol *via* RT-*q*PCR and Western Blot.

A: Comparisons of gene expression between the farnesol-treated and untreated groups of *C. albicans* biofilms at all studied phases; **B:** The levels of RAS1 in the strains with or without farnesol-treated were assessed using western blot at all studied phases; **C:** The bands in B were quantified and analyzed using grayscale values. C1: farnesol-untreated *SC5314*; F1: farnesol-treated *SC5314*; C2: farnesol-untreated resistant strain; F2: farnesol-treated resistant strain. Farnesol: 200 μM. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

Effects of Farnesol on the Susceptibility of Biofilms of *RAS1* Mutant Strains to Antifungals

Significant SMIC reductions were observed when farnesol was added to the biofilms of *RAS1*OE strains. Compared with the untreated biofilms, the resistance of *RAS1*OE biofilms to fluconazole (at 6 and 12 h biofilms), itraconazole (at 6, 12, 24, and 36 h biofilms), amphotericin B (at 12, 24 and 36 h biofilms), caspofungin (at 6, 12, 24, and 36 h biofilms), terbinafine (at 6, 12, and 36 h biofilms), 5-flurocytosine (at 6, 12, 24, and 36 h biofilms), and nystatin (at 12 h biofilm) were decreased after farnesol treatment. Moreover, the inhibitory effects of farnesol on the antifungal (including fluconazole, itraconazole, amphotericin B, nystatin, caspofungin, and 5-flucytosine) resistance of *RAS1*OE were more obvious than that of control strain (PCaEXP-*CAI4*) (P<0.05) (Table 3-2 Part B, C, and D). Further, compared with the untreated control, farnesol did not change the resistance of *RAS1*Δ/Δ biofilms to fluconazole, itraconazole, and 5-flurocytosine; however, farnesol decreased the resistance of *RAS1*Δ/Δ

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

Table 3-2: The SMIC Values of Antifungals in *C. albicans* Biofilms of Mutant Strains with Farnesol.

Part B	SMIC50 of antifungals (μg/ml) with 100 μM farnesol						
	6 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	>16	0.5	0.0625	>256	1	4
*RAS1*OE	256	16	4	0.0625	256	4	4
SN152	128	16	8	0.25	>512	512	8
RAS1Δ/Δ	8	4	1	0.25	>512	128	8
	12 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	512	>16	0.5	>8	>256	1	4
*RAS1*OE	512	64	8	0.125	256	1	4
SN152	1024	256	4	0.5	>512	512	8
RAS1Δ/Δ	128	4	4	0.5	>512	512	8
	24 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	1024	>16	1	>8	>256	2	2
*RAS1*OE	>1024	>64	8	16	512	4	8
SN152	1024	>256	16	0.5	>512	512	4
RAS1Δ/Δ	1024	64	16	0.5	>512	512	8
	36 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	>16	0.25	0.25	>256	0.5	4
*RAS1*OE	>1024	>64	2	0.125	64	0.5	8
SN152	1024	>256	2	1	>512	512	8
RAS1Δ/Δ	1024	>256	2	0.5	>512	512	8
Part C	SMIC50 of antifungals (μg/ml) with 200 μM farnesol						
	6 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	>16	1	0.0625	>256	2	2
*RAS1*OE	128	1	4	0.25	256	2	2
SN152	64	8	4	0.25	>512	512	4
RAS1Δ/Δ	4	4	4	0.25	>512	256	8
	12 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	512	>16	0.5	0.25	>256	4	2
*RAS1*OE	256	1	4	0.25	512	4	2
SN152	1024	>256	4	0.5	>512	16	4
RAS1Δ/Δ	128	4	4	1	>512	64	8
	24 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	512	>16	0.5	>8	>256	2	2
*RAS1*OE	512	16	8	0.25	>512	2	2
SN152	1024	>256	8	0.25	>512	512	4
RAS1Δ/Δ	1024	16	8	0.5	>512	512	8
	36 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	0.125	0.125	0.125	>256	16	2
*RAS1*OE	1024	32	16	0.5	512	2	2
SN152	1024	>256	2	0.5	>512	512	4
RAS1Δ/Δ	1024	>256	2	0.5	>512	512	8
Part D	SMIC50 of antifungals (μg/ml) with 300 μM farnesol						
	6 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	>16	1	0.0625	>256	16	4
*RAS1*OE	128	16	2	0.0625	256	16	4
SN152	64	4	2	0.25	>512	512	8
RAS1Δ/Δ	4	4	8	0.25	>512	128	16
	12 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	512	>16	0.5	0.25	>256	16	4
*RAS1*OE	256	64	16	0.0625	512	0.5	4
SN152	1024	4	2	0.25	>512	512	16
RAS1Δ/Δ	1024	4	2	0.5	>512	512	32
	24 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	512	>16	1	>8	>256	64	4
*RAS1*OE	512	64	16	2	512	0.5	4
SN152	1024	>256	8	0.5	>512	512	16
RAS1Δ/Δ	1024	128	8	0.5	>512	512	16
	36 h biofilms						
	Flu	Itr	AmB	Cas	Terb	5-Fc	Nys
pCaEXP-*CAI4*	>1024	0.5	0.125	0.0625	>256	64	0.5
*RAS1*OE	>1024	64	16	16	512	64	1
SN152	1024	>256	2	0.25	>512	512	16
RAS1Δ/Δ	1024	>256	2	0.5	>512	512	16

