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ORIGINAL ARTICLE

Proteomic Analysis of Cellular and Membrane Proteins in Fluconazole-Resistant *Candida glabrata*

Jae Il Yoo [a], Chi Won Choi [b], Hwa Su Kim [a], Jung Sik Yoo [a], Young Hee Jeong [a], Yeong Seon Lee [a,*]

[a]*Division of Antimicrobial Resistance, Korea National Institute of Health, Osong, Korea.*
[b]*Proteome Research Team, Korea Basic Science Institute, Daejeon, Korea.*

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Abstract
Objectives: *Candida glabrata* is one of the most common causes of *Candida* bloodstream infections worldwide. Some isolates of *C glabrata* may be intermediately resistant to azoles, with some strains developing resistance during therapy or prophylaxis with fluconazole. In this study, we used a proteomic approach to identify differentially expressed proteins between fluconazole-resistant and -susceptible strains.
Methods: Membrane and cellular proteins were extracted from fluconazole-susceptible and fluconazole-resistant *C glabrata* strains. Differentially expressed proteins were compared using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins with >1.5-fold difference in expression were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS).
Results: A total of 65 proteins were differentially expressed in the cellular and membrane fractions. Among the 39 cellular proteins, 11 were upregulated and 28 were downregulated in fluconazole-resistant strains in comparison with fluconazole-susceptible strains. In the membrane fraction, a total of 26 proteins were found, of which 19 were upregulated and seven were downregulated. A total of 31 proteins were identified by LC-MS/MS that are involved in glycolysis, carbohydrate transport, energy transfer, and other metabolic pathways. Heat shock proteins were identified in various spots.
Conclusion: Heat shock and stress response proteins were upregulated in the membrane fraction of the fluconazole-resistant *C glabrata* strain. Compared with susceptible strains, fluconazole-resistant strains showed increased expression of membrane proteins and decreased expression of cellular proteins.

*Corresponding author.
E-mail: yslee07@nih.go.kr

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1. Introduction

The incidence of life-threatening fungal infections has increased steadily over the most recent decade [1,2]. In the most recent years, there has been increased interest in the opportunistic pathogen, *Candida*, which is responsible for the majority of fungal infections. Indeed, the increase in *Candida* blood infections among hospitalized patients and the rising incidence of infections caused by non-*albicans Candida* species, including *C glabrata*, *C tropicalis*, and *C parapsilosis*, have been noted [3]. In particular, *C glabrata* has emerged as a major cause of mucosal and invasive fungal infection in the United States, second only to *C albicans* [4]. The rise in the number of *C glabrata* systemic infections deserves a great deal of attention due to the high mortality rate associated with *C glabrata* fungemia [5]. Because the frequency of fungal infections is increasing, the need for antifungal agents has correspondingly increased, but antifungal agents are restricted to a few compounds. In particular, fluconazole is a highly effective wide-spectrum antifungal agent that is widely prescribed for the treatment of superficial and systemic candidiasis because it can be orally administered. However, the widespread and prolonged use of azoles in recent years has led to the rapid development of drug resistance in *Candida* species [6,7]. *C glabrata* infections are difficult to treat and are often resistant to many azole antifungal agents, especially fluconazole [8,9]. Fluconazole has limited activity against *C glabrata* because *C glabrata* has an intrinsically low susceptibility to azole antifungal agents. Furthermore, acquired azole resistance has recently been reported in oral isolates of *C glabrata* from hematopoietic stem cell transplant recipients and patients who have received radiation for the treatment of head and neck cancer [9,10]. The mechanisms underlying *C albicans* resistance to azole antifungal agents have been well elucidated. Nonetheless, the molecular basis for the intrinsically low susceptibility of *C glabrata* remains unclear. Several mechanisms of acquired resistance to azole antifungal agents have been proposed, including the upregulation of the genes that encode the ATP-binding cassette (ABC) transporters (*CDR1* and *CDR2*) [11] and the target of azole antifungal agents, lanosterol 14-α-demethylase (*ERG11*) [12]. Recently, a proteomic analysis of multiple matched sets of azole-susceptible and azole-resistant *Candida* isolates was undertaken in order to understand the mechanisms underlying azole antifungal resistance. In this study, we investigated changes in the expression levels of cellular and membrane proteins between fluconazole-resistant and -susceptible *C glabrata* strains. Here, we report several proteins that were found to be associated with fluconazole resistance.

