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Missense mutation in *CgPDR1* regulator associated with azole-resistant *Candida glabrata* recovered from Thai oral candidiasis patients

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ABSTRACT

Objectives: Non-*albicans Candida* (NAC) species are increasingly identified as pathogens causing oral candidiasis. Incidence rates for azole resistance among NAC species have been continuously reported. This study aimed to evaluate the azole susceptibility profiles and to characterise the azole resistance mechanisms of oral clinical NAC isolates.

Methods: In vitro susceptibility patterns of 85 NAC species isolates were determined by the broth microdilution method. Azole resistance-related genes (*ERG3*, *ERG11* and *PDR1*) of *Candida glabrata* isolates were sequenced to determine the presence of nucleotide substitutions. Expression levels of various resistance-related genes were also evaluated by RT-qPCR in azole-susceptible, susceptible dose-dependent (SDD) and resistant *Candida* isolates.

Results: Two *C. glabrata* isolates (2.4% of all NAC isolates) were resistant to all three azoles tested (fluconazole, itraconazole and ketoconazole). All clinical isolates of *Candida tropicalis* and *Candida kefyr* were susceptible to azoles. Silent mutations were found in the *CgERG11* and *CgERG3* genes of clinical *C. glabrata* isolates. Interestingly, two missense mutations in *CgPDR1* (N768D and E818K) were identified only in resistant *C. glabrata* isolates. The presence of a *CgPDR1* missense mutation in resistant isolates is associated with overexpression of its own product as well as multidrug transporters including ABC and MFS transporters.

Conclusion: A gain-of-function (GOF) mutation in *CgPDR1* is associated with upregulation of various drug transporters, which appears to serve as a primary mechanism for azole resistance in the detected *C. glabrata* isolates. Therefore, analysis of GOF mutations in the *PDR1* regulator provides a better understanding of the development of antifungal resistance.

1. Introduction

Species of the genus *Candida* are among the most frequent and significant microorganisms causing human fungal infections. These organisms have the ability to colonise various anatomical sites of the human body. The mucosal membrane of the oropharynx is one of the most common sites for *Candida* colonisation and infection [1]. In addition, an increased incidence of oropharyngeal candidiasis is correlated with several predisposing factors, including advanced age, weakened immune condition [e.g. cancer, human immunodeficiency virus (HIV) infection, diabetes mellitus, chemotherapy treatment] and wearing dentures [1,2]. *Candida albicans* is the most prevalent species causing oral candidiasis, followed by non-*albicans Candida* (NAC) species such as *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida kefyr* and *Candida dubliniensis* [1]. Over the past several decades, the occurrence of NAC species associated with oral candidiasis has gradually increased [3,4]. The contribution of NAC species to oral candidiasis is an important finding as many studies have demonstrated reduced susceptibility to commonly used antifungal agents, especially azole drugs, for NAC isolates [5–7]. Therefore, antifungal resistance among *Candida* spp. is a growing medical concern for the treatment of oral candidiasis and other fungal infections.

Azoles are antifungal drugs commonly used in the treatment and prophylaxis of fungal infections, especially for oropharyngeal candidiasis in HIV patients. This class of drugs inhibits fungal cytochrome P450 enzymes required for ergosterol biosynthesis

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[7,8]. Two important enzymes in the ergosterol synthesis pathway are *ERG11* (lanosterol 14-α-demethylase) and *ERG3* (C-5 sterol desaturase). Lanosterol 14-α-demethylase (ERG11) has been identified as a target enzyme of azole drugs. While the systems for azole resistance in *Candida* spp. vary, three major mechanisms for resistance to azoles have been identified [9,10]. The first mechanism involves missense mutations in the *ERG11* and *ERG3* genes. A second resistance mechanism is increased expression of efflux pumps including ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter proteins. These drug transporters are responsible for export of azoles from inside the fungal cell to the external environment. Several reports have revealed that multidrug resistance regulators (*MRR1* and *TAC1* in *C. albicans* and *PDR1* in *C. glabrata*) control the expression levels of various drug transporters [11–13]. Besides, mutations identified in these regulators are associated with azole resistance. The last mechanism is intrinsic resistance to azoles was identified in *C. krusei.* This *Candida* species possesses an ERG11 protein that has low affinity for azoles, preventing inhibition of the enzyme. Azole resistance mechanisms in *C. albicans* have been extensively investigated in previous studies [9,14]. In contrast, investigation of azole resistance mechanisms of NAC species has not been thoroughly examined.