* "Flu" is an acronym for Fluconazole; "Itr" is an acronym for Itraconazole; "AmB" is an acronym for Amphotericin B; "Cas" is an acronym for Caspofungin; "Terb" is an acronym for Terbinafine; "5-Fc" is an acronym for 5-Flucytosine; "Nys" is an acronym for Nystatin. *Part B: SMIC50 of antifungals (μg/ml) to *C. albicans* biofilms with 100 μM farnesol; Part C: SMIC50 of antifungals (μg/ml) to *C. albicans* biofilms with 200 μM farnesol; Part D: SMIC50 of antifungals (μg/ml) to *C. albicans* biofilms with 300 μM farnesol.

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

biofilms (at 6, 12, 24, and 36 h biofilms) to amphotericin B and caspofungin and increased that to nystatin (at 6, 12, 24, and 36 h biofilms) and terbinafine (at 6 and 12 h biofilms) (Table 3-2 Part B, C, and D).

The Morphologic Changes of Biofilms

Both CLSM and SEM observations showed that the inhibitory effects of farnesol on hyphal growth of *RAS1*OE were more obvious than that of the control strain (pCaEXP-*CAI4*) (Figure 3 and 4), while the inhibitory effects of farnesol on hyphal growth of *RAS1*Δ/Δ were less obvious than that of the control biofilm (*SN152*) (Figure 3 and 4). The CLSM showed that the biofilms of *RAS1*OE and *RAS1*Δ/Δ exposed to farnesol had fewer hyphae but more pseudohyphae and blastospores than the control biofilms without farnesol (Figure 3 C1D1, C2D2, C3D3, C4D4; G1H1, G2H2; G3H3; G4H4), respectively. In addition, the biofilms formed from *RAS1*OE had more extensively grown hyphae and pseudohyphae with few blastospores than the biofilms formed from the control strain (pCaEXP-*CAI4*) (Figure 3 A1C1, A2C2, A3C3, A4C4). Moreover, the biofilms formed from *RAS1*Δ/Δ had fewer hyphae but more pseudohyphae and blastospores than the biofilms formed from the control strain (*SN152*) (Figure 3 E2G2, E3G3).

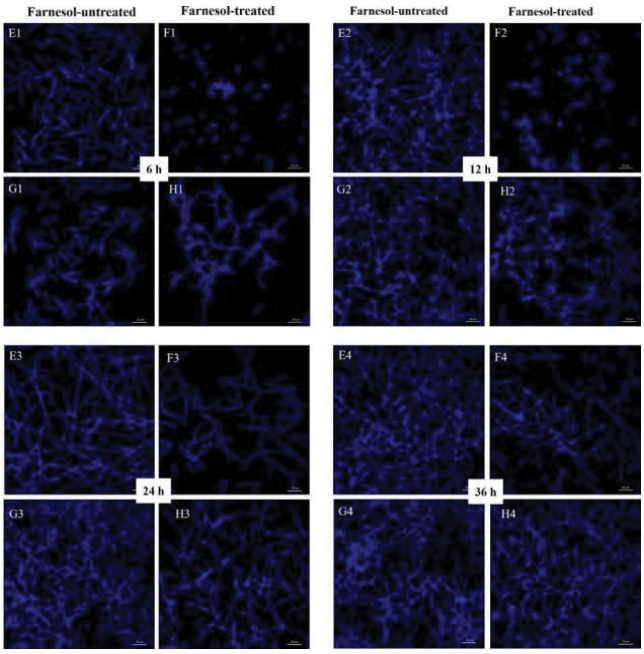

Figure 3: The Morphological Changes in *C. albicans* Biofilms of Mutant Strains by CSLM.

A: farnesol-untreated pCaEXP-*CAI4*; B: farnesol-treated pCaEXP-*CAI4*; C: farnesol-untreated *RAS1*OE; D: farnesol-treated *RAS1*OE. E: farnesol-untreated *SN152*; F: farnesol-treated *SN152*; G: farnesol-untreated *RAS1*Δ/Δ; H: farnesol-treated *RAS1*Δ/Δ. A1, B1, C1, D1 E1, F1, G1, and H1: 6 h biofilms; A2, B2, C2, D2, E2, F2, G2, and H2: 12 h biofilms; A3, B3, C3, D3, E3, F3, G3, and H3: 24 h biofilms; A4, B4, C4, D4, E4, F4, G4, and H4: 36 h biofilms. The biofilms of C1C2 had fewer hyphae but more pseudohyphae and blastospores than that of D1 and D2. The inhibitory effects of farnesol were more obvious in D1, D2, D3, and D4 than that in B1, B2, B3, and B4. The biofilms of G1 and G2 had fewer hyphae but more pseudohyphae and blastospores than that of H1and H2. The inhibitory effects of farnesol in H1, H2, H3, and H4 were less obvious than that in F1, F2, F3, and F4. Magnification: 400×; farnesol: 200 μM

