ORIGINAL ARTICLE

Gene Expression and Identification Related to Fluconazole Resistance of *Candida glabrata* Strains

Jae Il Yoo, PhD [1], Chi Won Choi, MA [2], Kyeong Min Lee, MA [1],
Yeong Seon Lee, PhD [1,*]

[1]Division of Antimicrobial Resistance, Center for Infectious Diseases,
Korea National Institute of Health, Seoul, Korea
[2]Proteome Research Team, Korea Basic Science Institute, Daejeon 3, Korea

Abstract

Objectives

Candida glabrata has become one of the most common causes of *Candida* bloodstream infections worldwide. Some strains of *C. glabrata* may be intermediately resistant to all azoles. The several possible mechanisms of azole resistance have been reported previously, but the exact resistant mechanism is not clear. In this study, we identified differentially expressed genes (DEGs) of fluconazole-resistant *C. glabrata* and compared the gene expression of fluconazole-resistant strains with that of fluconazole-susceptible strains to identify gene corresponding to fluconazole resistance.

Methods

Using antifungal susceptibility test, several *C. glabrata* strains were selected and used for further study. The expression of *CgCDR1* and *CgCDR2* genes was investigated by slot hybridization against fluconazole-susceptible, -resistant, and resistant-induced strains. In addition, *ERG3* and *ERG11* genes were sequenced to analyze DNA base substitution. DEGs were identified by reverse transcription-polymerase chain reaction using DEG kit composed of 120 random primers.

Results

In slot hybridization, *CgCDR1* gene was expressed more than *CgCDR2* gene in resistant strains. Though base substitution of *ERG11* and *ERG3* genes was observed in several base sequences, just one amino acid change was identified in resistant strain. In the results of reverse transcription-polymerase chain reaction, 44 genes were upregulated and 34 genes were downregulated. Among them, adenosine triphosphate-binding cassette transporter-related genes, fatty acid desaturase, lyase, and hypothetical protein genes were upregulated and

*Corresponding author. Division of Antimicrobial Resistance, Center for Infectious Diseases, Korea National Institute of Health, 194, Tongil-ro, Eunpyeong-gu, Seoul 122-701, Korea.
E-mail: ysleenih@korea.kr

aldehyde dehydrogenase, oxidoreductase, and prohibitin-like protein genes were downregulated. Other DEGs were also identified.

Conclusion

This study showed that *CgCDR1* gene was more closely related to fluconazole resistance of *C. glabrata* than *CgCDR2* gene. In addition, several other genes related with fluconazole resistance of *C. glabrata* were identified.

1. Introduction

Since the early 1980s, fungi have emerged as major causes of human infectious diseases, especially among immunocompromised patients and those hospitalized with serious underlying disease.[1−3] *Candida* is the agent most frequently implicated in invasive fungal infections, and it now ranks as the fourth most common cause of nosocomial bloodstream infections, accounting for 8% of all hospital-acquired bloodstream infections in the United States.[4,5] Among intensive care unit patients, it is the third most common cause of nosocomial bloodstream infections.[6] A recent trend noted in many hospitals is an increase in the prevalence of *Candida glabrata* as a cause of serious *Candida* infections.[7,8] The rise in the number of *C. glabrata* systemic infections deserves a great deal of concern because of the high mortality rate associated with *C. glabrata* infection. The incidence of invasive candidiasis has increased worldwide in recent decades.[9] Multiple antifungal agents are available for the treatment of candidiasis. Azole drugs, one of the four classes of antifungals, especially fluconazole, were commonly used in clinical practice. The extensive use of fluconazole has led to the increasing occurrence of resistant isolates. Especially, *C. glabrata* is yeast with intrinsically low susceptibility to fluconazole and is often recovered from clinical samples originating from AIDS or cancer patients.[10] Four main mechanisms of azole resistance have been described:[11] mutations in *ERG11* gene; increases in the copy number of the azole target; the blockage of the ergosterol biosynthesis pathway; and the overexpression of genes coding some adenosine triphosphate (ATP)-binding cassette or major facilitator superfamily efflux pumps, leading to the increasing efflux of azole drugs. Although several possible mechanisms of azole resistance have been reported previously, the exact resistant mechanism is not clear. And there is little gene information about fluconazole resistance in *C. glabrata*. In this study, we investigated gene expression of fluconazole-resistant strains compared to susceptible strains to identify gene corresponding to fluconazole resistance. We used gene fishing primers, including 120 random primers, to discover gene for fluconazole resistance and identified gene.