Therefore, the aim of this study was to determine the in vitro azole susceptibility profiles of clinical NAC isolates recovered from Thai oral candidiasis patients. In addition, the molecular mechanisms of azole resistance in *C. glabrata* isolates were identified.

2. Materials and methods

2.1. Collection of clinical Candida isolates

NAC isolates used in this study were obtained from a previous study [15]. A total of 85 clinical isolates, comprising *C. glabrata* (40 isolates), *C. tropicalis* (28 isolates), *C. kefyr* (9 isolates) and *C. parapsilosis* (8 isolates), were used. *Candida* isolates were obtained from various manifestations of oral candidiasis, including erythroplakia and leukoplakia, denture stomatitis, angular cheilitis, and complaints of a burning sensation. The study protocol was approved by the Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (Bangkok, Thailand).

2.2. Antifungal susceptibility testing

Candida isolates were grown on Sabouraud dextrose agar (Difco, Detroit, MI) at 37 °C for 48 h. Antifungal susceptibility testing was performed by the broth microdilution method in 96-well plates (Corning Costar, Corning, NY) according to a modified Clinical and Laboratory Standards Institute (CLSI) guideline [16]. Briefly, colonies of *Candida* spp. were re-suspended in RPMI 1640 medium (GE Healthcare, Pittsburgh, PA) containing 0.165 M MOPS buffer (Bio Basic Inc., Toronto, Canada) resulting in a final concentration of 0.5×10^3 to 2.5×10^3 cells/mL in the inoculum. Samples in microplates were incubated at 37 °C for 48 h and the minimum inhibitory concentration (MIC) for each antifungal drug was evaluated spectrophotometrically at A_{600} using a microplate reader (model EL340; Mandel Scientific). The tested antifungal drugs in this study were fluconazole (Pfizer, Sandwich, UK), itraconazole (Janssen, Beerse, Belgium) and ketoconazole (Merck, Darmstadt, Germany). The tested drugs were dissolved and diluted according to CLSI recommendations [16]. MIC endpoints were assigned as the lowest concentration of antifungal drug that resulted in 50% growth inhibition compared with the drug-free control well for itraconazole, ketoconazole and fluconazole. The MIC_{50} and MIC_{90} of each antifungal drug represent the concentrations required for inhibition of 50% and 90% of *Candida* isolates within a tested population, respectively. *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *C. albicans* ATCC 90028 were used as quality control organisms [16].

2.3. Interpretation of resistance

The MIC endpoint was used to interpret resistance breakpoint values of the antifungal drugs according to CLSI guidelines [17] and previous reports [18]. MIC breakpoint values are as follows: for itraconazole, resistant, ≥1.0 μg/mL; susceptible dose-dependent (SDD), 0.25–0.5 μg/mL; and susceptible, ≤0.125 μg/mL; for fluconazole, resistant, ≥64 μg/mL; SDD, ≤32 μg/mL for *C. glabrata*; and resistant, ≥8.0 μg/mL; SDD, 4.0 μg/mL; susceptible, ≤2.0 μg/mL for *C. parapsilosis* and *C. tropicalis*; and for ketoconazole, resistant, ≥1.0 μg/mL.

2.4. PCR amplification and sequencing of the ERG11, ERG3 and PDR1 genes

The *ERG3*, *ERG11* and *PDR1* genes from 11 *C. glabrata* isolates (2 fluconazole-resistant and 9 SDD isolates) were amplified by PCR using the primers shown in Supplementary Table S1 in the online version at DOI: 10.1016/j.jgar.2019.01.006 [19–22]. PCR was performed in a 50 μL reaction volume containing 5 μL of 10× PCR buffer, 10 ng of genomic DNA, 10 μM of dNTPs, 0.5 μM of each primer and 1 U of *Pfu* DNA Polymerase enzyme (QIAGEN, Valencia, CA). PCR products were analysed by 1% agarose gel electrophoresis for expected size. Sequencing was performed by Macrogen, Inc. (Seoul, South Korea). The nucleotide sequences of amplified genes were compared with sequences deposited in the GenBank database as follows: *CgERG11* (accession no. **L40389.1**); *CgERG3* (accession no. **L40390.1**); and *CgPDR1* (accession no. **NC005967**; location 47 557–50 880).