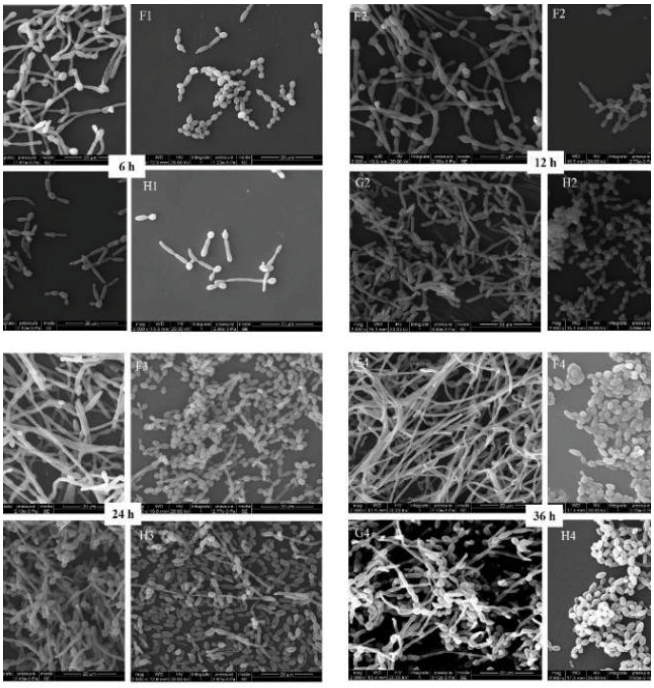

Figure 4: The Morphological Changes in *C. albicans* Biofilms of Mutant Strains by SEM.

A: farnesol-untreated pCaEXP-*CAI4*; B: farnesol-treated pCaEXP-*CAI4*; C: farnesol-untreated *RAS1*OE; D: farnesol-treated *RAS1*OE. E: farnesol-untreated *SN152*; F: farnesol-treated *SN152*; G: farnesol-untreated *RAS1*Δ/Δ; H: farnesol-treated *RAS1*Δ/Δ. A1, B1, C1, D1, E1, F1, G1, and H1: 6 h biofilms; A2, B2, C2, D2, E2, F2, G2, and H2: 12 h biofilms; A3, B3, C3, D3 E3, F3, G3, and H3: 24 h biofilms; A4, B4, C4, D4, E4, F4, G4, and H4: 36 h biofilms. The biofilms of D1 to D4 appeared in short pseudohyphae and had a rough cell surface, while C1 to C4 appeared in long hyphae and had smooth surfaces. The inhibitory effects of farnesol in D1, D2, D3, and D4 were more obvious than that in B1, B2, B3, and B4. The biofilms of F1 to F4 and H1 to H4 appeared in short pseudohyphae and had a rough cell surface, while E1 to E4 and G1 to G4 appeared in long hyphae and had smooth surfaces. The inhibitory effects of farnesol in H1, H2, H3 and H4 were less obvious than that in F1 F2, F3, and F4. Magnification: 2000×; farnesol: 200 μM.

SEM analysis showed that the cells in farnesol-treated biofilms of *RAS1*OE and *RAS1*Δ/Δ appeared in the short pseudohyphae and had a rough cell surface than those in the biofilms without farnesol (Fig. 4 C1D1, C2D2, C3D3, C4D4, G1H1, G2H2, G3H3, G4H4). In addition, the biofilms formed from *RAS1*OE had more hyphae than those formed from the control strain (pCaEXP-*CAI4*) (Fig. 4 A1C1, A2C2, A3C3, A4C4), while the biofilms formed from *RAS1*Δ/Δ had fewer hyphae and more pseudohyphae than those formed from the control strain (*SN152*) (Fig. 4 E1G1, E2G2, E3G3, E4G4). The surfaces of cells in *RAS1*OE biofilms were similar to the biofilms of *RAS1*Δ/Δ.

Citation: Lulu Zuo, Zhenzhen Zhang, Wei Duan, Yun Huang, Zheng Xu, Qinqin Zhang, Ying Lv, Xin Wei. *RAS1* is Involved in the Antifungal Resistance of *Candida Albicans* and the Inhibition of Farnesol to the Resistance of Biofilms. Archives of Clinical and Biomedical Research6 (2022): 694-706.

