

Mechanisms of azole resistance in clinical isolates of *Candida glabrata* from two hospitals in China

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Purpose: *Candida glabrata* has emerged as the second or third most common non-*albicans* species responsible for an increasing number of systemic infections. Moreover, its high-level of resistance to azole is associated with a high mortality rate. This study aimed to evaluate nosocomial infections and resistance characteristics of *C. glabrata* and to explore the mechanism of azole resistance in *C. glabrata*.

Patients and methods: Fifty-nine clinical *C. glabrata* isolates were collected from two hospitals in China. The susceptibility of the strains to antifungal agents was determined by both the ATB Fungus 3 strip and CLSI M27 broth microdilution method. Efflux of rhodamine 6G was examined to evaluate the effects of efflux pumps. The expression levels of *CgCDR1*, *CgCDR2*, *CgSNQ2*, *CgERG11*, and *CgPDR1* were examined by real-time PCR. The sequences of *CgERG11* and *CgPDR1* were determined by PCR-based DNA sequencing.

Results: All 59 isolates of *C. glabrata* were susceptible to flucytosine and amphotericin B. Twelve (20.3%) isolates were determined to be fluconazole-resistant, whereas 13 (22.0%) and 27 (45.7%) isolates were categorized as non-wild-type for itraconazole and voriconazole, respectively. Efflux pumps in azole-resistant isolates showed stronger effects than those in azole-susceptible-dose dependent isolates, which is consistent with the significant upregulation of *CgCDR1* and *CgCDR2* ($P < 0.05$), whereas no obvious differences were found for *CgSNQ2*, *CgERG11*, and *CgPDR1* ($P > 0.05$). Sequencing of *CgERG11* showed no alteration favoring the hypothesis that *CgERG11* is not involved in the azole resistance of *C. glabrata*. Four *CgPDR1* missense mutations were found in azole-resistant isolates, of which the high frequency of the *CgPDR1* mutation, A848V, has not been reported previously.

Conclusion: Efflux pump function is the main mechanism of resistance to fluconazole in our collected clinical isolates of *C. glabrata*, and further studies of the related gene disruption and genome-wide expression are needed to verify the function.

Keywords: *Candida glabrata*, antifungal susceptibility, azole, resistance mechanisms, efflux pump

Introduction

Candida species have become important opportunistic pathogens responsible for a growing number of candiduria and systemic infections.¹ The morbidity and mortality caused by non-*albicans Candida* species are rising, although *Candida albicans* remains the commonest *Candida* species.² Recent studies showed that *C. glabrata* has emerged as the second most common non-*albicans* species and causes fungemia in many countries,^{3,4} representing the highest proportion in

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Northern and Central Europe,⁵ and accounting for 21% of all *Candida* bloodstream isolates in the United States,⁶ 18.1–40.7% in North America and 8.5–31.0% in northern Europe.⁷ A study of 814 yeast isolates in the China Hospital Invasive Fungal Surveillance Net (CHIF-NET) showed that *Candida* spp. isolates were broadly distributed and constituted 92.3% of blood culture isolates, while the *C. glabrata* species complex was the third most common non-*albicans* species in China.⁸ Fungal infections can be treated with several antifungal agents including fluconazole, itraconazole, and voriconazole, which are widely used for *Candida* treatment. However, *C. glabrata* exhibits intrinsically low susceptibility and can develop resistance frequently during prolonged exposure to the class of azole antifungals.^{9,10} Azole resistance among *Candida* spp. arises from the overexpression of efflux pumps, while up-regulation of transporters, overexpression of drug targets and mutations are increasingly becoming common, and alternative mechanisms, such as mitochondrial defects and biofilm formation, have also been recently documented. In *C. glabrata*, studies of azole-resistant isolates have demonstrated frequent constitutive overexpression of multidrug transporters of the ATP-binding cassette transporter. Several genes encoding these transporters contribute to this phenotype and have been identified, including *C. glabrata* *CDR1* (*CgCDR1*), *CgCDR2*, and *CgSNQ2*.^{11,12} These transporters are regulated by the zinc finger transcription factor *CgPdr1*, which functionally resembles the *Saccharomyces cerevisiae* *Pdr1* (*ScPdr1*) and *ScPdr3* transcription factors.¹³ Vermitsky et al¹⁴ previously reported that expression of *CgPDR1* was elevated in one of seven *C. glabrata* fluconazole resistant mutants. Compared to the wild type *Pdr1*, the strain showing a higher *CgPDR1* expression had a single mutation, P927L, which caused azole resistance in the mutant strain. Additionally, azole resistance may be caused by increased expression of the gene coding for the azole target (*CgERG11*).^{15,16} In contrast, point mutations in *CgERG11* that are involved in the resistance of *C. glabrata* to azoles have not been identified. The aim of the present study was to explore whether the molecular mechanisms described above, alone or in combination, are enough to fully interpret the phenotype of azole resistance in clinical isolates of *C. glabrata* or if other mechanisms are correlated with azole resistance. Thus, we set out to explore these mechanisms in 59 clinical isolates of *C. glabrata* collected in China by

focusing on their efflux pumps, transporter and azole antifungal target enzyme.

