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Gene Expression Analysis of Key Players Associated with Fluconazole Resistance in *Candida albicans*

Hongxia Zhang [1], Qiufang Xu [2], Suchen Li [1], Ying Ying [1], Zhiqin Zhang [3], Lingbing Zeng [4], Xiao-Tian Huang [1] and Shibo Huang [1, 5, *]

[1] Jiangxi Medical College, Nanchang University, Nanchang, China
[2] Qingpu District Center for Disease Control and Prevention, Shanghai, China
[3] Pingxiang Second People's Hospital, Pingxiang, China
[4] Department of Clinical Laboratory, The First Affiliated Hospital of Nanchang University, Nanchang University, Nanchang, China
[5] Jiangxi Province Key Laboratory of Tumor Pathogens and Molecular Pathology and Department of Pathology, Schools of Basic Medical Sciences and Pharmaceutical Sciences, Nanchang University Medical College, Nanchang, China

[*] *Corresponding author*: Jiangxi Medical College, Nanchang University, 461 Bayi Road, Nanchang 330006, China. Tel: +86-79186360654, Email: hsb@ncu.edu.cn

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Abstract

Background: Fluconazole resistance in *Candida albicans* has become a serious public health problem. Most previous studies have focused on deciphering the relationship between fluconazole resistance and amino acid substitutions in *ERG11*, which encodes cytochrome P450 lanosterol 14α-demethylase whose enzymatic activity is inhibited by azoles. However, azole resistance in *C. albicans* is a multifactorial phenomenon and several lines of evidence indicate that other genes and mechanisms may contribute to the development of fluconazole resistance.

Objectives: The present study aimed to investigate the underlying role of six genes in fluconazole-resistant clinical strains of *C. albicans*, including *ERG11, RTA2*, and the efflux pump genes *CDR1, CDR2, MDR1*, and *FLU1*.

Methods: We collected 40 fluconazole-resistant isolates and 40 susceptible isolates from patients with *Candida* infections in the First Affiliated Hospital of Nanchang University in China from 2005 to 2008. The susceptibility of the isolates to antifungal agents was tested by the M27-A3 broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Then, the gene expression levels of several key players in azole resistance were quantified.

Results: Most fluconazole-resistant strains analyzed in this study were found to be cross-resistant to ketoconazole, itraconazole, and clotrimazole. We observed that the *FLU1* gene expression significantly increased ($P < 0.05$) and exhibited major changes in most of the fluconazole-resistant isolates (75.0%). In addition, the expression of a novel gene, *RTA2*, was remarkably upregulated ($P < 0.05$). Interestingly, we found that 10% of the fluconazole-resistant isolates were simultaneously associated with *ERG11* mutation and overexpression of *RTA2, CDR1*, and *FLU1* genes. Unlike other studies, we did not find any difference in the expression of *CDR2, MDR1*, and *ERG11* genes between the fluconazole-susceptible and resistant isolates.

Conclusions: Our findings suggested that the overexpression of *FLU1* and *RTA2* genes may cause azole resistance; this finding had not been reported previously in clinical isolates of *C. albicans*. The upregulation of *FLU1* and *RTA2* genes was the predominant mechanism of fluconazole resistance in *C. albicans* in China.

Keywords: *Candida albicans*, Fluconazole, Drug resistance, ATP-Binding Cassette Transporter

1. Background

Candida albicans is a predominant fungal opportunistic pathogen responsible for both mucosal and systemic infections, and is especially prevalent in immunodeficient hosts (1-4). Infections caused by *C. albicans* are mainly treated with azoles, which include both imidazoles (miconazole, clotrimazole, and ketoconazole) and triazoles (fluconazole and itraconazole). However, the long-term, repeated usage of azoles has resulted in the emergence of resistant isolates (5, 6). Drug resistance is a serious complication that can be challenging to clinicians, posing a major hurdle in the success of antifungal therapy (7). Previous studies have shown that decreased susceptibility to azoles is mainly associated with alterations in the *ERG11* gene and/or constitutive upregulation of multidrug efflux pumps (8, 9). Azoles act as non-competitive *ERG11* inhibitors through coordination with the iron atom of the heme group located in the active site of 14-α-sterol demethylase.

