Expression of fluconazole resistance-associated genes in biofilm from 23 clinical isolates of *Candida albicans*

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Abstract

This study aimed to establish the influence of biofilm from clinical isolates of *Candida albicans* on fluconazole resistance, focusing on efflux pumps and azole-targeted enzymes. Twenty-three *C. albicans* clinical isolates were collected from two hospitals in Shanghai, China. Antifungal susceptibility tests were performed on biofilm and planktonic cells. A crystal violet assay was used to monitor biofilm growth. Real-time RT-PCR was performed to quantify the expression of the transporter-related genes *MDR1*, *CDR1*, and *CDR2* as well as *ERG11*, a gene encoding an enzyme targeted by antifungal drugs. Fluconazole resistance was shown to increase in biofilm in a time-dependent manner. No significant differences were observed between different strains of *C. albicans*. Genes encoding efflux pumps were overexpressed in early stages of biofilm formation and could also be induced by fluconazole. While *ERG11* was not upregulated in biofilm, it was overexpressed upon the addition of fluconazole to biofilm and planktonic cells. Gene expression also appeared to be related to the original genotype of the strain. The upregulation of genes encoding efflux pumps demonstrates their role in the development of fluconazole resistance during the early stages of *C. albicans* biofilm formation.

Keywords *Candida albicans* · Biofilm · Fluconazole resistance · Efflux pump

Introduction

In recent years, the rates of infection by opportunistic and pathogenic fungi have increased in immunocompromised patients. According to the US SENTRY Antimicrobial Surveillance Program, which examined 2085 bloodstream infection cases involving *Candida* species from five different regions between 2008 and 2009, *Candida albicans* was the predominant species (48.4%) [1]. Outcomes of infection with *C. albicans* can range from superficial to systemic life-threatening. *C. albicans* cells can adhere to the surface of frequently used medical devices, forming a biofilm which drastically reduces the susceptibility of *C. albicans* to antifungal agents.

According to reports by Ramage et al. [2], biofilm development on catheters can occur in up to 30% of cases.

Triazoles are the most commonly used clinical antifungal agents, with fluconazole recommended as the first-line option for the treatment of *Candida* infections. Fluconazole, however, was shown to be inactive against biofilms [3, 4]. To elucidate the mechanism of drug resistance by biofilms and to address the problem, many studies have focused on *C. albicans* biofilm. Several mechanisms underlying increased resistance in biofilm have been described. The upregulation of drug targets is one mechanism of resistance. When mature biofilms were challenged with high concentrations of fluconazole, *ERG11* was found to be significantly overexpressed [5]. Additionally, the overexpression of efflux pumps has also been described as contributing to resistance of *C. albicans* biofilms to antifungal agents. When compared to planktonic cells, the expression of *CDR1*, *CDR2*, and *MDR1* was increased in biofilm-associated cells [6–8]. The production of an extracellular matrix by *C. albicans* biofilm, which is mainly composed of β -1,3-glucans and proteins, has also been reported to contribute to the resistance to antifungal agents [9–11]. It was shown that β -1,3-glucans can bind azoles and increase resistance [12]. Overall, it is hypothesized that

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resistance of *C. albicans* biofilm to antifungal agents arises from multiple factors.

While many studies have been performed, the strains used were generally not clinical isolates [6–8]. In this study, we aimed to identify the mechanisms underlying fluconazole resistance of biofilms from 23 clinical isolates of *C. albicans* by monitoring the growth of biofilm, drug susceptibility of the biofilm, and expression of resistance-associated genes.

Materials and methods

Strains and medium

All 23 *C. albicans* isolates were collected from two hospitals in Shanghai. The clinical isolates came from vaginal specimen, skin specimen, sputum specimen, and stool specimen. The isolates were plated on chromagenic medium (Chromagar, Pairs, France) to identify *C. albicans*. Carbohydrate assimilation patterns (API21C, Pairs, France) were also used for identification. Isolates were maintained in 25% glycerin YPD (yeast extract peptone dextrose) liquid medium at -20°C . RPMI 1640 was used for antifungal susceptibility tests.

Biofilm formation

According to Hawser's study [11], cells were grown in YPD agar at 37°C for 24 h, then suspended in normal saline at 1.0×10^8 cells/ml. Cells were then diluted twofold with YPD and added to a 96-well plate (200 μl) or 24-well plate (1 ml). After incubation at 37°C for 90 min, plates were washed three times with sterile phosphate buffered saline (PBS). Finally, 200 μl or 1 ml YPD medium was added to each well and plates incubated at 37°C for biofilm formation.