Material and methods

Strains and medium

A total of 59 *C. glabrata* isolates were collected from the Renji hospital affiliated to Shanghai Jiao Tong University School of Medicine and Dongfang hospital affiliated to Shanghai Tongji University. The isolates were identified based on standard clinical microbiological methods, involving the assessment of the carbohydrate assimilation pattern (API 21C, Paris, France) and colony characteristics on chromogenic medium (Chromagar, Paris, France) and then confirmed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Skycray, JiangSu, China). All isolates were cultured on yeast extract-peptone-glucose agar and preserved in broth containing 20% glycerol at -80°C . The study samples were collected from the samples that were received routinely in the laboratory. It was not collected separately for this project; hence, informed consent from the patients was not required, and the Research Ethics Committee of Longhua Hospital, Shanghai University of Traditional Chinese Medicine approved to conduct this study.

Antifungal susceptibility testing

The ATB Fungus 3 strip (bioMérieux, Marcy l'Etoile, France) was used to determine the antifungal susceptibility (flucytosine, amphotericin B, fluconazole, voriconazole, and itraconazole) of the 59 clinical isolates.^{17,18} Quality control was ensured by testing strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22,019. Susceptibility to fluconazole was also assessed for all 59 *C. glabrata* strains using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). The minimal inhibitory concentrations (MICs) were determined in RPMI 1640 buffered to a PH of 7.0 with an inoculum size of approximately 1.5×10^3 cells/mL. The MIC for amphotericin B and flucytosine was defined as the lowest concentration at which no visible growth was detected. Recently approved CLSI breakpoints were used:¹⁹ strains with fluconazole MICs ≤ 32 $\mu\text{g/mL}$ were defined as susceptible-dose dependent, while those with MICs ≥ 64 $\mu\text{g/mL}$ were defined as resistant. There were no clinical break points for voriconazole and itraconazole,

and thus, species-specific epidemiological cut-off values (ECVs) were used to define isolates as wide-type (WT) or non-WT. The epidemiological cut off values (ECVs) for voriconazole and itraconazole were 0.25 µg/mL and 4 µg/mL, respectively.

Flow cytometric analysis of the efflux of rhodamine 6G

The efflux of rhodamine 6G (Sigma-Aldrich, St Louis, MO, USA), a fluorescent dye that uses the same membrane transporter as azoles in some *Candida* spp.,²⁰ was measured to evaluate the activity of efflux pumps by flow cytometry, as described previously.²¹ Briefly, isolates cultured overnight were adjusted to a concentration of 5×10^7 cells/mL in 2 mL phosphate-buffered saline (PBS) and incubated at 30 °C for 4 h with shaking for deprivation. Rhodamine 6G was then added at a final concentration of 10 µM and shaken at 30 °C for 2 h. After washing twice with cold sterile PBS, the uptake of fluorescence by the cells was first measured at 535 nm with an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Glucose at a final concentration of 4 mM was added and shaken at 30 °C for another 2 h. The fluorescence of the cells was then evaluated. The fluorescence of cells incubated without rhodamine 6G served as a control. The data of geometric mean values presented correspond to fluorescence frequency distribution histograms (relative number of cells vs relative fluorescence intensity, expressed in arbitrary units on a logarithmic scale).

Quantitative real-time RT-PCR

Total RNA was extracted from isolates grown to the mid-exponential phase in YPD medium with the Yeast RNAiso Reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time-PCR of *CgCDR1*, *CgCDR2*, *CgSNQ2*, *CgPDR1*, and *CgERG11* was performed with the SYBR Premix Ex Taq Kit (TaKaRa) and LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland), as described previously.²² All reactions were performed as follows: denaturation at 95 °C for 1 min, followed by 40 cycles that consisted of 15 s at 95 °C and 60 s at 60 °C. The primers used in the real-time RT-PCR are shown in Table 1. Quantification of each target gene was performed by the $2^{-\Delta\Delta CT}$ method using the housekeeping gene *ACT1* as a control.²³ Each reaction was performed in triplicate.

Table 1 Primers used for real-time PCR

Gene	Nucleotide sequence (5'-3')
<i>CgCDR1</i>	F: ACACCAACAACAGCATCT R: ATTCTCCGCTTACCTACG
<i>CgCDR2</i>	F: CAACGCTATGAGGGAAAA R: AACATAAGTGGCGTGGGT
<i>CgSNQ2</i>	F: ACCATGTGTTCTGAATCAATCAAT R: TCGACATCATTACAATACCAGAAA
<i>CgERG11</i>	F: TGGAAAGCAGTGAAGATAGT R: AGTGTTCGGTAAAGGTGT
<i>CgPDR1</i>	F: AGCCTTGCCGATAGTCATAC R: AAGGTCAGGGCATACTTCAG
<i>ACT1</i>	F: AGAAGTTGCTGCTTTAGTT R: GACAGCTTGAATGGAAAC

Sequencing of *CgERG11* and *CgPDR1*

The genomic DNA of azole-resistant and azole-susceptible-dose dependent isolates was extracted with the DNeasy plant mini kit (Qiagen, Hilden, Germany). Two pairs of oligonucleotide primers were designed according to the *CgERG11* GenBank sequence (accession number L40389) to cover the whole gene (Table 2). *CgPDR1* coding sequences (accession number AY700584) were amplified using 6 pairs of primers shown in Table 2. Amplification conditions were as follows: 5 min of denaturation at 94 °C, followed by 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and finally 7 min of elongation at 72 °C. The PCR products were purified with the High Pure PCR product purification kit (Roche Diagnostics