The nucleotide sequences of *ERG3*, *ERG11* and *PDR1* of 11 clinical *C. glabrata* isolates in this study have been deposited in the GenBank database under accession no. **MH734294**–**MH734304**, **MH734272**–**MH734282** and **MH734283**–**MH734293**, respectively.

2.5. RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

C. glabrata isolates were grown in YPD (yeast extract–peptone–dextrose) medium to early exponential phase [optical density at 600 nm (OD_{600}) of 0.4–0.5]. *Candida* cells were lysed by mechanical disruption using zirconia beads and TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was purified using an RNeasy Mini Kit (QIAGEN), followed by treatment with TURBO™ DNase enzyme (Ambion, Austin, TX) to remove contaminating DNA. RNA concentration and purity were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was verified by gel electrophoresis. First-strand cDNA was converted from 1 μg of purified RNA with oligo(dT) and random hexamer primers using SuperScript™ III Reverse Transcriptase enzyme (Invitrogen). Negative controls (samples without reverse transcriptase enzyme, 'no RT') were performed for all RNA samples. The cDNA of samples was diluted 1:10 in nuclease-free water and was kept in aliquots at −20 °C prior to use.

The expression levels of *CgPDR1*, *CgERG11*, *CgCDR1*, *CgCDR2*, *CgSNQ2*, *CgYOR1*, *CgYBT1*, *CgQDR2* and *CgRTA1* of two fluconazole-resistant and nine SDD *C. glabrata* isolates were evaluated by RT-qPCR carried out in a 20 μL reaction volume using PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers (Supplementary Table S1 in the version at DOI: 10.1016/j.jgar.2019.01.006). Reactions were performed in 96-well plates using a 7500 Real-Time PCR System (Applied

Biosystems). *RDN5.8* was used as a reference gene for RT-qPCR analysis of *C. glabrata* gene expression [22]. No-RT and no-template controls were included and served as negative controls. All samples were analysed in duplicate in each experiment and the results are from three independent experiments. A dissociation analysis for each run was performed to examine the specificity of the primers. Gene expression levels of target genes were determined using the relative quantification ($2^{\Delta\Delta CT}$) method compared with reference gene expression levels.

2.6. Statistical analysis

Statistical analyses and graph creation were performed using GraphPad Prism software v.5.0 (GraphPad Software Inc., La Jolla, CA). Statistical differences between two groups (resistant and SDD) were evaluated with the non-parametric Mann–Whitney *U*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Azole susceptibility profile of clinical non-albicans Candida species

In this study, *C. glabrata* clinical isolates showed decreased susceptibility to azole drugs (fluconazole, itraconazole and ketoconazole). The MIC_{50} and MIC_{90} values of fluconazole in *C. glabrata* were 8 μg/mL and 32 μg/mL, respectively (Table 1). All clinical isolates of *C. tropicalis* and *C. kefyr* recovered from oral samples were susceptible to azoles. Of the 85 NAC isolates examined in this study, 2 *C. glabrata* isolates (2.4% of all NAC isolates) were resistant to all three types of azole drug, and 1 *C. parapsilosis* isolate was considered SDD to fluconazole.

3.2. Sequence analysis of CgERG3, CgERG11 and CgPDR1 genes

Two *C. glabrata* isolates exhibited cross-resistance to azoles. To better understand these observations, the molecular mechanisms of azole resistance in clinical *C. glabrata* isolates were investigated. Various azole resistance-related genes, including *CgERG3*, *CgERG11* and *CgPDR1*, were examined. Genes from two fluconazole-resistant and nine SDD isolates were amplified and sequenced to search for missense mutations altering amino acid sequences.

All nucleotide substitutions identified in the *CgERG3* and *CgERG11* coding sequences from all tested *C. glabrata* isolates (Table 2) were silent and did not change amino acid sequences. However, many amino acid substitutions in the *CgPDR1* coding sequence were observed in resistant and SDD isolates. Two distinct missense mutations in *CgPDR1* (N768D and E818K) were identified in the two azole-resistant *C. glabrata* isolates. Interestingly, these substitutions were absent in SDD isolates of *C. glabrata* (Table 2). One resistant isolate (CG25) had an amino acid substitution in CgPDR1 at position 768 from asparagine to aspartic acid (N768D). The other resistant isolate (CGL4) carried two missense mutations in CgPDR1, a leucine to isoleucine substitution at amino acid 139 (L139I) and a glutamic acid to lysine substitution at position 818 (E818K). However, the L139I substitution was also identified in one SDD isolate.