Antifungal susceptibility tests

The minimum inhibitory concentration (MIC) of fluconazole (Pfizer Pharmaceuticals, Shanghai, China) for the *C. albicans* clinical isolates was determined based on standard guidelines described in the Clinical and Laboratory Standards Institute document M27-S4 using the microdilution reference method. Biofilms were grown for 2, 4, 8, 24, or 48 h. Media was removed and the biofilms washed three times with sterile PBS. Subsequently, 200 μl RPMI 1640 medium with serial dilutions of fluconazole (256 to 0.5 mg/L) were added to 96-well plates. Results were analyzed after a 48-h incubation. The MIC was defined as the lowest antifungal concentration that inhibited cell growth by 80% versus the control. The MICs of fluconazole for resistant and susceptible strains were ≥ 8 and ≤ 2 mg/L, respectively. ATCC 90028 was used as control.

Biofilm growth curve

A crystal violet (CV) assay was used to establish the growth curve of biofilm. After biofilm formation, medium was aspirated, and the biofilms washed three times with sterile PBS. An adjusted CV assay was used to measure the metabolic activity of biofilm formation, based on a protocol described by Peeters and colleagues [13]. The biofilms were fixed with methanol for 15 min, followed by 0.02% (v/v) CV for 5 min, and then 33% (v/v) acetic acid for 5 min. Optical density (OD) at 590 nm was measured. The time points examined were 2, 4, 8, 24, and 48 h after biofilm formation. Each experiment was performed in triplicate.

Real-time RT-PCR

Eight isolates (32, 35, 64, 74, 84, 509, 201, and 64,110) were picked to evaluate gene expression. Total RNA from planktonic cells grown to the exponential phase and biofilms grown to particular time points was extracted using the Yeast RNAiso Reagent Kit (Takara, Tokyo, Japan) following the manufacturer's instructions. A Nanodrop 8000 nucleic acid quantitative analysis instrument (Thermo Scientific, Waltham, MA, USA) was used to quantify total RNA. RNA was reverse transcribed to cDNA using the Primer Script RT-PCR Kit (Takara, Tokyo, Japan). The real-time RT-PCR of *CDR1*, *CDR2*, *MDR1*, *ERG11*, and *TAC1* was performed using SYBR Premix Ex TaqTM II (Takara, Tokyo, Japan) and LightCycler Real-Time PCR System (Roche, Shanghai, China). 18S rRNA was used as an internal control and *ACT1* was used to observe whether the experimental protocol influenced gene expression. The primers used in this study are listed in Table 1. To compare the effects of fluconazole on planktonic cells and biofilm, 32 mg/L of fluconazole was added to each to establish whether any differences occurred. For planktonic cells, cells were grown in YPD agar at 37°C for 24 h, then suspended in YPD at 1.0×10^6 cells/ml, fluconazole was added, the final concentration of fluconazole was 32 mg/L, then cells were shaking cultured at 200 rpm for a period of time. As to biofilm cells, biofilms were formed as previously mentioned. After planktonic cells were washed off, YPD within 32 mg/L fluconazole was added, and then cells were incubated to particular points in time. Then, cells were harvested and experiments were performed as previously mentioned.

Statistical analysis

Data were analyzed using SPSS 17.0. Statistical significance was determined using Student's *t* test. Statistical significance was determined as $P < 0.05$.

Table 1 Primers used in the study

Gene	Sequence (5'-3')
TAC1-F	gaa ctc acc ctt caa ctt tta aca ac
TACT-R	ctc cga caa cta tca cat gcc act ct
ERG11-F	aac tac ttt tgt tta taa ttt aag atg gac tat tga
ERG11-R	aat gat ttc tgc tgg ttc agt agg t
MDR1-F	tta cct gaa act ttt ggc aaa aca
MDR1-R	act tgt gat tct gtc gtt acc g
CDR2-F	ggt att ggc tgg tcc taa tgt ga
CDR2-R	gct tga atc aaa taa gtg aat gga ttac
CDR1-F	ttt agc cag aac ttt cac tca tga tt
CDR1-R	tat tta ttt ctt cat gtt cat atg gat tga
ACT1-F	ttg gtg atg aag ccc aat cc
ACT1-R	cat atc gtc cca gtt gga aac a
18S-F	gag aaa cgg cta cca cat
18S-R	att cca att aca aga ccc