Numerous publications have shown that non-

synonymous point mutations in *ERG11* may lead to conformational changes, resulting in the decreased affinity of azoles for the 14-α-sterol demethylase enzyme (9-11). In addition, elevated gene expression levels of efflux transporters can reduce the intracellular accumulation of certain drugs, and is often the main mechanism of azole resistance in clinical *Candida* (12). There are two families of membrane-associated efflux pumps, including ATP-binding cassette (ABC) superfamily (12) and major facilitator superfamily (MFS) (13), which have been found to be upregulated in resistant isolates. Two major ABC transporter genes, *CDR1* (14) and *CDR2* (Candida Drug Resistance1 and 2), are well-documented in clinical drug resistance. Both genes have shown to be upregulated in azoles-resistant *C. albicans* isolates and the genetic deletion of both genes in *C. albicans* results in hypersensitivity to azoles (15-19).

The expression levels of *MDR1* (Multidrug resistance1) and *FLU1* (Fluconazole resistance1), which are the members of the MFS, have also shown to be specifically upregulated in azole-resistant *C. albicans* strains (20, 21). In addition, several other factors can contribute to *C. albicans* azole resistance, including processes involved in biofilm formation and mutations in other enzymes in the ergosterol pathway (22). More recently, a novel gene (resistant to 7-aminocholesterol, *RTA2*), which has also shown to contribute to the development of azole resistance, has been identified in a mutant strain lacking the *CDR1, CDR2*, and *MDR1* genes (23). Taken together, it is apparent that drug resistance is a multifactorial phenomenon. Due to the increased number of resistant strains, it is increasingly important to investigate the underlying mechanisms of azole resistance for the development of new antifungal treatments for *C. albicans*.

2. Objectives

In our previous study, we sequenced several fluconazole-resistant clinical isolates and found that 63.27% of the isolates did not contain mutated *ERG11* (24). Our findings strongly suggested that other resistance mechanisms exist that require further research. In this study, we first analyzed the susceptibility of these isolates to other antifungal agents, and then investigated the relationship between fluconazole resistance, alternations in the *ERG11* gene, and the expression levels of several membrane-associated efflux pumps (*CDR1, CDR2, MDR1, FLU1*) and *RTA2* genes to decipher the potential molecular mechanisms leading to fluconazole-resistance in these clinical *C. albicans* strains.

3. Methods

3.1. Strains

We selected 40 fluconazole-resistant *C. albicans* isolates from our previous study using standard drug susceptibility analysis assays, defined herein as the fluconazole-resistant group. Additionally, 40 *C. albicans* isolates, which were susceptible to all drugs evaluated in our study, were defined as the susceptible group. All the *C. albicans* isolates were obtained from patients undergoing treatment for respiratory, genital, bloodstream, urinary, and digestive tract infections from 2005 to 2008 at the First Affiliated Hospital of Nanchang University in China. For drug susceptibility analysis, we used *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 as quality control reference strains. Fluconazole-sensitive *C. albicans* ATCC 90028 was included as a control for the analysis of gene expression.

3.2. Antifungal Agents

Ketoconazole, itraconazole, 5-flucytosine, amphotericin B, clotrimazole, and nystatin were obtained from Sigma-Aldrich (USA). The measurable concentrations of ketoconazole, itraconazole, amphotericin B, clotrimazole, and nystatin ranged from 0.0313 to 16 μg/mL and dilutions of 5-flucytosine used herein ranged from 0.125 to 64 μg/mL.

3.3. Drug Susceptibility Analysis

The antifungal agent susceptibility analysis was performed according to the CLSI Broth Microdilution Susceptibility Method (M 27-A3 Document) for antifungal susceptibility of yeast. Briefly, stock inoculum suspensions of *C. albicans* were prepared and diluted with RPMI 1640 broth to obtain two inoculums (1×10^3 to 5×10^3 CFU/mL). Next, we plated 100 μL of each suspension into wells already containing 100 μL of the given antifungal agent (final inoculum size of 5×10^2 to 2.5×10^3 CFU/mL). Then, the plates were cultured at 35°C for 48 h. The resistance breakpoint categories of 5-flucytosine, itraconazole, and fluconazole were used according to the CLSI M27-A3 criteria (25) while ketoconazole and clotrimazole were based on previous investigations (26), as shown in Table 1. The minimal inhibitory concentrations (MICs) were determined as the lowest concentration causing at least 80% growth inhibition compared to control wells not containing the antifungal agent. The MICs for amphotericin B and nystatin were identified as the minimum concentration yielding a complete growth inhibition.