Table 2 Primers used for PCR

Primer	Nucleotide sequence (5'-3')
<i>CgERG11-1</i>	F: ACAATAACATGTCCACTGAAAACAC R: AGCTTGTGGGCATGATCTCT
<i>CgERG11-2</i>	F: GGACAAGGGTTTACCCCAA R: TTAGCAGGGCAGTTGGTAG
<i>CgPDR1-1</i>	F: TCGTATTGCCATTGTGATATGGA R: TGAGTTCATTAAGTGCCTGTGT
<i>CgPDR1-2</i>	F: ATGAGGAATGGTGACTCGGA R: GCTCTTTGTACTAGCACTTTGTGG
<i>CgPDR1-3</i>	F: TCGATTGCCAACCCGTTAGA R: ACAGTTGACAGTAGCATCATCA
<i>CgPDR1-4</i>	F: CCGTTGGGAGTTTTACGTCG R: CTGCGTAGCTAAACACAGACA
<i>CgPDR1-5</i>	F: TGGTGAACCAAAATCAGATTGGC R: AAGCCCCGATAAGGGAGATGC
<i>CgPDR1-6</i>	F: TGGACTTCAATTACGACCGCA R: TCGCTAATTTGAGGTAGTCTAAGT

Table 3 Information and MIC values for the 59 *Candida glabrata* isolates

Hospital	Isolate number	Origin	Sex/ Age	Ward	MIC($\mu\text{g/mL}$)						MLST	Group
					5-FC	AMB	VRC	ITR	FLC ^a	FLC ^b		
RJ	1	swab	F/26	Gynaecology	<4	<0.5	1	1	32	16	ST-3	
RJ	2	venous catheter	F/75	General surgery	<4	<0.5	1	0.125	8	8	ST-7	
RJ	3	sputum	F/73	Gastroenterology	<4	<0.5	0.25	0.125	2	4	ST-7	
RJ	4	sputum	M/86	Respiratory	<4	<0.5	0.5	0.125	2	2	ST-7	SDD
RJ	5	sputum	M/62	Hepatology	<4	<0.5	0.5	0.125	4	4	ST-7	
RJ	6	sputum	M/62	Neurosurgery	<4	<0.5	0.5	0.125	8	8	ST-7	SDD
RJ	7	drain	F/60	General surgery	<4	<0.5	0.5	1	16	16	ST-7	
RJ	8	feces	M/85	Emergency	<4	<0.5	1	0.5	8	4	ST-7	
RJ	9	sputum	F/88	Neurology	<4	<0.5	1	0.125	8	8	ST-7	
RJ	10	sputum	M/74	ICU	<4	<0.5	0.25	0.5	2	2	ST-10	SDD
RJ	11	sputum	M/25	Neurosurgery	<4	<0.5	0.5	0.5	8	8	ST-7	
RJ	12	feces	M/87	Health Care	<4	<0.5	0.5	0.125	8	8	ST-7	SDD
RJ	13	urine	F/33	Urology	<4	<0.5	0.25	0.25	4	4	ST-15	
RJ	14	sputum	F/53	Respiratory	<4	<0.5	0.125	0.125	2	2	ST-10	
RJ	15	sputum	M/64	Cardiology	<4	<0.5	0.25	0.125	4	4	ST-15	
RJ	16	sputum	M/67	Respiratory	<4	<0.5	0.125	0.25	2	2	ST-7	SDD
RJ	17	sputum	F/77	Neurosurgery	<4	<0.5	0.25	0.5	4	4	ST-10	
RJ	18	debridement	M/34	Neurosurgery	<4	<0.5	0.25	0.5	2	2	ST-7	
RJ	19	sputum	M/84	Gastroenterology	<4	<0.5	0.25	0.125	4	4	ST-7	
RJ	20	feces	F/78	General surgery	<4	<0.5	0.25	0.25	1	1	ST-7	SDD
RJ	21	sputum	F/71	Neurosurgery	<4	<0.5	4	>4	64	64	ST-7	R
RJ	22	sputum	M/45	Nephrology	<4	<0.5	0.25	0.125	4	2	ST-7	
RJ	23	blood	M/46	Hepatology	<4	<0.5	0.06	0.125	2	1	ST-7	
RJ	24	sputum	F/59	Hepatology	<4	<0.5	0.25	0.25	4	4	ST-7	
RJ	25	blood	F/47	Emergency	<4	<0.5	0.5	0.125	8	8	ST-7	
RJ	26	sputum	M/68	Neurosurgery	<4	<0.5	0.5	0.5	8	4	ST-55	SDD
RJ	27	blood	M/34	Neurosurgery	<4	<0.5	0.125	0.125	4	4	ST-7	
RJ	28	sputum	M/20	Neurosurgery	<4	<0.5	0.25	0.5	4	4	ST-7	
RJ	29	sputum	F/50	Neurosurgery	<4	<0.5	0.06	0.125	2	2	ST-43	SDD
RJ	30	feces	M/58	Neurosurgery	<4	<0.5	0.125	0.125	2	2	ST-7	
RJ	31	sputum	M/64	Radiology	<4	<0.5	0.125	0.125	2	2	ST-7	
RJ	32	throat	M/85	Health Care	<4	<0.5	0.125	0.125	2	2	ST-7	
RJ	33	sputum	M/82	Orthopedics	<4	<0.5	0.25	0.125	4	4	ST-7	SDD
RJ	34	sputum	M/61	ICU	<4	<0.5	>8	2	32	32	ST-7	
RJ	35	leucorrhoea	F/24	Gynaecology	<4	<0.5	8	>4	128	128	ST-7	R
RJ	36	leucorrhoea	F/25	Gynaecology	<4	<0.5	0.125	0.25	4	2	ST-7	SDD
RJ	37	leucorrhoea	F/28	Gynaecology	<4	<0.5	0.125	0.5	2	4	ST-7	
RJ	38	blood	F/87	General surgery	<4	<0.5	0.125	0.25	2	2	ST-7	
RJ	39	leucorrhoea	F/26	Gynaecology	<4	<0.5	0.125	0.125	4	4	ST-7	SDD
RJ	40	urine	F/57	Urology	<4	<0.5	0.125	0.25	2	2	ST-10	
RJ	41	leucorrhoea	F/57	Urology	<4	<0.5	0.06	0.25	2	2	ST-10	