Results

Fluconazole susceptibility of *C. albicans* planktonic cells and biofilm

Of the 23 isolates of *C. albicans* tested in this study, 8 isolates were susceptible to fluconazole, and 15 were resistant. As shown in Table 2, following biofilm formation, the susceptibility of *C. albicans* to fluconazole decreased to different levels. With prolonged incubation times, the susceptible isolates gradually became more resistant, and finally became highly resistant. The final MICs were more than 256 times higher than initial MICs. Of the resistant isolates, the changes occurred more quickly, with resistance appearing at the earlier stages, including at the 2-h point, with the MIC changing from 16 to 128. There was no change in the MICs of isolates that were initially highly resistant to fluconazole.

C. albicans biofilm growth curves

A CV assay was used to monitor biofilm growth at five time points (Fig. 1). The susceptible strains showed an approximate logarithmic growth up to 24 h, after which the amount of biofilm decreased. The resistant strains showed slow growth between 4 and 8 h after which growth resumed with the same trend as the susceptible strains. Due to the initial lag in growth, the susceptible strains showed more biofilm formation at 8 h than resistant strains.

Gene expression

To evaluate the expression levels of resistance-associated genes, real-time RT-PCR was used. Relative gene expression

levels (ΔCt) were established using the following calculation: $\Delta\text{Ct} [\text{test gene}] = \text{Ct} [\text{test gene}] - \text{Ct} [18\text{S}]$ and the $2^{-\Delta\Delta\text{Ct}}$ method was used. No differences in the expression levels of ACT1 were found between any two isolates ($P > 0.05$), confirming that the experimental protocol had no effect on gene expression. Only three time points were measured as biofilm formation at the earlier two points was low, making it difficult to obtain sufficient amounts of RNA. As shown in Fig. 2, MDR1 was expressed in biofilm in the absence of fluconazole at 8 h ($P < 0.05$). Expression was induced by fluconazole both in biofilm ($P < 0.05$) and planktonic cells ($P < 0.05$). In mature resistant planktonic cell strains, gene expression was upregulated in the absence of fluconazole after 48 h ($P < 0.05$). Expression of CDR1, CDR2, and TAC1 exhibited similar trends (Fig. 3). Gene expression was upregulated at 8 h in biofilm cells in the absence of fluconazole ($P < 0.05$ for CDR1, $P < 0.05$ for CDR2, $P < 0.05$ for TAC1). The addition of fluconazole to biofilms also induced expression at 8 h ($P < 0.05$ for CDR1, $P < 0.05$ for CDR2). After 24 h, CDR1 was more highly expressed in moderately resistant strains of planktonic cells ($P < 0.05$), especially in the absence of fluconazole. Fluconazole did not induce overexpression of CDR1 in biofilms. CDR2 was induced at 8 h ($P < 0.05$) and 24 h ($P < 0.05$), while TAC1 was upregulated at 8 h ($P < 0.05$). In planktonic cells, overexpression of CDR1 was induced with the addition of fluconazole at 8 h ($P < 0.05$). Expression of TAC1 followed a similar trend ($P < 0.05$). With regard to ERG11 (Fig. 4), biofilm did not affect expression. Fluconazole could induce expression ($P < 0.05$); however, ERG11 was downregulated in resistant isolates after 24 h ($P < 0.05$).

Discussion

In recent years, rates of infection with opportunistic pathogens have increased, with *C. albicans* infection most frequently reported. Like other microorganisms, *C. albicans* can adhere to implanted medical devices and form a biofilm. A report by Caliskan and colleagues identified *C. albicans* strains in vaginal and IUD string samples in 14.8% (8/54) and 45.5% (30/66) of samples, respectively [14]. It is estimated that 65% of microbial infections are caused by biofilm rather than planktonic cells [15, 16]. A study by Uppuluri demonstrated that biofilms display high levels of resistance to fluconazole, and although high concentrations of amphotericin B could reduce viability of cells within the biofilms, only caspofungin exhibited potent activity against biofilms [17]. In the present study, we investigated the fluconazole susceptibility of 23 clinical isolates of *C. albicans* after biofilm formation.