Table 1. The Interpretive Breakpoints of Drug Susceptibility to Fluconazole, 5- Flucytosine, Itraconazole, Ketoconazole, and Clotrimazole

Drugs	S, μg/mL	S-DD, μg/mL	R, μg/mL
Fluconazole (25)	$\leq$ 8	16 ~ 32	$\geq$ 64
5-flucytosine (25)	$\leq$ 4	8 ~ 16	$\geq$ 32
Itraconazole (25)	$\leq$ 0.125	0.25 ~ 0.50	$\geq$ 1.0
Ketoconazole (26)	$\leq$ 0.125	0.25 ~ 0.50	$\geq$ 1.0
Clotrimazole (26)			$\geq$ 0.5

3.4. RNA Extraction

Total cellular RNA was isolated from *C. albicans* at mid-exponential growth phase (OD_{600} = 0.8) using the hot phenol method (27). Briefly, cells were collected by centrifugation at 4000 rpm for 5 min at room temperature, the supernatant was removed, and then the pellets were spun down a second time to remove all liquid. Next, cell pellets were resuspended in 800 μL of AE buffer (50 mm sodium acetate, 10 mm EDTA, pH 5.2). Then, 80 μL of 10% SDS and 880 μL of pre-warmed phenol (pH 5.2) were added and the samples were vortexed thoroughly. The tubes were transferred to a 65°C water bath and vortexed for 5 s once every 1 min for 5 min. Lysates were transferred and kept in a dry ice/ethanol bath for 2 - 3 min, followed by centrifugation at 13,200 rpm for 10 min at room temperature.

The aqueous layers were carefully transferred to new microcentrifuge tubes and an equal volume of phenol (pH 5.2): chloroform: isoamyl alcohol (25: 24: 1) was added. Then, samples were mixed by vortexing and centrifuged again at 13,200 rpm for 10 min at room temperature. The aqueous layers were then carefully transferred to new microcentrifuge tubes. RNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of chilled 100% ethanol, followed by incubation at -20°C overnight. Precipitated RNA was collected by centrifugation at 13,200 rpm for 15 min at 4°C. The entire supernatant was decanted; the RNA pellet was washed with 1 mL of chilled 75% ethanol and centrifuged again. The pellet containing RNA was air-dried in a hood for 5 - 10 min and then resuspended in 100 μL of DEPC water; then, it was treated with RNase-free DNase (ThermoFisher, USA) to prevent genomic DNA contamination. The quantity and quality of RNA were assessed by the measurement of the OD 260/280 absorption ratio and running the denaturing formaldehyde agarose gel.

3.5. Quantitative Real-Time PCR

The cDNA was synthesized using 2 μg of total RNA according to the manufacturer's instruction of the PrimeScript™ RT Reagent kit with gDNA Eraser (Takara, Japan). Gene-specific PCR primers for *RTA2, CDR1, CDR2, ERG11, FLU1*, and *MDR1* genes and 18S rRNA were designed using Primer Premier 5.0 (Table 2). The 18S rRNA was included as an internal reference gene. The qRT-PCR analysis was performed using the 7500 Real-time PCR System (Applied Biosystems, USA) and the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Japan). All samples contained 10 μL of 2X SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (including TaKaRa Ex Taq® HS, dNTP Mixture, Mg^{2+}, Tli RNaseH, SYBR® Green I), 0.8 μL of forward and reverse primers, 1 μL of cDNA template, 0.4 μL of ROX Reference Dye, and 7.2 μL of nuclease-free water.