3.3. Determination of expression level of azole resistance-related genes in C. glabrata

To determine whether missense mutations identified in the *CgPDR1* gene correlated with expression of genes related to azole resistance, RT-qPCR analysis was performed. Previous studies reported a set of drug transporters that were regulated by *CgPDR1* [23,24]. Therefore, expression levels were examined for related genes, including *CgPDR1* and *CgERG11* as well as the multidrug transporter genes *CgCDR1*, *CgCDR2*, *CgSNQ2*, *CgYOR1*, *CgYBT1*, *CgQDR2* and *CgRTA1* in 11 clinical *C. glabrata* isolates (2 fluconazole-resistant and 9 SDD isolates). Expression of the *CgPDR1* regulator and all drug transporters was significantly higher in resistant isolates compared with the SDD isolates (Table 3). An approximate three-fold increase in expression of *CgPDR1* was observed in resistant strains carrying mutations in *CgPDR1*, suggesting that the *CgPDR1* regulator controls its own expression. The N768D and E818K mutations in *CgPDR1* were likely associated with quite high expression of *CgCDR1* and *CgCDR2*, which were upregulated >10-fold compared with the SDD isolates (Table 3). The expression levels of resistance-related genes of two azole-resistant isolates are shown in Supplementary Fig. S1 in the version at DOI: 10.1016/j.jgar.2019.01.006. These data suggest that both mutations in *CgPDR1* act as gain-of-function (GOF) mutations.

4. Discussion

C. glabrata is a haploid yeast with no ability to produce true hyphae and has fewer virulence factors compared with *C. albicans* and *C. tropicalis.* Furthermore, a low percentage of *C. glabrata* strains have the ability to produce proteinase and phospholipase enzymes [25,26]. Nevertheless, *C. glabrata* is the second most

Table 1
In vitro azole antifungal susceptibility of 85 non-*albicans Candida* (NAC) species isolates recovered from Thai oral candidiasis patients.

NAC species (no. of isolates)	Antifungal agent	MIC (μg/mL)			No. (%) of isolates		
		Range	MIC_{50}	MIC_{90}	S	SDD	R
C. glabrata (40)	FLU	0.125–64	8	32	0	38 (95.0)	2 (5.0)
	ITR	0.03–1.0	0.125	0.125	37 (92.5)	1 (2.5)	2 (5.0)
	KET	0.03–2.0	0.25	0.25	38 (95.0)	0	2 (5.0)
C. tropicalis (28)	FLU	0.125–2.0	0.25	1	28 (100)	0	0
	ITR	0.03–0.125	0.125	0.125	28 (100)	0	0
	KET	0.03–0.25	0.03	0.25	28 (100)	0	0
C. kefyr (9)	FLU	1.0–2.0	1	2	9 (100)	0	0
	ITR	0.03–0.125	0.125	0.125	9 (100)	0	0
	KET	0.03–0.25	0.06	0.25	9 (100)	0	0
C. parapsilosis (8)	FLU	0.25–4.0	1	2	7 (87.5)	1 (12.5)	0
	ITR	0.03–0.125	0.06	0.125	8 (100)	0	0
	KET	0.03–0.06	0.03	0.06	8 (100)	0	0

MIC, minimum inhibitory concentration; $MIC_{50/90}$, concentrations required to inhibit 50% and 90% of *Candida* isolates within a test population, respectively; S, susceptible; SDD, susceptible dose-dependent; R, resistant; FLU, fluconazole; ITR, itraconazole; KET, ketoconazole.

Table 2
Nucleotide (nt) and amino acid (AA) alterations in CgERG3, CgERG11 and CgPDR1 in 11 *Candida glabrata* isolates.