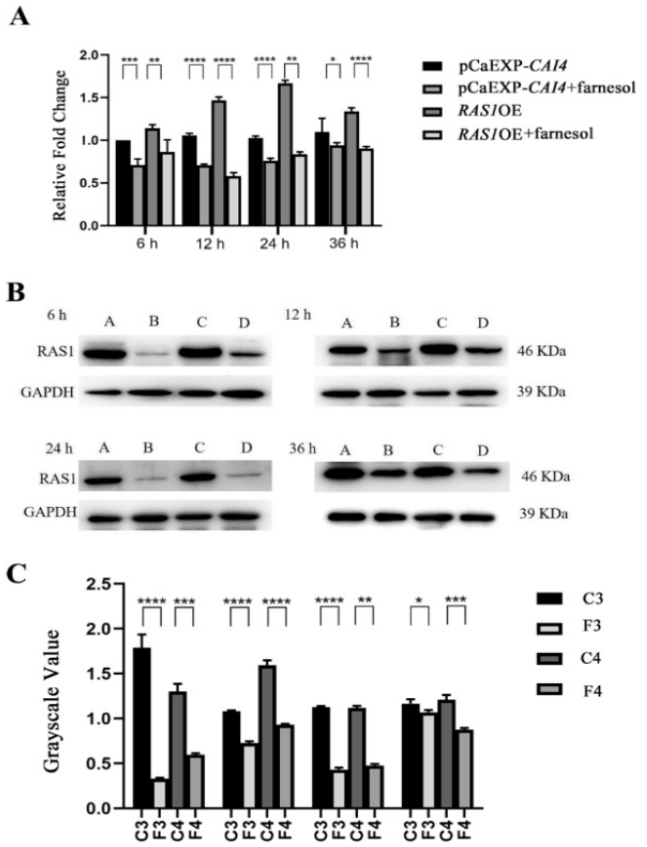

Figure 5: The Expression of the *RAS1* Gene and the RAS1 Protein in the Farnesol-treated *RAS1*OE.

A: Comparison of gene expression between farnesol-treated and untreated groups of *C. albicans* biofilms at all studied phases (6, 12, 24, and 36 h); B: The level of RAS1 in the strains with or without farnesol-treated was assessed using western blot at all studied phases; C: The bands in B was quantified and analyzed using grayscale value. C1: farnesol-untreated pCaEXP-*CAI4*; F1: farnesol-treated pCaEXP-*CAI4*; C2: farnesol-untreated *RAS1*OE; F2: farnesol-treated *RAS1*OE. Farnesol: 200 μM; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

Farnesol Decreased the Expression of *RAS1* in the Biofilms formed from the *RAS1*OE Strains

RT-*q*PCR and western blotting showed that farnesol significantly reduced the expression of *RAS1* and the level of RAS1 in the biofilms formed by *RAS1*OE and the control strains (PCaEXP-*CAI4*), as compared with that of the farnesol-untreated biofilms at all studied phases (at 6, 12, 24, and 36 h biofilms) ($P<0.05$) (Figure 5), respectively. Moreover, farnesol reduced the expression of *RAS1* and the level of RAS1 more obviously in the biofilms of *RAS1*OE than that of control strains (PCaEXP-*CAI4*) ($P<0.05$). Meanwhile, there were no expression of *RAS1* in the biofilms formed by *RAS1*Δ/Δ, with or without farnesol.

Discussion

Previous investigations have clearly showed that *RAS1* gene is required for the induction of hyphal growth and the maintenance of hyphal state [12, 14], and also has a close relationship with pathogenic processes [27], such as cell adhesion and biofilm formation. RAS1 is a highly conserved signaling protein that might play the roles in the antifungal resistance of *C. albicans* and the relevant farnesol regulation mechanisms of the biofilm resistance, which are discussed in the present study. The study found that *RAS1* is involved in the antifungal resistance of *C. albicans*. For the *C. albicans* in planktonic form, *RAS1*-overexpressing strain increased the resistance of *C. albicans* to fluconazole and itraconazole, and *RAS1* deletion strain reduced that to all the tested antifungals. For the *C. albicans* in biofilm form, *RAS1*-overexpressing strain increased the resistance of biofilms to all the tested antifungal drugs, and *RAS1* deletion strain reduced that. The findings indicates that *RAS1* is associated with the antifungal resistance of *C. albicans* in planktonic and biofilm forms, which has not been reported in the previous studies.

Currently, azoles, polyenes, echinocandins and miscellaneous antifungals are the main antifungal families used in clinical practices. In the present study, the antifungal drugs for susceptibility test were fluconazole, itraconazole, amphotericin B, nystatin, caspofungin, terbinafine, and 5-flurocytosine. Fluconazole/itraconazole impairs ergosterol synthesis and leads to a cascade of membrane abnormalities in the fungus [28]. Amphotericin B/nystatin induces the ergosterol sequestration after binding to ergosterol and acts through pore formation at the cell membrane [29]. Caspofungin blocks the synthesis of β-(1,3)-D-glucan which is an essential component of the fungal cell wall [30]. Terbinafine prevents the conversion of squalene to lanosterol by inhibiting squalene epoxidase and inhibits the ergosterol synthesis [31]. 5-flurocytosine works by being converted into 5-fluorouracil inside the fungal, and blocks its ability to make protein [32]. Each of the antifungal drugs has its own mechanism of action, thus, the effects of antifungals to the mutant strains might be complex and different. The results that *RAS1* overexpression increased the antifungal susceptibility to the drugs, and *RAS1* deletion decreased that, regarding multiple antifungals with different mechanisms, implies that *RAS*1 has an ability in antifungal resistance and might be involved in a non-specific mechanism for the resistant regulation by *C. albicans*.