Table 2. The Primer Sequences Used for Quantitative Real-time PCR

No.	Primer Name	Sequence (5' - 3')
1	CaERG11a-F	CAAGAAGATCATAACTCAAT
	CaERG11a-R	CAGAACACTGAATCGAAAGA
2	CaERG11b-F	TTTGGTGGTGGTAGACATAGAT
	CaERG11b-R	TAATCAGGGTCAGGCACTTT
3	CaCDR1-F	GATTCTCAAACTGCCTGGTC
	CaCDR1-R	CCAAAATAAGCCGTTCTTCCAC
4	CaCDR2-F	AAAAAGGTGGAAGAACGGC
	CaCDR2-R	TTGGCATGAGATCCTGGTG
5	CaMDR1-F	TGCGTCAAGAACAGGTTITC
	CaMDR1-R	AAGCAGTAGTAGCAGCACC
6	CaFLU1-F	TGGATAGTCCCCGTCATTGG
	CaFLU1-R	GGCAAAAAGTGGGAAAACAGC
7	CaRTA2F	ATGTCAAATCGGTAAGAGGTC
	CaRTA2R	AGCCAATTCTGCCACTCTAT
8	18S RNA-F	TCTTTCTTGATTTTGTGGGTGG
	18S RNA-R	TCGATAGTCCCTCTAAGAAGTG

The PCR conditions were as follows: 40 cycles of denaturation for 5 s at 95°C, annealing for 34 s at 60°C for 18S rRNA, *MDR1, CDR2, CDR1, FLU1, RTA2*, and *ERG11*, and a melt curve step (from 60°C, gradually increasing at 0.5°C/s to 95°C, with acquisition data every 1 s). Fluorescent data were collected during the annealing step and analyzed with ABI software. The threshold cycle (ΔCT) value was obtained by calculating the difference between the CT values of the target gene and the normalizer (18S rRNA). The mean mRNA levels for each gene were calculated from at least three independent biological replicates. The expression levels of target genes were analyzed according to the $2^{-\Delta\Delta Ct}$ method (28). Differences between resistant and susceptible groups were analyzed with independent sample *t*-test.

3.6. Statistical Analysis

The results are expressed as means ± standard deviation (SD). Comparisons between the sensitive and resistant groups were made using independent-samples *t*-tests by SPSS 13.0 software. The differences were considered statistically significant when the P values were less than 0.05.

4. Results

4.1. Antifungal Susceptibility

As described in Table 3, all fluconazole-resistant strains were susceptible to 5-flucytosine. Moreover, 25 (67.74%) fluconazole-resistant isolates were also resistant to ketoconazole, 28 (70.97%) were resistant to itraconazole, and 23 (64.52%) were resistant to clotrimazole. Interestingly, the fluconazole-resistant isolates, which were associated with the mutation of *ERG11*, exhibited a higher MIC for nystatin, while the isolates that were not associated with the mutation of *ERG11* were cross-resistant to other azoles.

4.2. Expression of ERG11

The melting peaks and curves of the *ERG11* amplicon indicated that there were no non-specific amplification products or primer dimers produced during the reactions. Further, a single band representing the PCR product with the expected length on an agarose gel confirmed the specificity of the PCR (data not shown). Gene expression levels of *ERG11* from 40 fluconazole-resistant and -susceptible isolates were analyzed by the real-time PCR. The mean ΔCt values of the *ERG11* gene in the fluconazole-resistant group and the susceptible group were 16.80 ± 0.18 and 17.16 ± 0.26, respectively, with no statistically significant difference (P = 0.25).

4.3. The Expression of Efflux Pumps

The *CDR1* expression level was 3.68 folds higher in the fluconazole-resistant group than in the fluconazole-susceptible group (Figure 1). The mean ΔCt values of *CDR1* expression were 15.92 ± 0.28 and 16.80 ± 0.24 in the resistant and susceptible groups, respectively. The expression of *CDR1* was significantly different between the fluconazole-susceptible and resistant groups (P = 0.0193). Among the 40 fluconazole-resistant isolates, isolate 59679 displayed the highest level of *CDR1* gene expression (23.07 folds) (Figure 1B). In our previous work, we found that only 36.73% (19/49) of the fluconazole-resistant isolates were associated with the mutation in the *ERG11* gene. In this study, we further analyzed the differences between isolates related and unrelated to the mutation in the *ERG11* gene and found that *CDR1* expression was altered in 80.00% of the fluconazole-resistant isolates containing a mutant variant of *ERG11*, and that the expression level of *CDR1* was more than 1.5-fold higher in these isolates (Figure 1A).