C. glabrata isolates	MIC (μg/ml)*			*CgERG3*	*CgERG11*	*CgPDR1*
	FLC	ITC	KTC	AA change	AA change	AA change (NT change)
CG25	**64.00**	**1.00**	**2.00**	-	-	**N768D** (A2302G)
CGL4	**64.00**	**1.00**	**1.00**	-	-	L139I, **E818K** (C415A, G2452A)
CG29	32.00	0.50	0.13	-	-	D243N (G727A)
CG13	32.00	0.125	0.25	-	-	D243N (G727A)
CG20	32.00	0.125	0.03	-	-	D243N (G727A)
CG179	32.00	0.03	0.03	-	-	E259G (A776G)
CG325	8.00	0.125	0.25	-	-	-
CGJ6	8.00	0.125	0.25	-	-	E259G (A776G)
CGJ7	8.00	0.125	0.125	-	-	G189V (G566T)
CGK85	8.00	0.03	0.03	-	-	-
CGN3	0.50	0.125	0.06	-	-	L139I (C415A)

MIC, minimum inhibitory concentration; FLU, fluconazole; ITR, itraconazole; KET, ketoconazole.
[a]Shading represents azole susceptibility category: dark grey, resistant; light grey, susceptible dose-dependent; no shading, susceptible.

Table 3
Comparison of relative mRNA expression levels of nine azole resistance-related genes between fluconazole-resistant and susceptible dose-dependent (SDD) *Candida glabrata* isolates.[a,b]

Gene	Fluconazole susceptibility category	
	Resistant[c]	SDD
CgPDR1	**3.04 ± 0.29 (2.87)**	1.12 ± 0.07 (1.14)
CgERG11	1.12 ± 0.04 (1.06)	1.32 ± 0.11 (1.36)
CgCDR1	**24.79 ± 4.56 (21.56)**	1.18 ± 0.09 (1.20)
CgCDR2	**16.42 ± 2.30 (15.09)**	1.17 ± 0.09 (1.04)
CgSNQ2	**2.29 ± 0.22 (2.32)**	1.03 ± 0.04 (1.00)
CgQDR2	**2.26 ± 0.12 (2.20)**	1.25 ± 0.12 (0.89)
CgYOR1	**5.07 ± 0.78 (5.27)**	1.11 ± 0.07 (1.01)
CgYBT1	**3.16 ± 0.45 (2.99)**	1.09 ± 0.06 (1.01)
CgRTA1	**5.76 ± 0.82 (5.34)**	1.25 ± 0.11 (1.09)

[a] Data are the mean ± standard error of the mean (median).
[b] Fluconazole-resistant and SDD groups comprised two and nine *C. glabrata* isolates, respectively.
[c] Bold indicates increased expression of azole resistance-related genes in the resistant group compared with the SDD group ($P \leq 0.01$).

frequently isolated *Candida* spp. from oral candida infections. The reason for the high occurrence of *C. glabrata* may be due to increased resistance to azole drugs [27,28]. In vitro susceptibility testing demonstrated greater cross-resistance to fluconazole, itraconazole and ketoconazole in *C. glabrata* compared with other NAC species. However, the detection rate of azole cross-resistance in NAC isolates (approximately 2.4% of NAC isolates in the current study) was quite low compared with previous reports [29,30], possible due to different sources of clinical isolates tested and history of previous exposure to azoles.

Fluconazole and itraconazole are broad-spectrum antifungal drugs that exhibit low toxicity to patients, resulting in both drugs being commonly used for the treatment and prophylaxis of candidiasis. At present, ketoconazole is not used as a first-line drug for fungal infections owing to its adverse effects related to severe liver injury [31]. However, topical ketoconazole is still used in the treatment of *Candida*-infected denture stomatitis [32]. Cross-resistance to itraconazole and fluconazole was shown in the current study, therefore prescription of itraconazole against fluconazole-resistant *Candida* infection may only have limited benefits.

Previous studies have reported on the molecular mechanisms of azole resistance in *C. glabrata* [30,33,34]. The major route for azole resistance involves GOF mutations in the *CgPDR1* gene, which are associated with overexpression of several drug transporters and virulence genes [33]. The *CgPDR1* gene encodes a regulatory factor and contains a Zinc(2)–Cys(6) cluster DNA-binding domain, inhibitory region and activation domain [33,35]. These functional domains have been classified as hotspot regions for mutations leading to drug resistance. Several amino acid substitutions identified within these regions exhibit GOF phenotypes, resulting in enhanced activity of *CgPDR1* [24,33]. The current investigation identified two missense mutations leading to N768D and E818K substitutions in CgPDR1. Consistent with two previous studies [36,37], the N768D and E818G mutations in CgPDR1 were only found in fluconazole-resistant *C. glabrata* isolates. Amino acid position 818 of PDR1 is polymorphic; substitution of glutamic acid with glycine was reported in the study by Hou et al. [37], whilst in the current study it was found that this position was replaced with lysine and likely led to fluconazole resistance. Interestingly, the E818K mutation lies within a putative nuclear localisation signal (NLS) (spanning from amino acids 793–836) [24]. The change results in alteration from a negatively charged to positively