In the antifungal susceptibility test, we found different levels of drug susceptibility between strains. Susceptible strains gradually became resistant and by 48 h showed

Table 2 The MICs of fluconazole for planktonic cells and biofilm

Isolate	MIC (mg/L)					
	Planktonic cells	Biofilm 2 h	Biofilm 4 h	Biofilm 8 h	Biofilm 24 h	Biofilm 48 h
13	0.5	2	4	16	32	256
32	0.5	4	16	32	64	> 256
35	0.5	8	16	32	64	> 256
64	2	8	16	32	128	> 256
68	0.5	4	16	32	128	256
74	0.5	4	8	16	32	128
82	1	8	16	32	64	128
84	0.25	2	8	32	128	> 256
<i>509</i>	16	64	128	256	256	> 256
<i>70045</i>	32	64	256	> 256	> 256	> 256
<i>24308</i>	16	64	128	256	256	> 256
<i>208</i>	32	128	128	256	256	> 256
<i>141</i>	16	128	128	256	> 256	> 256
<i>201</i>	16	128	256	> 256	> 256	> 256
<i>205</i>	32	64	128	256	256	> 256
<i>210</i>	32	128	128	256	256	> 256
<i>206</i>	16	32	128	> 256	> 256	> 256
<i>13139</i>	128	256	> 256	> 256	> 256	> 256
<i>64110</i>	> 128	> 256	> 256	> 256	> 256	> 256
<i>21897</i>	> 128	> 256	> 256	> 256	> 256	> 256
<i>H11</i>	> 128	2556	> 256	> 256	> 256	> 256
<i>H49</i>	> 128	> 256	> 256	> 256	> 256	> 256
<i>H66</i>	> 128	> 256	> 256	> 256	> 256	> 256
ATCC 90028	0.25					

Resistant isolates are shown in italics

complete resistance to fluconazole. A second group of strains, the moderately resistant group, developed the resistance more quickly, with MICs of 256 mg/L at 8 h. Highly resistant strains

Fig. 1 Growth curves of *C. albicans*. Growth was measured using a CV assay at 2, 4, 8, 24, and 48 h after biofilm formation. The average is shown in the Figure. Student's *t* test showed a difference in OD₅₉₀ between susceptible and moderately resistant strains at 8 h ($P < 0.05$), and between susceptible and highly resistant strains ($P < 0.05$). (a) susceptible strains; (b) moderately resistant strains; (c) resistant strains

did not show differences in the MICs over time and were highly resistant throughout. Although the method we used was not entirely accurate in identifying the MICs for biofilms (because the initial concentration of biofilm cells was not strictly according the guidelines), it reflected resistance of biofilms to fluconazole *in vivo*. It showed the trend that biofilms become resistant to fluconazole in a time-dependent manner, and illustrate the difficulties in using fluconazole to treat infection once biofilms have matured for 24 h. These findings are important for physicians, who will need to use caution in cases where *C. albicans* or other microorganisms that can form biofilms are detected on the surface of medical devices.

Growth curves showed the biofilm grew rapidly up to 24 h, with the exception of resistant strains which displayed less growth at time points of 4 and 8 h. Unlike a report by Vavala et al. [18], which demonstrated that resistant strains exhibited increased metabolic activity, our study found that susceptible strains formed more biofilm at 8 h, although the differences did not extend to other time points. It is possible that different strains exhibit different growth patterns which

Fig. 2 Fold change in expression levels of *MDRI* in *C. albicans* planktonic cells and biofilm. Quantification of each gene was determined by the $2^{-\Delta\Delta C_t}$ method. An expression level of 1.0 was assigned to the 8-h group of planktonic cells. The letter B indicates the

sample is biofilm and the letter F indicates the addition fluconazole. Error bars represent the standard deviation. (a) susceptible strains; (b) moderately resistant strains; (c) resistant strains

Fig. 3 Fold change in expression levels of *CDR1*, *CDR2*, and *TAC1* in *C. albicans* biofilm and planktonic cells. *C. albicans*. Quantification of each gene was determined by the $2^{-\Delta\Delta C_t}$ method. An expression level of 1.0 was assigned to the 8-h group of planktonic cells. The letter B

indicates the sample is biofilm and the letter F indicates the addition fluconazole. Error bars represent the standard deviation. (a) susceptible strains; (b) moderately resistant strains; (c) resistant strains

Fig. 4 Fold change in expression levels of *ERG11* in *C. albicans* planktonic cells and biofilm. Quantification of each gene was determined by the $2^{-\Delta\Delta C_t}$ method. An expression level of 1.0 was assigned to the 8-h group of planktonic cells. The letter B indicates the

sample is biofilm and the letter F indicates the addition fluconazole. Error bars represent the standard deviation. (a) susceptible strains; (b) moderately resistant strains; (c) resistant strains

are independent of their susceptibility to fluconazole. Further studies will be needed to establish whether drug susceptibility is correlated in any way to growth, although in this study it appeared the two were not related. Growth of all biofilms decreased after 24 h, suggesting that the medium could not provide sufficient nutrients to maintain biofilm growth.