Among the 40 fluconazole-resistant isolates, 31 (77.50%) isolates exhibited 1.29 to 10.50-fold upregulation of the *FLU1* gene (Figure 2). The mean ΔCt values of the *FLU1* gene were 17.64 ± 0.16 and 18.39 ± 0.15, respectively, in the resistant and susceptible groups. The difference in *FLU1* gene expression was 0.0010 between fluconazole-resistant and susceptible groups, which was statistically significant (P = 0.0010). Compared to the susceptible isolates, the expression levels of *FLU1* were not upregulated in most resistant isolates, which carried a mutant variant of *ERG11*. The mean ΔCt values of the *CDR2* and *MDR1* genes were 15.96 ± 0.25 and 21.51 ± 0.31, respectively, in the resistant group and 16.27 ± 0.45 and 21.72 ± 0.17, respectively, in the susceptible group. The mRNA expression levels of *CDR2* and *MDR1* were not statistically different between fluconazole-resistant and susceptible groups (P = 0.55 and 0.56, respectively).

4.4. Expression of RTA2

Among 40 fluconazole-resistant isolates, 19 (47.5%) isolates exhibited more than 1.5-fold upregulation in the *RTA2* gene when compared to the fluconazole susceptible isolates, and the mean *RTA2* gene expression in resistant isolates was 4.91 folds higher (Figure 3). The mean ΔCt values of the *RTA2* gene were 16.08 (SD = 0.28) and 17.19 (SD = 0.20), respectively, in the resistant and susceptible groups, which indicated that *RTA2* gene expression was significantly different between these two groups (P = 0.0017).

5. Discussion

Azole is a widely used antifungal agent for the treatment of both superficial mucosal and deep disseminated *Candida* infections. However, the widespread application of azoles and the structural similarity of these molecules have resulted in the development of cross-resistance to various members of this class of drugs (29). Previous studies demonstrated that fluconazole resistance was correlated with cross-resistant to other azoles (30). Recently, researchers found that about 74% of fluconazole-resistant strains were also resistant to ketoconazole and itraconazole (31). Our present study demonstrated that most fluconazole-resistant isolates were cross-resistant to other azoles.

Resistance to a variety of drugs is defined as multidrug resistance (MDR). In infectious bacteria, such as *Mycobacterium tuberculosis*, the emergence of MDR is caused by a series of point mutations in different target genes (32). A similar process can be found in fungal infections, as well.

Table 3. The Minimal Inhibitory Concentrations for Antifungal Agents and *ERG11* Gene Mutations of Clinical *Candida albicans*

Isolate	FlZ	MIC, μg/mL						Amino Acid Change (s) in Erg11p
		KETO	ITR	CLOT	5-FC	NYS	B	
56388	64	8	4	2	2	16	4	A114S Y257H
49372	64	0.5	16	0.5	2	8	1	A114S Y257H
49922	64	0.5	4	0.25	0.125	8	1	A114S Y257H
56539	64	0.25	0.5	0.25	1	8	1	A114S Y257H
57451	64	1	2	4	0.25	4	4	A114S Y257H
57800	64	0.0625	0.5	0.625	0.125	4	2	A114S Y257H
58181	64	1	4	0.25	0.125	4	2	A114S Y257H
59161	64	0.25	2	0.25	0.125	4	2	A114S Y257H
59182	64	0.5	1	2	4	8	1	A114S Y257H
56472	64	16	2	0.25	0.25	0.125	0.5	D116E K128T Y132H G465S
59690	64	16	8	0.25	0.5	8	2	D116E K128T Y132H G465S
51527	64	1	0.5	0.25	4	2	2	G450E Y132H
58614	64	0.0313	0.0313	0.25	0.125	16	4	Y132H G450E
56392	64	2	16	0.5	0.125	8	4	Y132H G488E
56292	64	8	16	1	0.125	4	1	A114S Y257H
54535	64	0.5	0.125	0.125	0.125	2	2	-[a]
49340	64	8	4	2	1	2	1	-[a]
49345	64	0.5	16	0.5	0.25	4	1	-[a]
56350	64	16	16	2	0.125	1	4	-[a]
56525	64	8	16	1	0.125	4	4	-[a]
57598	64	8	16	0.5	0.25	4	4	-[a]
49312	64	16	0.25	0.25	0.125	2	1	D116E
49477	64	0.5	16	0.25	0.125	4	1	D116E
56452	64	16	4	0.0625	0.25	0.125	2	D116E
56477	64	16	16	0.25	0.125	4	4	D116E
56507	64	8	16	0.5	0.5	4	4	D116E
56533	64	16	16	0.5	1	8	4	D116E
59145	64	16	1	1	0.25	4	2	D116E
57464	64	16	1	0.25	0.25	2	4	D116E E266D
59537	64	0.0625	0.125	0.625	0.125	4	0.5	D116E E266D V488I
59407	64	1	0.25	0.25	0.25	4	2	D116E V488I
57856	64	0.125	1	0.25	0.125	4	4	D11DE E266D V488I
56682	64	16	16	1	0.125	4	1	D225H E266D
56517	64	16	16	2	0.125	4	2	E266D
56689	64	0.0313	0.125	0.625	0.125	4	2	E266D
56262	64	1	16	1	8	4	4	E266D V437I V488I
59679	64	16	0.5	2	0.25	4	2	E266D V488I
56214	64	4	16	0.5	0.25	8	4	E266D V488I
57442	64	0.125	0.5	1	0.25	4	0.5	K342R
55475	64	64	0.25	0.25	0.125	0.125	4	V437I