In the present study, farnesol decreased the expression of *RAS1* gene and the level of RAS1 protein in *C. albicans* biofilms of *RAS1*-overexpressing strain and the strain with wild-type phenotype. Meanwhile, the inhibitory effects by farnesol on the resistance of biofilms of *RAS1*-overexpressing strain were more obvious than that of the wild-type phenotype (PCaEXP-*CAI4*) and *RAS1*-deletion strains. The results suggested that *RAS1* is involved in the antifungal resistance regulated by farnesol. On the other hand, farnesol decreased

the resistance of biofilms from *RAS1*-deletion strain to amphotericin B and caspofungin, while increased that to nystatin and terbinafine. The possible reason for this might be the complexity of multiple mechanisms of antifungals, which leads to the variations of antifungal resistance as the *RAS1*-deletion strain exposed to farnesol.

A previous study showed that *RAS1*-deletion strain was impaired in hyphal development under many different induction conditions [33]. In the present study, morphological observations showed that *RAS1*-deletion decreased the hyphal growth of the *C. albicans* biofilms and *RAS1*-overexpression increased the hyphal growth of *C. albicans* biofilms, which was in accordance with the previous study[33]. Furthermore, both CLSM and SEM observations showed that the inhibitory effects of farnesol on *RAS1*-overexpression were more obvious than that on the control strain with wild-type phenotype, and the inhibitory effects of farnesol on *RAS1* deletion were less obvious than that on the control strain with wild-type phenotype. The morphological results suggest that farnesol is involved in the antifungal resistance of *C. albicans* biofilms via decreasing the hyphal growth caused by *RAS1* regulation.

The formation of mature biofilms was composed of a series developed steps. *C. albicans* biofilms started with the initial adherence of yeast cells (0-2 h), followed by germination and micro-colony formation (2-4 h), filamentation (4-6 h), monolayer development (6-8 h), proliferation (8-24 h), and maturation (24-48 h) [34]. The main stages of biofilms were investigated here to clarify the effects of farnesol on the *RAS1* regulation in *C. albicans* biofilms. A phase-specific mechanism might be involved in biofilm resistance, which appeared of the increased resistance of *C. albicans* with the mature biofilms (24 and 36 h). Because of this phase-specific resistance, the inhibitory effects of farnesol were reduced following with the mature of biofilms. In addition, the study showed that the inhibitory effects of farnesol on RAS1 level and the antifungal resistance of biofilms were consistent with the growth phases of *RAS1*-overexpressing strain, which further confirmed that *RAS1* is involved in the antifungal resistance caused by farnesol. On the other hand, the effects of farnesol on the resistance of biofilms from the *RAS1*-deletion strain are not accordance with the mature of biofilms, other complicated and multiple antifungal mechanisms might affect the resistance of biofilms fromed from the *RAS1*-deletion strain, which need to be discussed in the future.

Conclusion

The extensive use of antifungals has led to the increased resistance of *C. albicans* biofilms to the few existing antifungals. Farnesol enhances the susceptibility of *Candida albicans* biofilms to antifungals, while the mechanisms of this behavior are poorly understood. Thus, it is crucial to explore the mechanisms of preventive strategies for the farnesol-mediated increase in susceptibility to various antifungals targeting biofilm-related infections. For the first time, this study demonstrated that *RAS1* is involved in the antifungal resistance of *C. albicans* and the inhibition regulated by farnsol on the resistance of biofilms. Additional research regarding the specific molecular mechanisms should be further pursued to elucidate the antifungal resistance mechanisms of *C. albicans* biofilms.

Declarations

Ethics Approval

Not applicable

Consent to Participate

Not applicable

Consent for Publication

Not applicable

Acknowledgments

Not applicable

Competing Interests

Lulu Zuo, Zhenzhen Zhang, Wei Duan, Yun Huang, Zheng Xu, Qinqin Zhang, Ying Lv, and Xin Wei declare that they have no conflict of interest.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

XW conceived the study, participated in study design, and data analysis, and was responsible for writing and submitting the final manuscript. LLZ structured the *RAS1* overexpressing and deletion strains and performed statistical analysis and drafted the manuscript. ZZZ carried out the XTT reduction assay and spot assay. WD and QQZ participated in statistical analysis. YL participated in RT-*q*PCR and western blot analysis. YH and ZX participated in morphological observations by CLSM and SEM. All authors read and approved the manuscript.