(Continued)

Table 3 (Continued).

Hospital	Isolate number	Origin	Sex/ Age	Ward	MIC($\mu\text{g/mL}$)						MLST	Group
					5-FC	AMB	VRC	ITR	FLC ^a	FLC ^b		
RJ	42	blood	M/59	ICU	<4	<0.5	0.125	0.25	2	2	ST-55	
RJ	43	blood	M/56	ICU	<4	<0.5	0.125	0.5	2	2	ST-55	
RJ	44	leucorrhea	F/29	Gynaecology	<4	<0.5	8	4	128	128	ST-7	R
RJ	45	BALF	M/51	Respiratory	<4	<0.5	0.25	0.5	4	4	ST-3	
RJ	46	urine	M/47	ICU	<4	<0.5	0.125	0.25	2	2	ST-10	
RJ	47	blood	M/73	Emergency	<4	<0.5	0.125	0.25	2	4	ST-15	
RJ	48	urine	F/92	ICU	<4	<0.5	0.125	0.25	2	2	ST-7	
RJ	49	sputum	F/50	Neurosurgery	<4	<0.5	4	>4	32	32	ST-7	
DF	50	sputum	M/87	ICU	<4	<0.5	8	>4	64	128	ST-7	R
DF	51	urine	F/71	ICU	<4	<0.5	4	>4	64	64	ST-7	R
DF	52	sputum	F/69	ICU	<4	<0.5	4	>4	32	32	ST-10	
DF	53	sputum	M/70	ICU	<4	<0.5	8	>4	128	128	ST-7	R
DF	54	urine	M/79	ICU	<4	<0.5	8	>4	64	64	ST-7	R
DF	55	throat	M/88	ICU	<4	<0.5	4	>4	64	64	ST-7	R
DF	56	throat	F/71	ICU	<4	<0.5	4	>4	64	64	ST-7	R
DF	57	urine	F/74	ICU	<4	<0.5	4	>4	64	64	ST-7	R
DF	58	sputum	M/81	ICU	<4	<0.5	4	>4	64	64	ST-7	R
DF	59	throat	M/72	ICU	<4	<0.5	4	>4	64	64	ST-7	R
ATCC90030					<4	<0.5	0.125	0.125	8	8	ST-10	SDD

Notes: ^aThe ATB Fungus 3 strip was used for the susceptibility test; ^bthe CLSI M27-A2 broth microdilution method was used for the susceptibility test.

Abbreviations: RJ, Renji hospital; DF, Dongfang hospital; BALF, Bronchoalveolar lavage fluid; 5-FC, 5- flucytosine; AMB, amphotericin B; VRC, voriconazole; FLC, fluconazole; ITR, itraconazole; SDD, susceptible-dose dependent; R, resistant.

GmbH, Mannheim, Germany) and sequenced directly, as described previously.²⁴

Statistical analysis

Data were analyzed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined by independent samples *t*-test. Statistical significance was indicated when $P < 0.05$.

Results

Antifungal susceptibilities of *C. glabrata* isolates

The ATB Fungus 3 strip was used to determine the antifungal susceptibility of the 59 clinical isolates. For fluconazole, the susceptibility of all the isolates was also detected by the broth microdilution method, and the results were consistent with those of the ATB fungus 3 strip. All the 59 isolates of *C. glabrata*, were found to be susceptible to amphotericin B and flucytosine. According to the CLSI interpretive criteria, 20.3% (12/59) of the isolates were determined to be fluconazole-resistant (MICs $\geq 64 \mu\text{g/mL}$), with a high level of MICs ranging

from 64 to 128 $\mu\text{g/mL}$. MIC₅₀ and MIC₉₀ were 4 $\mu\text{g/mL}$ and 64 $\mu\text{g/mL}$ for fluconazole respectively. After applying epidemiological cutoff values, 22.0% (13/59) and 45.7% (27/59) of the isolates were categorized as non-wild-type for itraconazole ($>4 \mu\text{g/mL}$) and voriconazole ($>0.25 \mu\text{g/mL}$), respectively. The results of antifungal susceptibility testing are shown in Table 3.