Abbreviations: B, amphotericin B; CLOT, clotrimazole; FLZ, fluconazole; ITZ, itraconazole; KETO, ketoconazole; NYS, nystain.
[a] Isolates with no missense mutation in *ERG11*.

Azoles inhibit the enzyme lanosterol 14α-demethylase, which is encoded by the *ERG11* gene. A mutation in the *ERG11* gene or its overexpression may affect the enzyme's affinity for drugs, resulting in resistance (10). However, in our previous study, 63.27% (31/49) of the fluconazole-resistant isolates were not associated with point mutations in the *ERG11* gene, indicating that other factors are involved in azole resistance in these strains. In this study, we further investigated the relationship between *ERG11* expression/mutation and fluconazole resistance. The overexpression of the *ERG11* gene may increase the production of drug target enzymes to an extent exceeding the inhibitory capacity of antifungal drugs, which may, in turn, contribute to fluconazole resistance. However, the role of overexpression of *ERG11* in fluconazole resistance remains enigmatic. Some studies have suggested that the overexpression of *ERG11* is not significantly related to fluconazole resistance in *C. albicans* (33). Similar to previous reports, we did not find any significant difference in the expression of *ERG11* between multi-azole susceptible and resistant strains in this

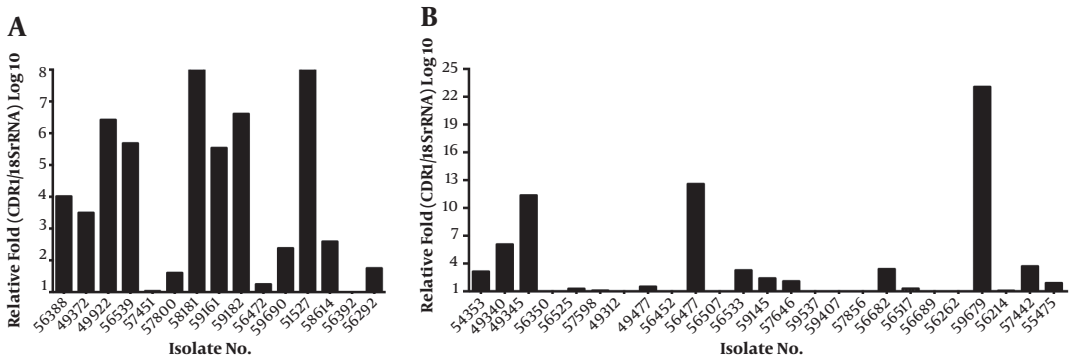

Figure 1. The CDR1 gene upregulation in fluconazole-resistant isolates. A, *CDR1* gene expression in fluconazole-resistant isolates that were associated with *ERG11* gene point mutations; B, *CDR1* gene expression in fluconazole-resistant isolates that were not associated with *ERG11* gene point mutations.