2. Materials and Methods

2.1. *C glabrata* strains and growth conditions

We previously reported the results of an antifungal susceptibility test [13]. Based on those results, we selected four *C glabrata* strains according to their fluconazole susceptibility for a comparative proteomic study. To minimize variation between strains, two strains of each type (fluconazole-susceptible and fluconazole-resistant) were used. All strains were stored at -80°C. Prior to testing, each strain was subcultured twice on Sabouraud dextrose agar (Difco, Co., USA) to ensure viability and purity. For the proteomic analysis, an aliquot of the glycerol stock of each strain was diluted in YPD broth (1% yeast extract, 2% peptone, and 1% dextrose) and cultured overnight at 30°C in a shaking incubator. The cultures were diluted to an optical density 0.2 at OD600 in 0.5 L of fresh YPD broth and grown as before to the logarithmic phase and an equivalent optical density.

2.2. Cellular and membrane protein extraction

C glabrata was cultured in YPD broth at 30°C to the late exponential phase of growth. Cells were harvested, washed with distilled water, and resuspended in homogenizing buffer (50 mM Tris-HCl, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride; pH 7.5). The cells were then disrupted using glass beads. Cell debris and any unbroken cells were removed by centrifugation at 5000× *g* at 4°C for 10 minutes. The supernatants were pooled, desalted, concentrated, and analyzed for cellular proteins. A crude membrane fraction was isolated from the cell-free supernatant (after cell disruption) by centrifugation at 30,000× *g* at 4°C for 30 minutes. The pellets were washed in GTE buffer (10 mM Tris-HCl, 0.5 mM EDTA, 20% [v/v] glycerol; pH 7.0), resuspended in GTE buffer, and stored at -80°C. The protein concentration was determined using a micro-Bradford assay in the protein assay kit II (Bio-Rad, Hercules, CA, USA).

2.3. Two-dimensional gel electrophoresis analysis

For the two-dimensional (2D) gel electrophoresis analysis, pH 3−10 immobilized pH gradient (IPG) strips (Amersham Biosciences, Amersham Co., USA) were rehydrated in a swelling buffer containing 7 M urea, 2 M thiourea, 0.4% (w/v) DTT, and 4% (w/v) CHAPS. The protein lysates (500 μg) were cup-loaded into the rehydrated IPG strips using a Multiphor II apparatus (Amersham Biosciences) at 57 kVh. Two-dimensional gel separation was performed on 8−16% (v/v) linear gradient sodium dodecyl sulfate (SDS) polyacrylamide gels. Then, the gels were fixed, stained, destained, and imaged using a GS-710 imaging calibrated densitometer (Bio-Rad). Protein spot detection and 2D pattern matching were

carried out using ImageMasterTM 2D Platinum software (Amersham Biosciences). To compare the protein spot densities between the control and treated samples, > 20 spots throughout all of the gels were correspondingly landmarked and normalized. To ensure the reproducibility of the 2D gel electrophoresis experiments, each sample was analyzed in duplicate.

2.4. In-gel protein digestion

Protein bands of interest were excised and digested in-gel with sequencing-grade, modified trypsin (Promega, Madison, WI, USA) as previously described [14]. In brief, each protein spot was excised from the gel, placed in a polypropylene (Eppendorf) tube, and washed 4−5 times (until the gel was clear) using 150 μL of a 1:1 mixture of acetonitrile:25 mM ammonium bicarbonate at pH 7.8. The gel slices were dried and rehydrated. The tryptic peptides that remained in the gel matrix were extracted for 40 minutes at 30°C using 20 μL of 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. The combination supernatants were evaporated and dissolved in 8 μL of 5% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) formic acid for mass spectrometry analysis.

2.5. Identification of proteins by liquid chromatography tandem mass spectrometry

The resulting tryptic peptides were separated and analyzed using capillary reversed-phase high-performance liquid chromatography (HPLC) that was directly coupled to a Thermo Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) [15]. The individual spectra from MS/MS were processed using TurboSEQUEST software (Thermo Quest, San Jose, CA, USA). The generated peak list files were used to query either the MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com, matrix science Ltd.). Modifications to methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by the MASCOT probability analysis were initially considered.