Uptake and efflux of rhodamine 6G

The accumulation and efflux of rhodamine 6G were quantitatively analyzed by flow cytometry. After 4 h of starvation and 2 h of incubation in PBS containing a fluorochrome, the mean fluorescence intensity of the cells was 13.16 ± 6.26 (Table 4) in the azole-resistant group and 31.81 ± 7.27 in the azole-susceptible-dose dependent group, with a significant difference between the two groups (Figure 1A). Moreover, after removing the free dye and incubating for another 1 h in PBS containing 4 mM glucose, the mean fluorescence intensity in the resistant isolate was significantly lower than that in the azole-susceptible-dose dependent isolate (Figure 1A), and the mean fluorescence intensity was

Table 4 Efflux pump activity of *Candida glabrata*

Isolate number	MIC ($\mu\text{g}/\text{mL}$)	Azole susceptibility category	Mean fluorescence intensity	
			Uptake	Efflux
21	64	R	28.7	24.4
35	128	R	18.7	3.74
44	128	R	11.7	8.48
50	128	R	9.72	2.36
51	64	R	13.07	5.67
53	128	R	7.32	1.72
54	64	R	11.2	4.14
55	64	R	9.51	8.29
56	64	R	12.4	6.06
57	64	R	9.26	6.06
Average			13.16 \pm 6.26	7.09 \pm 6.48
4	2	SDD	32.2	19.8
6	8	SDD	26	23.2
10	2	SDD	38.1	27.3
12	8	SDD	23	17.7
16	2	SDD	27	22.3
20	1	SDD	22.2	19.2
26	4	SDD	39.5	37
29	2	SDD	34.7	19.5
33	4	SDD	31.7	21.4
QC	8	SDD	43.7	23.5
Average			31.81 \pm 7.27	23.09 \pm 5.61

7.09 \pm 6.48 in the azole-resistant group and 23.09 \pm 5.61 in the azole-susceptible-dose dependent group. The visual variation of rhodamine 6G for azole-resistant isolate number 35 is presented in Figure 1B.

Expression of CgCDR1, CgCDR2, CgSNQ2, CgERG11, and CgPDR1

Quantitative RT-PCR experiments showed that the expression levels of *CgCDR1* and *CgCDR2* in the azole-resistant group were higher than those in the azole-susceptible-dose dependent group ($P<0.05$). On the contrary, there was no significant difference in the expression levels of *CgSNQ2*, *CgERG11*, and *CgPDR1* between the azole-resistant and azole-susceptible-dose dependent isolates ($P>0.05$) (Figure 2).

Mutations in CgERG11

DNA sequence analysis of the *CgERG11* open reading frame revealed 4 mutations (T1328C, T1394C, G1487A, and A1583G) in 24 isolates compared to the corresponding sequence in the GenBank database (GenBank accession number L40389). These point mutations did not change the amino acid sequence, indicating that this gene polymorphism was functionally silent. T1394C and A1583G were discovered in 11 azole-susceptible-dose dependent isolates and 7 azole-resistant isolates. T1328C was found in 9 azole-susceptible-dose dependent isolates and 4 azole-resistant isolates, while G1487A existed in only one azole-susceptible-dose dependent isolate.

Mutations in CgPDR1

DNA sequence analysis of the *CgPDR1* open reading frame revealed 11 mutations in 24 isolates compared to the corresponding sequence in the GenBank database (GenBank accession number AY700584), of which 5 were synonymous mutations and the others were missense mutation sites. The 5 synonymous mutations were T309C, C837T, C1563T, A3156G, and T3228C, which only appeared in azole-susceptible-dose dependent isolates. For the 6 missense mutations, 2 were found in azole-susceptible-dose dependent isolates, which were A776G (E259G) and A1665C (E555D), while the other 4 missense mutations were found in azole-resistant isolates and were G1042T (G348C), A2290G (N764D), G2626A (D876N) (found in one isolate, respectively) and C2543T (A848V) (found in 8 isolates). Among the 4 missense mutations, G1042T (G348C) and A2290G (N764D) have been reported previously.²⁵ Although G2626A (D876N) has also been reported, the amino acid mutation was different. C2543T (A848V) has not been reported and showed the highest frequency in this experiment (Table 5).

Discussion

The mechanisms of *Candida* resistance against azole antifungal drugs include the following: 1) an increase in lanosterol 14 α -demethylase, which increases the concentration of azole drug required to inhibit target enzyme production, 2) a decrease in the affinity between the azole drug and its target enzyme, 3) enhanced excretion effects against the drug, preventing the drug from collecting in the fungal cell, 4) inactivation of the sterol, $\Delta 5, 6$ - desaturase, leading to the dysfunction of ergosterol synthesis.