2. Materials and Methods

2.1. Clinical strains

In this study, a total of 56 *C. glabrata* strains were collected from tertiary hospitals. The source of *C. glabrata* strains were blood, urine, sputum, and others. And just one strain was collected per patient. The collected strains were subcultured onto Sabouraud dextrose agar and CHROMagar *Candida* medium (CHROMagar Co., France) to ensure their viability and purity. Cultures were routinely inoculated from single colony and grown at 30°C in yeast extract peptone dextrose (YEPD) (10 g of yeast extract, 20 g of peptone, and 20 g of dextrose per liter) or on YEPD agar plates (10 g of yeast extract, 20 g of peptone, 20 g of dextrose, and 15 g of agar per liter), stored at 4°C, and subcultured weekly, or stored at −80°C in YEPD containing 10% glycerol. Using the source information and genetic background like restriction fragment length polymorphism, pulse-field gel electrophoresis pattern (data not shown), fluconazole-susceptible strain D-31 (minimal inhibitory concentration [MIC] 1 µg/mL), susceptible dose-dependent (SDD) strain D-284 (MIC 16 µg/mL), and resistant D-116 (MIC ≥256 µg/mL) strain were selected for further study.

2.2. Antifungal susceptibility testing

Antifungal susceptibility testing of *Candida* strains to fluconazole was performed in exact accordance with the reference broth microdilution method described in the M27-A2 guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS).[12]

2.3. Fluconazole-resistant induction

To investigate the expression signal change of *CgCDR1* and *CgCDR2* genes against fluconazole susceptibility step, fluconazole-susceptible D-31 strain was induced to be fluconazole resistant. *C. glabrata* D-31 strain was cultured in YEPD broth including fluconazole 1 µg/mL at the beginning, and the concentration of fluconazole was increased continuously up to 64 µg/mL. The MIC was confirmed with broth microdilution method and E-test experiment. The slot hybridization results were compared to results of susceptible, SDD, and resistant strains.

2.4. Expression of *CgCDR1* and *CgCDR2* genes

The *CgCDR1* and *CgCDR2* genes were used for slot hybridization to analyse the patterns of gene expression of *C. glabrata* strain. Using polymerase chain reaction (PCR), *CgCDR1*, *CgCDR2*, and 26S ribosomal RNA products were synthesized, purified, and labelled by a random prime labelling kit (Amersham, Co., PA, USA). For hybridization experiment, nitrocellulose membrane was soaked in distilled water and in 20× saline-sodium citrate solution for 1 hour at room temperature. Sample RNA was added to the membrane and washed two to three times with 10× saline-sodium citrate solution, and the membrane was dried with a vacuum instrument. The RNA was fixed on the membrane with ultraviolet and hybridized. Signals were detected using an enhanced chemiluminescence detection kit (Amersham, Co., PA, USA). In slot hybridization experiment, fluconazole resistant-induced strains were also tested against *CgCDR1* and *CgCDR2* gene expression.

2.5. *CgERG3* and *CgERG11* sequence analysis

Using three selected strains (fluconazole susceptible, SDD, resistant), *ERG3* and *ERG11* genes were detected by specific PCR with the use of *ERG3*-F, *ERG3*-R, *ERG11*-F, and *ERG11*-R primers complementary to the regions of *ERG3* and *ERG11* genes. The genomic DNA was used for PCR template. PCR ran in GeneAmp 2700 apparatus (Perkin-Elmer, Co., CA, USA) under the condition 25 cycles of PCR amplification with Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan). Annealing reaction was done at 50°C, and extension reaction was done at 72°C. The product was confirmed with electrophoresis at 1.2% agarose gels. The PCR products of *ERG3* and *ERG11* genes were purified by PCR purification kit (Qiagene GmbH, Hilden, Germany) and directly sequenced.