3. Results

3.1. Analysis of the 2D gel spots

Among the clinically isolated *C glabrata* strains, the *C glabrata* D-31 and D-33 strains were identified as fluconazole-susceptible strains and the *C. glabrata* D-116 and D-149 strains were identified as fluconazole-resistant strains. Using 2D gel electrophoresis, the differential expression of proteins between the fluconazole-susceptible and -resistant strains was investigated (Figure 1). Regarding the membrane proteins, a total of 26 protein spots were found to be differentially expressed (by 1.5-fold) in fluconazole-resistant *C glabrata* strains in comparison with fluconazole-susceptible strains. Among these proteins, 19 proteins were upregulated and 7 proteins were downregulated in the fluconazole-resistant strains. As for the cellular proteins, a total of 39 protein spots were found to be differentially expressed in fluconazole-resistant versus fluconazole-susceptible strains. Among these 39 proteins, 11 were upregulated and 28 were downregulated. The protein spots were evenly spread throughout the pH 3−10 IPG gel.

3.2. Identification of differentially expressed proteins

Using LC-MS/MS, a total of 31 proteins were identified in the cellular and membrane protein fractions. In the membrane protein fraction, the expression of stress

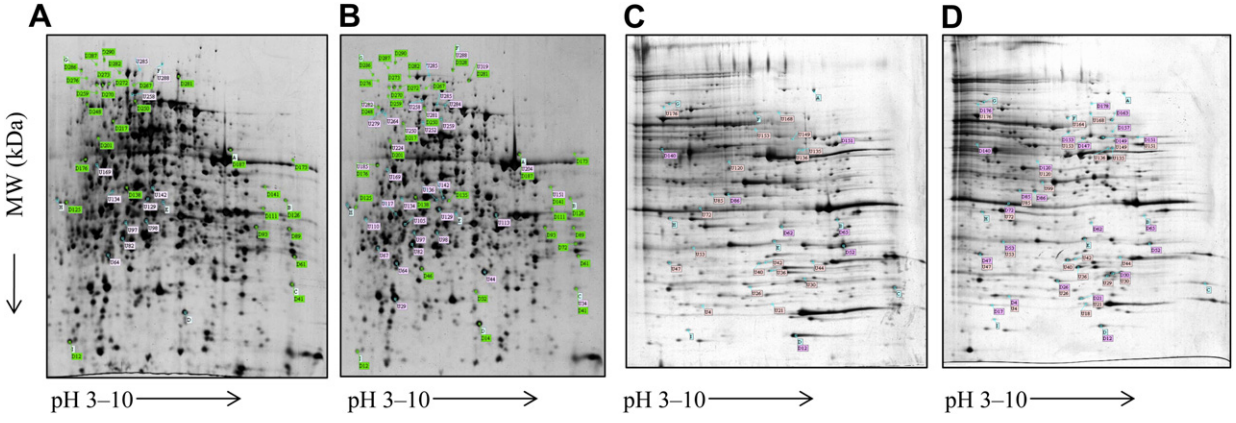

Figure 1. Cellular and membrane protein spots of *C glabrata* strains that were resolved using two-dimensional gel electrophoresis. Spots representing differentially expressed proteins were later identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptide mass fingerprinting. (A) Cellular protein spots of fluconazole-susceptible strains. (B) Cellular proteins spots of fluconazole-resistant strains. (C) Membrane protein spots of fluconazole-susceptible strains. (D) Membrane protein spots of fluconazole-resistant strains.

Table 1. Differentially expressed membrane proteins, as identified by LC-MS/MS between of fluconazole-susceptible and -resistant *C glabrata* strains

Spot	Protein	Molecular mass (kDa)	pI	Protein coverage (%)	Fold change (R/S)*
151	Ugp1	55.916	6.98	18	−4.04
86	Phosphoglycerate kinase	44.590	6.37	8	−2.33
65	Malate dehydrogenase	35.544	8.93	4	−2.02
149	Putative stress response protein	59.391	5.93	15	3.98
26	Nucleotide transport and metabolism hydrolase	25.680	5.96	15	3.07
153	Aldehyde dehydrogenase	27.079	5.77	5	3.89
176	Heat shock protein 70	69.806	5.06	13	2.98
72	Glyceraldehyde 3-phosphate dehydrogenase	35.871	6.46	2	2.12
136, 47	Methionine aminopeptidase	44.307	6.48	4	1.92