Funding

This work was supported by the National Natural Sciences Foundation of China under Grant Nos. 81970945 and 81371156 and the Foundation of the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, 2018-87). Xin Wei was the recipient of these funds.

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

References

1. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. Virulence 4 (2013): 119-128.
2. Gulati M, Nobile CJ. *Candida albicans* biofilms: development, regulation, and molecular mechanisms. Microbes and infection 5 (2016): 310-321.
3. Nobile CJ, Johnson A. *Candida albicans* Biofilms and Human Disease. Annual review of microbiology 69 (2015): 71-92.
4. Chen S, Xia J, Li C, et al. The possible molecular mechanisms of farnesol on the antifungal resistance of *C. albicans* biofilms: the regulation of *CYR1* and *PDE2*. BMC Microbiology 18 (2018).
5. Hall R, Turner KJ, Chaloupka J, et al. The Quorum-Sensing Molecules Farnesol/Homoserine Lactone and Dodecanol Operate via Distinct Modes of Action in *Candida albicans*. Eukaryotic Cell 10 (2011): 1034-1042.
6. Jabra-Rizk MA, Meiller TF, James CE, et al. Effect of farnesol on staphylococcus aureus biofilm formation and antimicrobial susceptibility. Antimicrobial Agents and Chemotherapy 50 (2006): 1463-1469.
7. Polke M, Leonhardt I, Kurzai O, et al. Farnesol signalling in *Candida albicans* – more than just communication. Critical Revies in Microbiology 44 (2018): 230-243.
8. Koo H, Hayacibara MF, Schobel BD, et al. Inhibition of Streptococcus mutans biofilm accumulation and polysaccharide production by apigenin and tt-farnesol. Journal of Antimicrobial Chemotherapy 52 (2003): 782-789.
9. Luo K, Rocheleau H, Qi P, et al. Indole-3-acetic acid in Fusarium graminearum: Identification of biosynthetic pathways and characterization of physiological effects. Fungal biology 120 (2016): 1135-1145.
10. Xia J, Qian F, Xu W, et al. *In vitro* inhibitory effects of farnesol and interactions between farnesol and antifungals against biofilms of *Candida albicans* resistant strains. Biofouling 33 (2017): 283-293.
11. Yu L, Wei X, Ma M, et al. Possible Inhibitory Molecular Mechanism of Farnesol on the Development of Fluconazole Resistance in *Candida albicans* Biofilm. Antimicrobial Agents and Chemotherapy 56 (2011): 770-775.
12. Davis-Hanna A, Piispanen AE, Stateva LI, et al. Farnesol and dodecanol effects on the *Candida albicans* *RAS1*-cAMP signalling pathway and the regulation of morphogenesis. Molecular Microbiology 67 (2008): 47-62.
13. Martin R, Walther A, Wendland J. *RAS1*-Induced Hyphal Development in *Candida albicans* requires the Formin Bni1. Eukaryotic Cell 4 (2005): 1712-1724.
14. Lindsay AK, Deveau A, Piispanen AE, et al. Farnesol and Cyclic AMP Signaling Effects on the Hypha-to-Yeast Transition in *Candida albicans*. Eukaryotic Cell 11 (2012): 1219-1225.
15. Piispanen AE, Bonnefoi O, Carden S, et al. Roles of *RAS1* Membrane Localization during *Candida albicans* Hyphal Growth and Farnesol Response. Eukaryotic Cell 10 (2011): 1473-1484.
16. Chen S, Xia J, Li C, et al. The possible molecular mechanisms of farnesol on the antifungal resistance of *C. albicans* biofilms: the regulation of CYR1 and PDE2. BMC microbiology 18 (2018): 203.
17. Noble S, Johnson A. Strains and Strategies for Large-Scale Gene Deletion Studies of the Diploid Human Fungal Pathogen *Candida albicans*. Eukaryotic Cell 4 (2005): 298-309.
18. Zuo L, Chengxi LI, Zheng L, et al. Comparison of knocking-out the *RAS1* gene in *candida albicans* using different auxotrophic markers. Stomatology (2018).
19. Xia J, Zhang Z, Xu S, et al. A study of modified lithium acetate transformation for plasmid transformation in *candida albicans*. Journal of Nanjing Medical University (Natural Sciences) (2017).
20. Ramage G, Saville S, Wickes B, et al. Inhibition of *Candida albicans* Biofilm Formation by Farnesol, a Quorum-Sensing Molecule. Applied and Envirnmental Microbiology 68 (2002): 5459-5463.
21. Xiao J, Moon Y, Li L, et al. *Candida albicans* Carriage in Children with Severe Early Childhood Caries (S-EC) and Maternal Relatedness. PLoS ONE 11 (2016).
22. Ku TS, Palanisamy SK, Lee S. Susceptibility of *Candida albicans* biofilms to azithromycin, tigecycline and vancomycin and the interaction between tigecycline and antifungals. International journal of antimicrobial agents 5 (2010): 441-446.
23. Manoharan RK, Lee J, Kim Y. Alizarin and Chrysazin Inhibit Biofilm and Hyphal Formation by *Candida albicans*. Fronters in Cellular and Infection Microbiology 7 (2017).
24. Richard M, Nobile CJ, Bruno V, et al. *Candida albicans* Biofilm-Defectiv Mutants. Eukaryotic Cell 4(2005): 1493-1502.
25. Barbier FF, Chabikwa TG, Ahsan M, et al. A phenol/chloroform-free method to extract nucleic acids from recalcitrant, woody tropical species for gene expression and sequencing. Plant Methods 15 (2019).