In *C. albicans*, two groups of efflux pumps have been shown to be involved in drug resistance: the ATP binding cassette (ABC) transporters encoded by the CDR genes and the MDR genes encoding the major facilitator (MF) superfamily [19–21]. Reports by Coste et al. showed that *TAC1* is involved in the regulation of *CDR1* and *CDR2* [22], and that a gain-of-function mutation could result in the upregulation of *CDR1* and *CDR2*. The study also demonstrated that the overexpression of *TAC1* could contribute to drugs resistance. Several groups have demonstrated that, in vitro, biofilm can upregulate the expression of *MDR1*, *CDR1*, and *CDR2* [6–8]. In our study, biofilm formation induced the expression of *MDR1* at 8 h, after which expression decreased. The expression was even higher in planktonic cells at 48 h, which is consistent with previous findings [6]. We also found that at 8 h, fluconazole could induce expression of *MDR1* in both types of cells. *MDR1* was involved in resistance of biofilm at early stages, and the addition of fluconazole enhanced expression of *MDR1*. Similarly, *CDR1*, *CDR2*, and *TAC1* played active roles in fluconazole-resistant biofilm in early stages. The addition of fluconazole to both biofilm and planktonic cells resulted in the overexpression of *CDR2* and *TAC1*, although higher expression levels of *CDR1* and *CDR2* were found in biofilm as compared to planktonic cells. These results suggest that the addition of drugs induces gene expression in biofilm for protective purposes. Interestingly, the moderately resistant strains were an exception. After 24 h, *CDR1* was more highly expressed in planktonic cells, which we proposed is likely due to the genotype of the isolates. In our previous studies, isolates 509 and 201 demonstrated an upregulation of *CDR1*, while *TAC1* contained mutations N776D and A736V

which was not found in any other isolates from this study (data not shown). The mutation A736V had been reported to be involved in regulation of CDR genes [22]. It is possible that mutations in the genes regulating *CDR1*, such as *TAC1*, result in the planktonic cells displaying higher expression levels after 24 h.

Studies have demonstrated that efflux pumps are not essential for resistance in biofilm [7, 23, 24]. In our study, efflux pumps appeared to play a role in resistance in the early stages of biofilm growth, although drug resistances did not increase significantly. It is possible that the efflux pumps are only involved in the early stages of biofilm formation, while in mature biofilm there are other mechanisms conferring resistance. Moreover, basal expression levels of the genes encoding efflux pumps may affect regulation in biofilm.

An enzyme targeted by fluconazole in *C. albicans* is encoded by *ERG11*. Mutations and the overexpression of this gene contribute to resistance [25]. The changes in expression of *ERG11* have been shown to be involved in drug resistance in biofilms [26]. In our study, however, there was no difference in expression between biofilm-associated cells and planktonic cells. However, upon the addition of fluconazole, both groups showed increased expression, which supports a study by Nailis and colleagues [27]. After 24 h, when challenged with fluconazole, the highly resistant strain did not show upregulation of *ERG11*, instead the expression decreased. Notably, the strain contained two mutations, Y132F and K143Q, in *ERG11*, which were confirmed to confer resistance. We hypothesize that resistance after 24 h was mainly due to structural changes in the target, rather than the overexpression, and that structural changes may affect the susceptibility to a larger extent. The overexpression of *ERG11* can decrease susceptibility, but it did appear to be involved in resistance in biofilm.

In conclusion, in our study, the 23 clinical isolates of *C. albicans* exhibited a high level of resistance to fluconazole after biofilm formation. We did not find any correlation

between the growth status and the susceptibility to drugs of the different strains. Efflux pumps displayed a role in resistance at early stages, and the addition of fluconazole induced essentially all the tested gene in each cell type. Further studies will be needed to fully elucidate the mechanism of resistance of biofilm to antifungal drugs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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