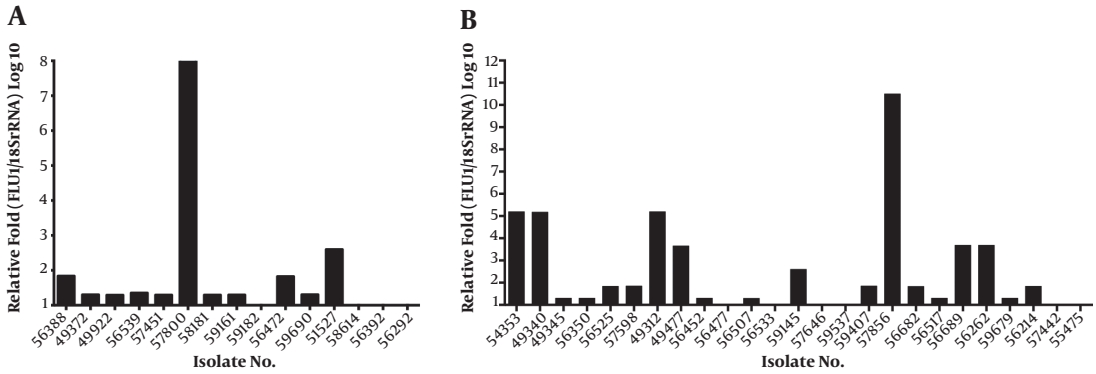

Figure 2. The *FLU1* gene upregulation in fluconazole-resistant isolates. A, *FLU1* gene expression in fluconazole-resistant isolates that were associated with *ERG11* gene point mutations; B, *FLU1* gene expression in fluconazole-resistant isolates that were not associated with *ERG11* gene point mutations.

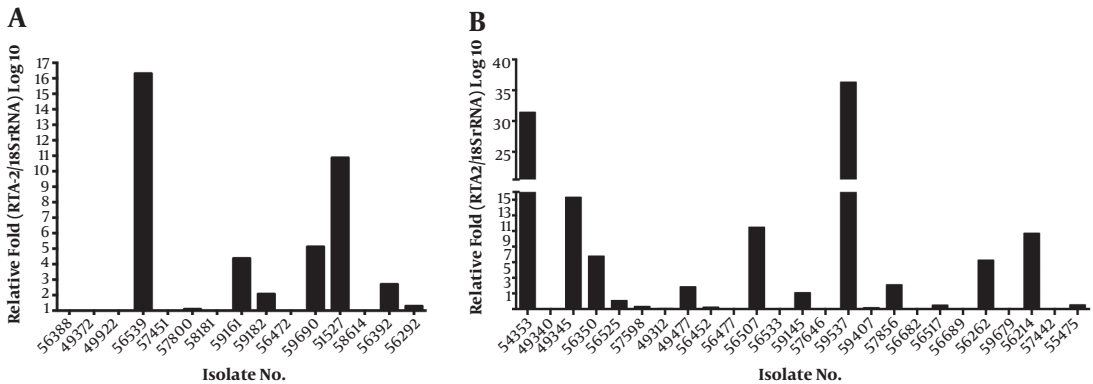

Figure 3. *RTA2* gene overexpression in fluconazole-resistant isolates. A, *RTA2* gene expression in fluconazole-resistant isolates that were associated with *ERG11* gene point mutations; B, *CDR1* gene expression in fluconazole-resistant isolates that were not associated with *ERG11* gene point mutations.

study.

In *Candida*, another important mechanism of drug resistance is associated with efflux transporters. In this study, we aimed to elucidate the role of efflux transporters in the development of fluconazole resistance. As previously reported, the overexpression of the *CDR* gene is one of the

most predominant mechanisms of MDR in azole-resistant *Candida* clinical isolates. Prior studies found that a drug efflux pump-encoding *CDR* gene contributed to the development of the cross-resistance phenotype in *C. glabrata* strains (34). Mdr1p, encoded by the *MDR* gene, is a member of the MFS transporters, and is able to pump several structurally unrelated compounds out of the cell, including fluconazole. The expression levels of *CDR1, CDR2*, and *MDR1* genes increased in most clinical isolates with fluconazole MICs of $\geq$ 64 μg/mL, while the disruption of these genes resulted in hypersensitivity to azoles (34). Furthermore, the increased expression of *CDR1, CDR2*, and *MDR1* genes was a major contributor to azoles resistance in clinical isolates (21, 35-38). In this study, our results suggested that the *CDR1* gene was upregulated in fluconazole-resistant isolates. However, unlike most reports, no correlation was observed between MDR and overexpression of *CDR2* and *MDR1* in this study.