Citation: Lulu Zuo, Zhenzhen Zhang, Wei Duan, Yun Huang, Zheng Xu, Qinqin Zhang, Ying Lv, Xin Wei. *RAS1* is Involved in the Antifungal Resistance of *Candida Albicans* and the Inhibition of Farnesol to the Resistance of Biofilms. Archives of Clinical and Biomedical Research6 (2022): 694-706.

26. Ma S, Li H, Yan C, et al. Antagonistic effect of protein extracts from *Streptococcus sanguinis* on pathogenic bacteria and fungi of the oral cavity. Experimental and Therapeutic Medicine 7 (2014): 1486-1494.

27. Katragkou A, McCarthy M, Alexander EL, et al. *In vitro* interactions between farnesol and fluconazole, amphotericin B or micafungin against *Candida albicans* biofilms. The Journal of antimicrobial chemotherapy 70 (2015): 470-478.

28. Martel CM, Parker J, Bader O, et al. Identification and Characterization of Four Azole-Resistant *erg3* Mutants of *Candida albicans*. Antimicrobial Agents and Chemotherapy 54 (2010): 4527-4533.

29. Gray KC, Palacios DS, Dailey I, et al. Amphotericin primarily kills yeast by simply binding ergosterol. Proceedings of the National Academy of Sciences 109 (2012): 2234-2239.

30. Hao B, Cheng S, Clancy C, et al. Caspofungin Kills *Candida albicans* by Causing both Cellular Apoptosis and Necrosis. Antimicrobial Agents and Chemotherapy 57 (2012): 326-332.

31. Koutsoulas C, Pippa N, Demetzos C, et al. Preparation of liposomal nanoparticles incorporating terbinafine in vitro drug release studies. Journal of nanoscience and nanotechnology 14 (2014): 4529-4533.

32. Gopinathan S, Janagond AB, Agatha D, et al. Detection of *FUR1* Gene in 5-Flucytosine Resistant *Candida* Isolates in Vaginal Candidiasis Patients. Journal of clinical and diagnostic research: JCDR 7 (2013): 2452-2455.

33. Martin R, Walther A, Wendland J. *RAS1*-Induced Hyphal Development in *Candida albicans* requires the Formin Bni1. Eukaryotic Cell 4 (2005): 1712-1724.

34. Mathé L, Dijck PV. Recent insights into *Candida albicans* biofilm resistance mechanisms. Current Genetics 59 (2013): 251-264.

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

The primers used to obtain the RAS1 deletion strain are listed in Table 1.

Primer	Sequences (5'-3')
Universal F	ccgctgctaggcgcgccgtgAGCTCGGATCCACTAGTAACG
Universal R	gcagggatgcggccgctgacGCCAGTGTGATGGATATCTGC
RAS1-F	GATGAATATGACCCAACT
RAS1-R	AAGAAACACCTCCATTAC
RAS UP-F	CTCCCTAACCCCAGTAAA
RAS UP-R	cacggcgcgcctagcagcggTCAAGGTCAATGTCCAAT
RAS DOWN-F	gtcagcggccgcatccctgcTGATTGTTTCCAAGTTAC
RAS DOWN-R	AAGGTATGAAGAGGATGT
RAS UP check	ATGACAATCCCTCCCTAA
RAS HIS left	TGCATAAACGGTGGCACATT
RAS LEU left	AAACCTCTTTCTTGACCC
RAS HIS right	TCCAATTCAACGACGGCTGA
RAS LEU right	GATTCTGATTGGCTCTTT
RAS DOWN check	GAGACAAAGGTATGAAGAGG

Table 1: Primer Sequences in Constructing Knockout Strain *RAS1*Δ/Δ.

Identification of *RAS1* Overexpressing Strain (*RAS1*OE)

The results of sequencing showed that results of overexpression strain (pCaEXP-*RAS1*) were the same as NCBI *C. albican RAS1* sequence (Fig. 1A). RT-PCR showed that the *RAS1* expression (at 12 and 24 h) increased significantly in the *RAS1*OE (pCaEXP-*RAS1*-*CAI4*) compared to the control strain (pCaEXP-*CAI4*) (Figure 1B).