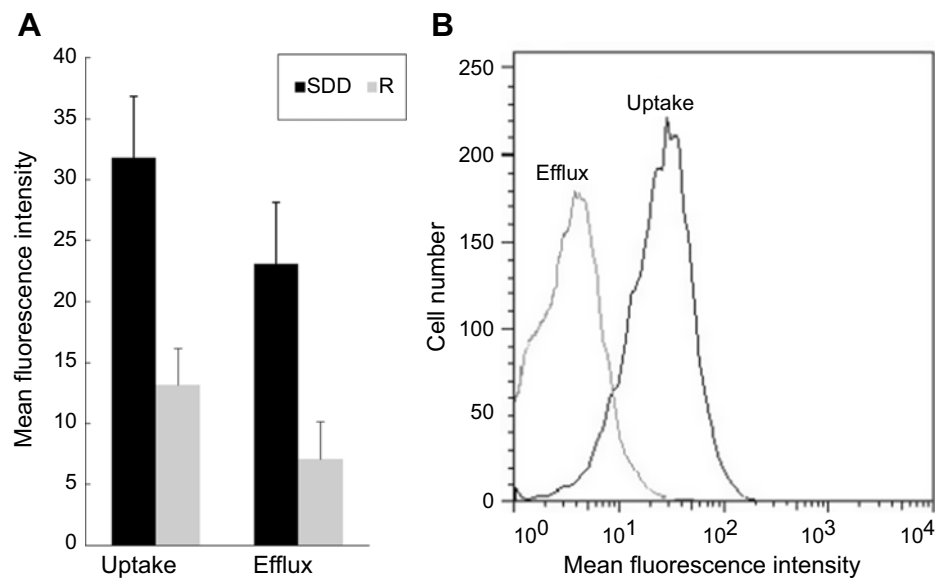


Figure 1 Rhodamine 6G uptake and efflux test. (A) Mean fluorescence intensity of rhodamine 6G for the azole- susceptible-dose dependent and azole-resistant groups; (B) Flow cytometric analysis of rhodamine 6G uptake and efflux for the number 35 azole-resistant isolates.

Abbreviations: SDD, susceptible-dose dependent; R, resistant.

Antifungal resistance can be acquired by *C. glabrata* during therapy.²⁶ In addition, *C. glabrata* is intrinsically resistant or less susceptible to specific antifungals.²⁷ One of the largest and a representative study that tested 1699 isolates of *C. glabrata* from two large antifungal surveys, the SENTRY Global Surveillance Program from 2006 to 2010 and the Centers for Disease Control and Prevention (CDC) population-based surveillance conducted in the Atlanta, GA, and Baltimore, MD, metropolitan areas between 2008 and 2010, showed that 162(9.7%) of the isolates were resistant to fluconazole, including 62 of 847 (7.3%) isolates from the SENTRY Program and 100 of 822 (12.2%) isolates from the CDC population-based surveillance.²⁸ Data of the global SENTRY Program in 2010 and 2011 show 8.8% of *C. glabrata* isolates were resistant to fluconazole and that voriconazole was active against all *Candida* spp. except *C. glabrata* (10.5% non-WT).²⁹ A study from China showed that resistance to fluconazole (MIC >32 µg/mL) was observed in 68 of 411 (16.5%) *C. glabrata* isolates from 11 hospitals participating in CHIF-NET (2010–2014), while MICs of >0.5 µg/mL for voriconazole and >2 µg/mL for itraconazole were observed for 28.7% and 6.8% of isolates, respectively.³⁰ Data from CHIF-NET (2015) showed that 14 of 158 (8.9%) *C. glabrata* isolates were resistant to fluconazole (MICs≥64 µg/mL) and the non-WT rates of

C. glabrata for voriconazole and itraconazole were 19.0% and 5.1%, respectively.³¹

In the present study, 20.3% (12/59) of the isolates were determined to be fluconazole-resistant (MICs≥64 µg/mL), and 22.0% (13/59) and 45.7% (27/59) of the isolates were categorized as non-wild-type for itraconazole (>4 µg/mL) and voriconazole (>0.25 µg/mL), respectively. According to the distribution of the strains, the number of *C. glabrata* isolates from the intensive care unit was the highest, accounting for 27.1% (16/59), followed by that from the neurosurgery ward (18.6%,11/59). This may be related to the frequent invasive operation and long-term bed rest of patients in these wards. Therefore, the prevention and control of nosocomial fungal infections in these wards should be strengthened, and attention should be paid to the examination of patients with high risk factors of invasive fungal infections. Narrow-spectrum antibiotics should be used according to the results of drug sensitivity to avoid unnecessary invasive operations, to reduce the incidence of nosocomial fungal infections. All the 59 clinical isolates of *C. glabrata* were genotyped by the MLST technique in our previous studies.^{32,33} Six ST genotypes (ST-7, ST-10, ST-3, ST-15, ST-43 and ST-55) were obtained with six housekeeping genes (*FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3*) as target genes. The discriminatory power index was 0.470. Among these isolates, 43 were ST-7,