2.6. Gene identification

Cells from 24-hour cultures grown in YEPD were inoculated in 200 mL of YEPD at starting concentration of 2×10^4 cells/mL. The cultures were grown overnight at 30°C with agitation. Total RNAs were prepared from the cultures of susceptible, SDD, resistant strains at optical density of 600 nm of 0.3 value using RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Total RNAs extracted from fluconazole-susceptible, -SDD, and -resistant strains were used for the synthesis of first-strand complementary DNAs (cDNAs) by reverse transcriptase. Reverse transcription was performed for 1.5 hours at 42°C in a final reaction volume of 20 μL containing 3 μg of the purified total RNA, 4 μL of 5′ reaction buffer (Promega, Madison, WI, USA), 5 μL of

deoxynucleotide triphosphates (each 2 mM), 2 μL of 10-μM deoxythiamine annealing control primer 1(ACP-1) (5′-CTGTGAATGCTGCGACTAC GATIIIIIT-3′), 0.5 μL of RNasiǹ RNase inhibitor (40 U/μL; Promega), and 1 μL of Moloney murine leukaemia virus reverse transcriptase (200 U/μL; Promega). First-strand cDNAs were diluted by the addition of 80 μL of ultra-purified water for GeneFishing PCR and stored at −20°C until use. Differentially expressed genes were screened by the ACP-based PCR method using GeneFishing differentially expressed gene kits (Seegene, Seoul, South Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 μL containing 3−5 μL (about 50 ng) of diluted first-strand cDNA, 1 μL of dT-ACP2 (10 μM), 1 μL of 10 μM arbitrary ACP, and 10 μL of 2′ Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 minute, followed by 50°C for 3 minutes, and 72°C for 1 minute. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 seconds, 65°C for 40 seconds, and 72°C for 40 seconds, followed by a 5-minute final extension at 72°C. The amplified PCR products were separated in 2% agarose gel and stained with ethidium bromide. The differentially expressed bands were re-amplified and extracted from the gel using GENCLEAǸ II Kit (Q-BIO gene, Carlsbad, CA, USA) and directly sequenced with ABI PRISM̀ 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Expression of *CgCDR1* and *CgCDR2* genes

In the results of hybridization experiments, the fluconazole-resistant D-116 strain showed increased expression of *CgCDR1* gene than the susceptible (D-31) and SDD (D-284) strains. *CgCDR2* gene expression also increased in the fluconazole-resistant strain than in the susceptible strain, but the signal was weaker than the *CgCDR1* gene signal (Figure 1A). The fluconazole resistant-induced strain showed increased expression of *CgCDR1* and *CgCDR2* compared to susceptible strain. But *CgCDR1* gene responded first to the fluconazole resistance induction environment (Figure 1B).

3.2. *CgERG3* and *CgERG11* sequence analysis

Three *C. glabrata* strains showed the same predicted product size against *ERG3* and *ERG11* genes (Figure 1C). Three point mutations of *ERG3* gene were observed in T435C, T317A, and G423A. In addition, two amino acid changes were also observed (Leu106Gln, Glu139Ala) including one in the
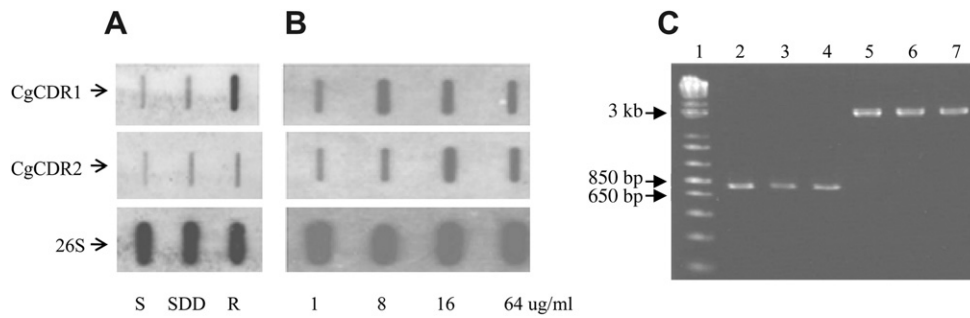
Figure 1 The results of RNA slot blot hybridization of fluconazole-susceptible, -SDD, and-resistant *Candida glabrata* strains. (A) *C. glabrata*-susceptible (D-31), -SDD (D-284), -resistant (D-116) strains, and 26s ribosomal RNA (quantity control). (B) Results of RNA expression from fluconazole resistant-induced isolate (MIC 1, 8: susceptible, MIC 16: SDD, MIC 64: resistant). (C) Lane 1: 1-kb marker, PCR product of *ERG3* (Lane 2−4) and *ERG11* (Lane 5−7) genes. Lane 2 and Lane 5: *C. glabrata* D-31 strain (susceptible), Lane 3 and Lane 6: *C. glabrata* D-284 strain (SDD), Lane 4 and Lane 7: *C. glabrata* D-116 strain (resistant). The PCR product was sequenced. SDD = susceptible dose dependent; MIC = minimal inhibitory concentration; PCR = polymerase chain reaction.

fluconazole-resistant strain. Moreover, five point mutations were shown in *ERG11* gene but no amino acid change (Table 1).