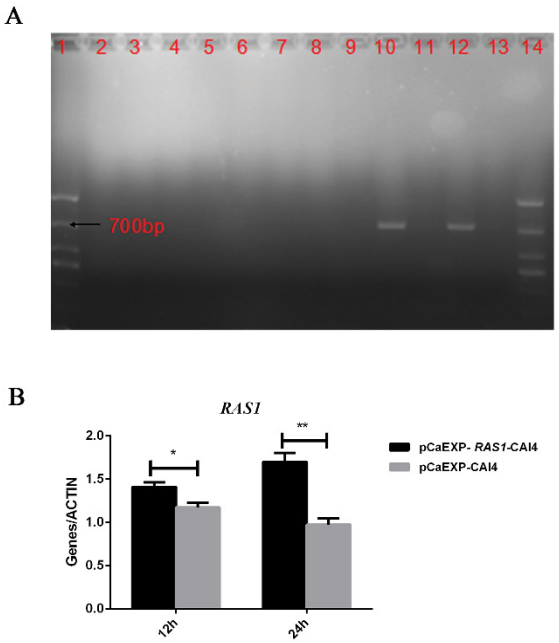

Figure 1: Identification of *RAS1* Gene Overexpressing Strains.

A: Agarose gel electrophoresis; 1: maker; 2-7: CaEXP-*CAI4*: 8-13: transformants; 10 and 12: postive transformations of PCaEXP-*RAS1*-*CAI4*; 14: maker; B: RT-PCR showed that the expression of *RAS1* (at 12 and 24 h) significantly increased compared to the control strain. *: $P<0.05$; **: $P<0.01$.

Citation: Lulu Zuo, Zhenzhen Zhang, Wei Duan, Yun Huang, Zheng Xu, Qinqin Zhang, Ying Lv, Xin Wei. *RAS1* is Involved in the Antifungal Resistance of *Candida Albicans* and the Inhibition of Farnesol to the Resistance of Biofilms. Archives of Clinical and Biomedical Research6 (2022): 694-706.

Lulu Zuo et al., Arch Clin Biomed Res 2022
DOI:10.26502/acbr.50170281

Identification of *RAS1* Deletion Strain (*RAS1*Δ/Δ)

The results of agarose gel electrophoresis showed that *C. albicans RAS1*Δ/Δ (*RAS1* double allelic deletion strain) was successfully constructed using *HIS-LEU-ARG* knocking-out strategy (Fig. 2A). As shown in Fig. 2B, only *C. albicans RAS1*Δ/Δ (*RAS1* double allelic deletion strain) could grow on selective medium (SD-Leu-His).

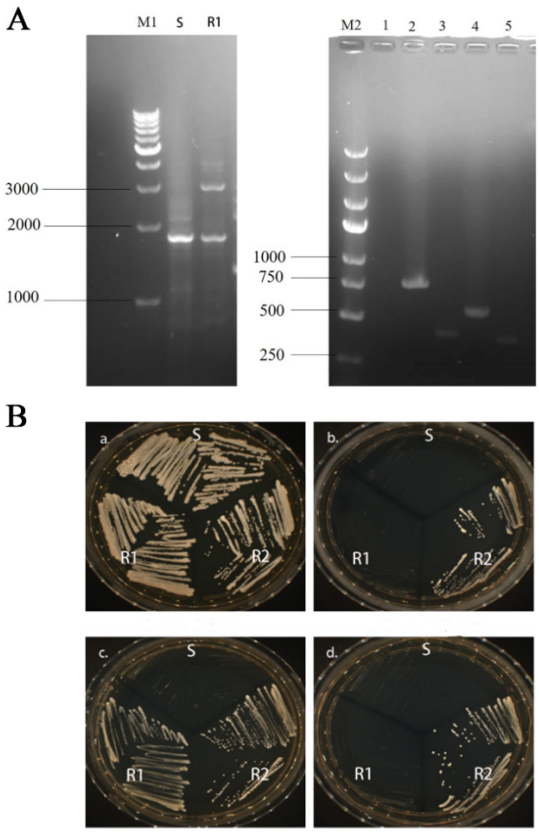

Figure 2: Identification of *RAS1* Gene Knockout Strains.

A: Agarose gel electrophoresis; M1: 1 kb DNA marker; S: *SN152* strain; R1: *RAS1*Δ; M2: DL5000 DNA marker; 1: *RAS1* gene; 2: *HIS1* upstream fragment; 3: *HIS1* downstream fragment; 4: *LEU2* upstream fragment; 5: *LEU2* downstream fragment; B: S: *SN152* strain; R1: *RAS1*Δ strain; R2: *RAS1*Δ/Δ strain. The growth of the control strains and mutant strains in the following media are shown: a: YPD agar; b: SD-*Leu* agar, c: SD-*His* agar; d: SD-*Leu-His* agar; The schematic drawing of a plate indicates the location of the plated strains (S, R1 and R2).