charged amino acid. The N768D substitution occurs near the NLS region. Asparagine (polar amino acid) is replaced with aspartic acid (negatively charged amino acid). The mutation L139I was found both in resistant and SDD strains therefore it would not be responsible for azole resistance. More than 50 missense mutations have been previously reported within the *CgPDR1* coding sequence [33,37], making the *CgPDR1* gene highly polymorphic among clinical *C. glabrata* isolates. This indicates that *C. glabrata* has evolved under strong selective pressure, requiring gradual mutation of genes involved in antimicrobial resistance as well as other related genes.

This work is the first report to demonstrate that azole cross-resistant *C. glabrata* isolates from Thai oral candidiasis patients possess specific mutations in *CgPDR1*. These mutations are associated with enhanced expression of several multidrug transporters. Moreover, *CgPDR1* variants identified in this study were associated with low susceptibility to azole drugs both from triazole (fluconazole and itraconazole) and imidazole (ketoconazole) groups. It is striking that the two resistant *C. glabrata* isolates carrying the GOF mutation in *CgPDR1* exhibited >10-fold upregulation of *CgCDR1* and *CgCDR2* multidrug transporters compared with SDD isolates. In addition, other drug transporters including *CgSNQ2*, *CgYOR1*, *CgYBT1*, *CgQDR2* and *CgRTA1* (putative transmembrane protein related to 7-aminocholesterol resistance) were significantly overexpressed in the two azole-resistant *C. glabrata* strains. Elevated gene expression in the resistant *C. glabrata* isolates is likely due the presence of pleiotropic drug response (PDRE) elements within the promoters of genes encoding drug efflux transporters [23,24]. The DNA binding site for the CgPDR1 regulator is the PDRE element, which has the consensus sequence TCC(A/G)(C/T)GGA. It is possible that the amino acid substitution in CgPDR1 may have a positive effect on gene expression mediated by increased binding between the CgPDR1 regulator and the PDRE element. This event promotes the recruitment of RNA polymerase enzyme to bind the promoter, which then increases the transcriptional level of the target genes. Another report also revealed that GOF mutations in *CgPDR1* are not only associated with reduced susceptibility to azoles but may also contribute to enhanced adhesion of *C. glabrata* to mammalian epithelial cells [12]. The significance of N768D and E818K substitutions in CgPDR1 on the adhesion of *C. glabrata* to oral mucosa has not been examined and warrants further investigation. Besides, increased adhesion to epithelial cells has also been observed in fluconazole-resistant *C. albicans* and *C. dubliniensis* isolates carrying GOF mutations in the MRR1 transcriptional factor, which is responsible for upregulation of the MDR1 efflux pump [11].

This study identified no missense mutations in *CgERG11* and *CgERG3* that were specific to azole-resistant *C. glabrata* isolates. Moreover, expression of *ERG11* was not significantly upregulated in resistant isolates compared with SDD isolates. These results are consistent with previous reports [21,30] that sequence alterations or increased expression of azole targets did not have major roles in azole resistance in *C. glabrata*. This is in contrast with *C. albicans* where the vast majority of clinical azole-resistant isolates possess missense mutations within ERG11 and ERG3 proteins. Besides, missense mutations within transcriptional regulators (*MRR1*, *UPC2* and *TAC1*) related to overexpression of drug transporters were responsible for fluconazole-resistant *C. albicans* [9,38]. The results of the current study indicate that in *C. glabrata* development of cross-resistance against different classes of azoles is primarily due to the presence of GOF mutations in the transcriptional factor *CgPDR1*, leading to overexpression both of ABC multidrug and MFS transporters. Therefore, analysis of GOF mutations in the *PDR1* regulator should provide a better understanding of the development of drug resistance and aid in the development of antifungal drugs against *Candida* strains with azole cross-resistance.

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Competing interests

None declared.

Ethical approval

The study protocol was approved by the Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy of Mahidol University [MU-DT/PY-IRB 2013/063.0810].

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