3.3. Gene identification

When 120 random primers were used, the expression of 258 genes increased or decreased (Figure 2). Among these genes, 78 genes showed more than two times expression difference between fluconazole-susceptible and -resistant strains. Finally, 44 genes were of higher expression and 34 were of lower expression in the fluconazole-resistant strain than in the susceptible strain. Among the 44 genes, 13 were identified as fumarate hydratase, manganese transporter, transcriptional regulation mediator, adenosine diphosphate/ATP carrier protein, and other proteins. All of the proteins were corresponding to *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, and *C. glabrata* protein. The 34 less expressed genes included aldehyde dehydrogenase, oxidoreductase, and prohibitin-like protein gene. The

ATP-binding cassette transporter was identified in both patterns (Table 2).

4. Discussion

Candida species is one of the major organisms of fungal infections. Recently, *C. glabrata* has emerged as a common cause of bloodstream and mucosal infections. The major mechanisms of fluconazole resistance among *C. glabrata* described to date have been primarily based on *CgCDR1*, *CgCDR2*, and ergosterol synthesis pathway enzymes. The *CgCDR1* and *CgCDR2* gene upregulation and the point mutation of *ERG3* and *ERG11* genes are known to have related with fluconazole resistance of *C. glabrata*. However, there is not much information on other genes contributing to the fluconazole resistance of *C. glabrata*. In this study, we discovered that 258 genes were expressed differentially between fluconazole-susceptible and -resistant strains. Among them, 73 genes showed higher or lower expression in the resistant strain compared to the

Table 1 Base substitution and amino acid changes of *ERG3* and *ERG11* genes from *Candida glabrata* fluconazole-susceptible, -susceptible dose-dependent, and -resistant strains

	Erg3		Erg11
	Base substitution	Amino acid change	Base substitution
Susceptible (*C. glabrata* D-31)	T435C	Glu139Ala	T768C
S-DD (*C. glabrata* D-284)	T317A	Leu106Gln	T768C
	T435C	Glu139Ala	T834C
			A1593G
Resistant (*C. glabrata* D-116)	G423A	Glu139Ala	C588T
	T435C		T768C
			C918T

The amino acid change was shown in *ERG3* sequence from all *C. glabrata* strains, but there was no amino acid change in *Erg11* gene sequence.
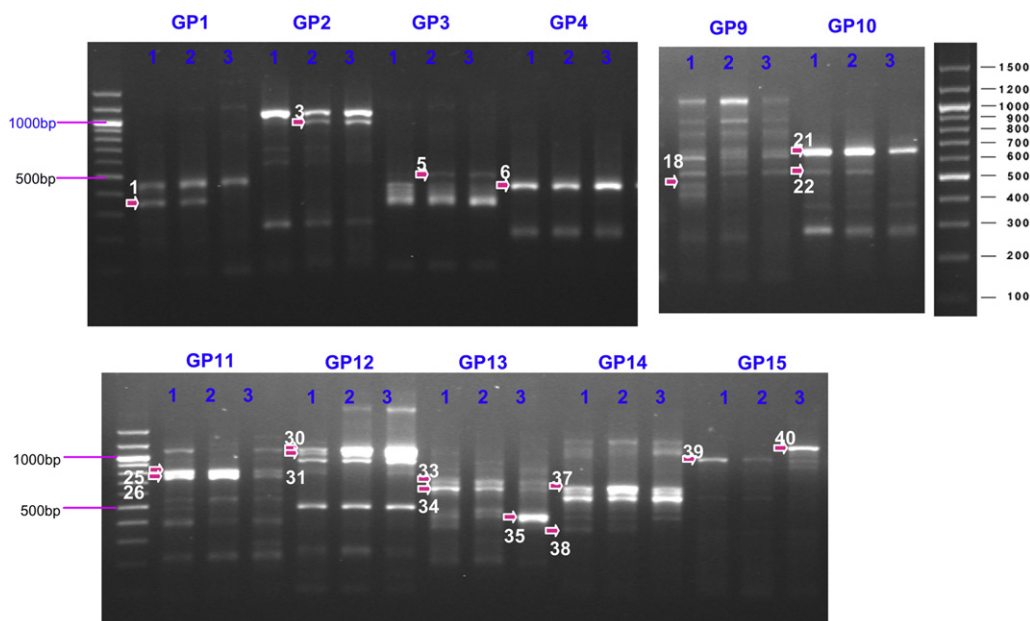*C. glabrata* = *Candida glabrata*.