**Expression ratio of fluconazole-resistant (R) over fluconazole-susceptible (S) strains. The minus sign (-) indicatesthe decreased protein expression of fluconazole-resistant strains in comparison with fluconazole-susceptible strains.*
LC-MS/MS: liquid chromatography-tandem mass spectrometry.

response protein, aldehyde dehydrogenase, and heat shock protein, among others, were increased, whereas the expression levels of phosphoglycerate kinase, malate dehydrogenase, and UgP1 protein were decreased. Of note, the expression levels of stress response protein and aldehyde dehydrogenase were 3.8-fold higher in the fluconazole-resistant strain compared with the fluconazole-susceptible strain (Table 1). In the cellular protein fraction, several proteins showed decreased expression, but only four were upregulated in the fluconazole-resistant strains: fructose bisphosphate aldolase, phosphoglycerate kinase, and another unnamed protein. Among the identified proteins, heat shock protein 70 was found in five protein spots and hexokinase was identified in three spots (Table 2).

4. Discussion

C glabrata has an intrinsic tendency to resist the antifungal agent, fluconazole. The exact mechanisms underlying fluconazole resistance are not well known. Recently, however, a proteomic analysis study revealed a series of proteins associated with antifungal agent

Table 2. Differentially expressed cellular proteins, as identified by LC-MS/MS, between fluconazole-susceptible and -resistant *C glabrata* strains

Spot	Protein	Molecular mass (kDa)	pI	Protein coverage (%)	Fold change (R/S)*
288	Unnamed protein	61.726	5.59	10	1.90
64	Fructose bisphosphate aldolase	39.322	5.49	4	1.90
82	Phosphoglycerate kinase	44.590	6.37	16	1.71
286	GroEL-like type I chaperonin	60.351	5.14	5	−2.50
287, 290, 217, 284, 248	Heat shock protein70	66.355	5.32	5	−2.31
272, 270, 273	Hexokinase	53.772	5.23	32	−2.30
187	Putative oxidoreductase	33.032	5.01	4	−2.15
282, 250	Putative pyruvate decarboxylase	61.531	5.43	6	−2.09
281	Pyruvate kinase	55.060	6.08	6	−2.03
41	DNA-dependent RNA polymerase II	38.917	7.7	7	−1.95
141	Mitochondrial aconitase hydratase	84.169	5.96	1	−1.83
138	CoA-transferase	57.167	8.46	5	−1.75
176	IPP1P inorganic pyrophosphatase	14.532	4.84	83	−1.66

**Expression ratio of fluconazole-resistant (R) over fluconazole-susceptible (S) strains. The minus sign (-) indicates decreased protein expression of fluconazole-resistant strains in comparison with fluconazole-susceptible strains.*
LC-MS/MS: liquid chromatography-tandem mass spectrometry.

resistance [16]. In this study, we determined that cellular and membrane proteins are differentially expressed between fluconazole-susceptible and -resistant strains. A total of 31 proteins were identified by LC-MS/MS that are involved in glycolysis, carbohydrate transport, energy transfer, and other metabolic pathways. Heat shock protein was one of the most frequently identified cellular proteins. Fluconazole-resistant strains exhibited several downregulated proteins in the cellular fraction, while those in the membrane fraction were mostly upregulated. The overall fold change was narrow in the cellular proteins (1.6−2.5-fold), but was larger in the membrane proteins (1.9−4.4-fold), indicating that membrane proteins responded more severely to fluconazole-induced stress than cellular proteins. The expression of heat shock protein in the cellular fraction was decreased 2.31-fold; in the membrane fraction, heat shock protein 70 was increased by 2.98-fold and one stress response protein was increased by 3.98-fold. Usually, the expression of heat shock protein is not only regulated by temperature but also a range of noxious stimuli, including stress in general. Stress response protein has been previously associated with *C glabrata* biofilm formation. The association between fluconazole resistance and heat shock protein/ stress response protein expression should be further investigated in future studies.

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