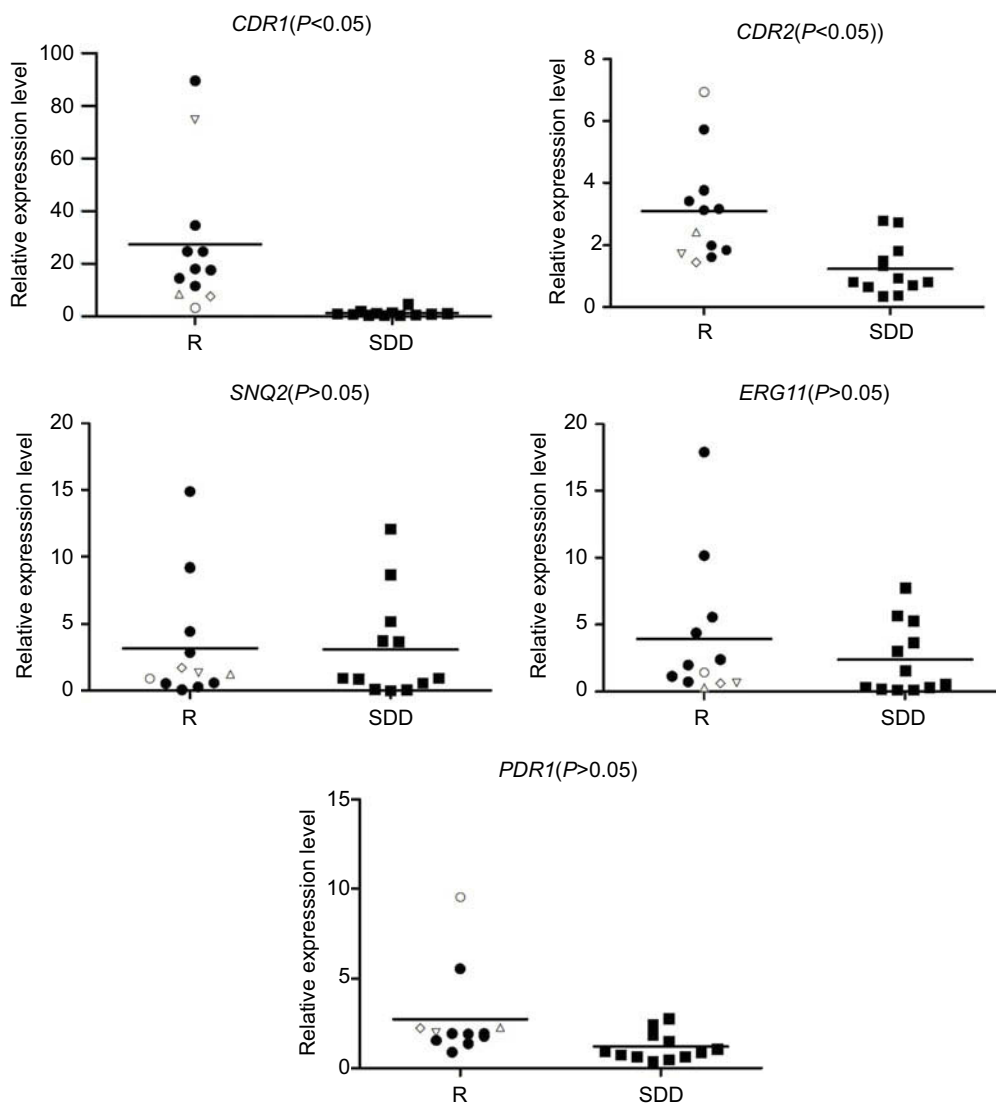


Figure 2 Relative expression of genes in the azole-resistant group and azole-susceptible-dose dependent group. Δ , N764D *CgPDR1* mutations; ∇ , G348C *CgPDR1* mutations; \diamond , D876N *CgPDR1* mutations; \bullet , A848V *CgPDR1* mutations; \circ , not *CgPDR1* mutations.

Abbreviations: SDD, susceptible-dose dependent; R, resistant.

accounting for 72.9% (43/59), and thus ST-7 was the dominant genotype. By comparing MLST genotype with the isolation sources, we found that, of the 11 strains of *C. glabrata* isolated from sterile sites (7 strains from blood and 4 strains from venous catheter, BALF, drain, and debridement, respectively), 7 isolates were ST-7, 2 were ST-55 and, the remaining 2 were ST-3 and ST-15. The sequence types (STs) were independent of isolation sources. Likewise, there was also no significant difference between different genotypes and drug susceptibility.

We attempted to evaluate different resistance mechanisms in the 59 clinical isolates of *C. glabrata*. Overexpression of the efflux pump increases drug efflux and prevents the drug from accumulating in the cell,

leading to drug resistance. Because the same protein transports the azole drug and the fluorescent dye rhodamine 6G, we used the latter to detect the function of the efflux pump in *C. glabrata*. Our results showed that *C. glabrata* excreted significant amounts of rhodamine 6G, indicating that efflux pump function plays an important role in the drug resistance of *C. glabrata*. Two types of efflux pumps are present on the fungal cell membrane: the ATP-binding cassette transporter and major facilitator superfamily. *CgCDR1*, *CgCDR2*, and *CgSNQ2* have been examined previously.^{34,35} Shin et al³⁶ discovered 2 strains of *C. glabrata* isolated from the same patient; one was resistant to fluconazole and the other was sensitive to fluconazole. The mRNA expression of *CgCDR1* and *CgCDR2* in