Figure 2   Differently expressed genes were screened by the reverse transcription-PCR method using GeneFishing DEG kits. RNA was purified from three *Candida glabrata* strains (1: susceptible, 2: susceptible dose dependent, 3: resistant). The DEGs were cloned and sequenced. A total of 258 genes showed higher or lower expression in the resistant strain compared to the susceptible strain. The PCR product with numbers is selected for the sequencing. PCR = polymerase chain reaction; DEG = differentially expressed gene.

susceptible strain. Most of the identified genes were assigned to *S cerevisiae* because of insufficient information on *C. glabrata* gene even though the full sequence of *C. glabrata* was recently published.[13] The identified gene products were corresponded to amino acid metabolism, cell wall synthesis, energy production

protein, tricarboxylic acid cycle enzyme-related products, oxidoreductase, prohibitin-like protein, and others. Among the identified genes, specifically ATP-binding transporters increased or decreased. Because ATP-binding transporters like *CgCDR1* and *CgCDR2* genes showed higher expression in the fluconazole-resistant

Table 2   Identification of differently expressed genes from reverse transcription-polymerase chain reaction

Increased expression	Decreased expression
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*
Tryptophanyl-tRNA synthetase	DNA-dependent ATPase/DNA helicase B
Carnitine O-acetyltransferase	GTP cyclohydrolase II
Peroxisomal long-chain fatty acid import protein 2	Prohibitin antiproliferative protein
Fumarate hydratase	Methionine aminopeptidase
Manganese transporter	Aldehyde dehydrogenase
Transcriptional regulation mediator	Oxidoreductase
General transcriptional adaptor or co-activator	prohibitin-like protein
Component of actin cortical patches	
Negative regulator of ras-cAMP pathway	*Candida glabrata*
AAC2 ADP/ATP carrier protein	ATP-binding cassette transporter
Involved in cell wall biogenesis and architecture	
Gamma-glutamylphosphate reductase	
Acetyl-CoA synthetase	
Candida glabrata	
Carnitine acetyltransferase gene	
ATP-binding cassette transporter	
Saccharomyces kluyveri	
Putative citrate synthase gene	

A total of 258 genes showed increased or decreased expression. Among them, 78 genes were selected for product cloning and sequencing.
tRNA = transfer RNA; cAMP = cyclic adenosine monophosphate; AAC2 ADP/ATP = adenosine diphosphate/adenosine triphosphate carrier 2; CoA = coenzyme A; GTP = guanosine triphosphate.

strain than in the susceptible strain, other ATP-binding transporters related to fluconazole resistance are of high interest. The expression of *CgCDR1* has higher fluconazole-resistant levels than in the concentration of *CgCDR2* expression.[10] In our study, the hybridization experiment of *CgCDR1* and *CgCDR2* genes also showed that the expression of *CgCDR1* gene was more increased than *CgCDR2* compared to susceptible strain. And the expression of fluconazole resistant-induced strain showed that *CgCDR1* gene was first increased to the fluconazole stress. This result supposes that *CgCDR1* gene is the main gene corresponding to fluconazole resistance. And the point mutation of *ERG3* gene was observed in three DNA bases. And it caused amino acid changes. The position of base substitution of *ERG3* gene was different from the position previously reported.[14−16] Usually, the point mutation of *ERG3* or *ERG11* gene is related with fluconazole resistance.[17,18] But the results of this study need more sequence analysis using more fluconazole-susceptible strains to confirm the results. Finally, in this study, we identified a few genes whose expression increased or decreased in fluconazole-resistant strain. But the exact mechanism of how resistance is mediated has not been answered. Thus, it is necessary to study the functions of meaningful genes including transporter-related gene except already known *CgCDR1* and *CgCDR2* genes.

Acknowledgement

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