FLU1, encoding Flu1p, is a multidrug efflux transporter implicated in mycophenolic acid resistance. Similar to *CDR, FLU1* was discovered in a genomic library screened for the complementation of fluconazole hyper-susceptibility in the Pdr5 (ABC transporter gene) mutant *Saccharomyces cerevisiae* isolates (20). When the *FLU1* gene was heterologously expressed in *S. cerevisiae*, it mediated both fluconazole and cycloheximide resistance (20). The deletion of *FLU1* leads to insignificant changes in susceptibility to fluconazole. However, the deletion of *FLU1* in a strain based on the disruption of other genes encoding multidrug efflux pumps (such as *CDR1, CDR2*, and MDR) may cause increased susceptibility to several azole derivatives (20). Similarly, the gene product of *FLU1* has been found to mimic Tpo1 from *S. cerevisiae*, which is a primary plasma membrane polyamine efflux transporter (39). Further study found that *FLU1* is able to pump Histatin 5 out of the cell and reduce the toxicity of Histatin 5 in *C. albicans* (40). However, the overexpression of *FLU1* is generally uncommon among clinical resistant isolates of *C. albicans*. In most studies, changes in the expression level of *FLU1* were not significant between azole-resistant and susceptible *C. albicans* isolates (21, 37). Interestingly, our findings showed that upregulation of *FLU1* was one of the dominant mechanisms in the fluconazole-resistant isolates analyzed in this study.

Several lines of evidence suggest that fluconazole resistance may involve many unknown mechanisms that have yet to be elucidated. Recently, studies found that calcium signaling plays an important role in the development of drug resistance and it may be a target for overcoming drug resistance (41, 42). A novel gene, *RTA2*, which mediates calcineurin-dependent resistance to azoles, was found to contribute to the development of fluconazole resistance (23, 43). The knockdown of *RTA2* leads to higher susceptibility of *C. albicans* to fluconazole. Conversely, ectopic expression of *RTA2* resulted in markedly decreased fluconazole efficacy in mice with systemic *Candida* infections (44). Furthermore, previous studies found that the *RTA2* gene was over-expressed in both laboratory and clinical resistant strains (45). Consistently, the *RTA2* expression levels elevated in our present study.

Our previous results showed that only 36.73% of fluconazole-resistant strains were associated with point mutations in *ERG11*. Interestingly, we also found that 75.0% of the fluconazole-resistant isolates exhibited the overexpression of *FLU1* gene, 62.5% were associated with upregulation of *CDR1* (more than 1.5-fold expression), and 45% showed high levels of *RTA2* expression. In addition, we found that 10% of the fluconazole-resistant isolates were associated simultaneously with *ERG11* mutation and overexpression of *RTA2, CDR1*, and *FLU1* genes.

These results indicate that multiple genes are associated with fluconazole resistance. Additionally, the upregulation of *CDR1* was a major mechanism in fluconazole-resistant isolates having point mutations in *ERG11*. Interestingly, we found that more than 90% of the fluconazole-resistant isolates with A114S and Y132H *ERG11* variants exhibited the upregulated expression of *CDR1*. It seems that the specific *ERG11* mutant variants A114S and Y132H may be associated with *CDR1* overexpression. However, further studies need to investigate the relationship between *CDR1* expression and *ERG11* variants A114S and Y132H. For the isolates that were not related to mutant variants of *ERG11*, the overexpression of *FLU1* and *RTA2* was a major contributor to drug resistance.

5.1. Conclusions

In conclusion, we demonstrated that the overexpression of *FLU1* and *RTA2* was correlated with azole resistance; this finding had not been reported previously in clinical isolates of *C. albicans*. Taken together, this study provides useful information for the treatment of candidiasis and indicates that clinicians should be cautious of cross-resistance within this class of antifungal drugs, especially for the treatment of patients with prior azoles prophylaxis or patients at high risk of *C. albicans* infections. Moreover, fluconazole resistance in *C. albicans* is a multifactorial phenomenon with complicated mechanisms. Therefore, it is important to notice that most of these mechanisms are frequently combined in a single isolate to contribute to a step-by-step acquisition of fluconazole resistance.

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Footnotes

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