Table 5 Positions of polymorphic nucleotide sites in *CgPDR1*

Isolate number	MIC ($\mu\text{g/mL}$)	Azole susceptibility category	mutation
26	4	SDD	A776G (E259G)
29	2	SDD	A1665C (E555D)
35	128	R	G2626A (D876N)
44	128	R	A2290G (N764D)
54	64	R	G1042T (G348C)
50	128	R	C2543T (A848V)
51	64	R	C2543T (A848V)
53	128	R	C2543T (A848V)
55	64	R	C2543T (A848V)
56	64	R	C2543T (A848V)
57	64	R	C2543T (A848V)
58	64	R	C2543T (A848V)
59	64	R	C2543T (A848V)

Abbreviations: SDD, susceptible-dose dependent; R, resistant.

the resistant strain was 13.2- and 3.5-fold higher than that in the sensitive strain. Tumbarello et al³⁷ also detected the mRNA expression of *CgCDR1*, *CgCDR2*, and *CgSNQ2* in approximately 35 strains of *C. glabrata*, finding that 6 resistant strains were obviously upregulated, 8 susceptible-dose dependent strains were slightly upregulated, and the other sensitive strains showed no change. Our results also show that compared to that in the susceptible-dose dependent group, the expression of *CgCDR1* and *CgCDR2* in the resistant group was upregulated, while the expression of *CgSNQ2* did not differ. In this study, comparison of the expression levels of *CgCDR1* and *CgCDR2* revealed the upregulation of *CgCDR1* (3.4–89.6 fold) was more obvious than that of *CgCDR2* (1.4–6.9 fold), which is consistent with the results of other studies. These findings support the view that *CgCDR1* is more closely related to azole resistance than *CgCDR2*.³⁸

Many studies have indicated that overexpression of *CgERG11* causes *C. glabrata* to be resistant to

fluconazole.^{39–41} However, in this study, we found that the expression difference between the resistant group and susceptible-dose dependent group was not significant. Sequencing of *CgERG11* revealed 4 synonymous mutation sites, in which T1328C, T1394C, and A1583G appeared in both resistant and susceptible-dose dependent strains, while G1487A was only observed in susceptible-dose dependent strains. These 4 synonymous sites were also reported by Shen et al⁴² Additionally, G1487A was detected in their resistant strains. Thus, our result was conducive to the hypothesis that *CgERG11* did not participate in the azole resistance of *C. glabrata*.

CgCDR1 and *CgCDR2* have the same transcriptional regulatory factor, *CgPdr1*. Some *CgPDR1* mutation promotes resistance to azole antibiotics by increasing the expression of *CgCDR1* and *CgCDR2* or enhancing the virulence of yeast and plays a central role in fluconazole resistance acquired by *C. glabrata*. Ferrari et al²⁵ investigated the incidence of *CgPDR1* mutations in a large collection of clinical isolates and identified 57 distinct single amino acid substitutions located at 50 locations, including the 3 mutations found in our research. Caudle et al⁴³ reported upregulation of *CgCDR1*, *CgCDR2*, and *CgSNQ2* and a mutation in *CgPDR1* T2837C (L946S) in *C. glabrata*. They introduced this mutation into the sensitive strain and found that the MIC increased from 2 to 64 $\mu\text{g/mL}$. Tsai et al⁴⁴ also introduced the gain of function mutation sites of *CgPDR1* C2465T (P822L) from resistant *C. glabrata* into the sensitive strain and observed that the MIC was increased. Therefore, mutation in *CgPDR1* is one mechanism leading to the resistance of *C. glabrata*. In the present study, we detected *CgPDR1* mutations in 12 azole resistant clinical isolates, among which we found that only one isolate (isolate 21) did not have *CgPDR1* mutation, while all the 11 remaining isolates carried a single missense mutation. Interestingly, the isolates number 21 showed a moderate upregulation of *CgCDR1* (3.4-fold) while *CgCDR2* was significantly upregulated (6.7-fold). The other 11 isolates carrying *CgPDR1* mutations seemed to result in higher *CgCDR1* expression levels, ranging from 7.5- to 89.6-fold increases. Compared with that of *CgCDR1*, the impact of *CgPDR1* mutations on *CgCDR2* was much weaker in these 11 isolates, with an increase of no more than 5.7-fold. Therefore, the *CgPDR1* mutation has different regulatory effects on the expression of *CgCDR1* and *CgCDR2*, and not all clinical isolates of *C. glabrata* showing azole

resistance can be explained by amino acid substitutions in *PDR1*. Additionally, we found 4 missense mutation sites in only resistant strains, among which C2543T (A848V) and the other three were found in 8 of the 11 isolates, and one isolate, respectively. The high mutation frequency of A848V in our present study has not been reported previously, and thus, further studies of the related gene disruption and genome-wide expression may help to determine the regulation of these mutations in *C. glabrata*.

Conclusion

In summary, efflux pump function is the main mechanism of resistance to fluconazole in *C. glabrata*. Resistance development is a complex process regulated by multiple factors, and thus, cannot be explained by a single resistance gene or a single mechanism; therefore, further studies are needed to understand the relationship.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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