Original Article

Differentially expressed proteins in fluconazole-susceptible and fluconazole-resistant isolates of *Candida glabrata*

Yinzhong Shen[1], Lijun Zhang[2], Xiaofang Jia[2], Yongxin Zhang[3], Hongzhou Lu[1,*]

[1] *Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China;*
[2] *Department of Science Research, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China;*
[3] *Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China.*

Summary   **The current study aimed to identify the differences presented in the proteome of fluconazole-susceptible isolates of *Candida glabrata* compared to those with fluconazole-resistant ones. Two-dimensional differential gel electrophoresis was applied to identify proteins that were differentially expressed in fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata*. Eight proteins including aspartyl-tRNA synthetase, translation elongation factor 3, 3-phosphoglycerate kinase, ribosomal protein L5, coproporphyrinogen III oxidase, pyruvate kinase, G-beta like protein, and F1F0-ATPase alpha subunit were found to be more abundantly represented, while four proteins including vitaminB12-(cobalamin)-independent isozyme of methionine synthase, microtubule-associated protein, adenylosuccinate synthetase, and aldose reductase were found to be less abundantly represented in fluconazole-resistant strains versus those with fluconazole-susceptible ones. These differentially expressed proteins were primarily associated with energy metabolism, stress response, and macromolecule synthesis. Proteins associated with energy metabolism, stress response, and macromolecule synthesis may play a role in the development of fluconazole resistance in the clinical isolates of *C. glabrata*. Multiple different mechanisms are involved in the development of fluconazole resistance in *C. glabrata*. These findings provide a scientific basis for discovering new genes and mechanisms associated with fluconazole resistance in *C. glabrata*.**

Keywords: *Candida glabrata*, proteomics, fluconazole, drug resistance

1. Introduction

In recent years, mucous membrane and invasive infections caused by *Candida glabrata* have increased significantly. In some areas, *C. glabrata* has become one of the three most frequent causes of candidiasis besides C. albicans (*1*). Currently, triazole antifungals are the most commonly used agents to treat *Candida* infections, however, *C. glabrata* exhibits intrinsically low susceptibilities to triazoles (*2*). The drug resistance of *C. glabrata* has become a major problem affecting the efficacy of clinical treatment of *C. glabrata* infections.

Recently, studies have been conducted to understand the mechanisms of fluconazole resistance in *C. glabrata*. It is relatively clear that the *ERG11*, *CDR1* and *CDR2* genes are associated with the development of fluconazole resistance in *C. glabrata* (*3,4*), but these genes can only partially explain the resistance in clinical isolates of *C. glabrata*. So far there have been no other genes reported to be responsible for the development of drug resistance. Therefore, it would be helpful to explain the mechanisms of fluconazole resistance in *C. glabrata* if more resistance-related genes and mechanisms could be identified. Over the years, genomic technologies have been the primary ways to study the resistance mechanisms of fungi. Recently, proteomics, defined as the global analysis of cellular proteins, is becoming a key area of research that is developing in the post-genome era. It has been demonstrated to be a powerful tool for the investigation of complex biochemical processes and the discovery

of new proteins. Proteomics provides a new tool for the study of fungal resistance. The evaluation of protein profiles in response to a modification of fungal resistance could represent a valid and useful approach for the development of new therapeutic strategies. Discovering resistance-related proteins through proteomics makes it possible to identify additional genes and mechanisms related to the development of fungal resistance. At present, proteomics are being applied to study the pathogenesis and drug resistance mechanisms of fungi.

In the present study, proteomic techniques were applied to identify proteins that are differentially expressed in fluconazole-resistant isolates of *C. glabrata* and fluconazole-susceptible ones. The study aimed to find new resistance-related proteins and thus provide a point of reference for the discovery and study of new fluconazole resistance related genes and mechanisms.

2. Materials and Methods

2.1. *Strains and antifungal susceptibility testing*

Clinical strains of *C. glabrata* were isolated from clinical specimens. The *in vitro* susceptibilities of *C. glabrata* isolates to fluconazole were determined by the broth microdilution method as described in the NCCLS document M27-A2 (*5*). All susceptibility tests were carried out in duplicate. The interpretive criteria for susceptibility to fluconazole were based on those published by the NCCLS (*5*) and were as follows: susceptible, ≤ 8 μg/mL; susceptible-dose-dependent (S-DD), 16 to 32 μg/mL; and resistant, ≥ 64 μg/mL. Nineteen isolates of *C. glabrata* were included in the present study, 10 of them were fluconazole-susceptible isolates while 9 of them were fluconazole-resistant isolates.

2.2. *Protein extraction*

Isolates of *C. glabrata* were cultured on Sabouraud dextrose agar containing chloramphenicol for 24 h. After that, each isolate was diluted in YPD broth (1% yeast extract, 2% peptone, 1% dextrose) and grown overnight at 30°C in a shaking incubator. Yeasts were grown in YPD at 30°C until they reached the late exponential phase of growth (optical density at 600 nm, approximately 7). Fungal cells were harvested, and washed with phosphate buffered saline (PBS) for 3 times. The 9 resistant isolates were then mixed with the same amount of cells for the isolation of proteins, while the 10 susceptible ones were also mixed with the same amount of cells to isolate proteins, respectively. The detailed extraction process was as follows: added an equal volume of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) lysis

buffer [8 mol/L urea, 2 mol/L thiourea, 65 mmol/L dithiothreitol (DTT), 0.5% pharmalytes pH 3-10, 0.5 mmol/L phenylmethanesulfonyl fluoride, 4% 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, 1% nonidet P-40, 0.05% nuclease mix] into the mixture of isolates, re-suspended the precipitation, then transferred into a mortar. Poured appropriate amount of liquid nitrogen and the sample immediately formed white lumps. Afterwards, ground the lumps into powder with continuously adding liquid nitrogen during the grinding process. After 10 min of grinding, the slurry was transferred into an EP tube. The residual liquid in the mortar was rinsed with a small amount of 2D-PAGE lysis buffer and the solution was transferred into the EP tube as well. Extracted on ice for half an hour by shaking the tube every several minutes. Centrifuge at 12,000 revolutions per minute (RPM) for 15 min. The resulting supernatant contained the soluble protein fraction. Soluble sodium dodecylsulphate (SDS) lysis buffer (100 mM Tris-HCl (pH = 6.8), 20% glycerol, 4% SDS, 100 mM DTT) was added into the insoluble precipitate after the extraction with 2D-PAGE lysis buffer. Ice extraction was conducted for 30 min by shaking every several minutes. Centrifuge at 12,000 RPM for 15 min. The resulting supernatant contained the less soluble protein fraction. Concentration of soluble protein was analyzed using a micro-Bradford assay using a Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). Concentration of less soluble protein was then determined by Bio-Rad's DC-RC kit (Bio-Rad, Hercules, CA, USA).

2.3. *2D-PAGE and imaging*

2D-PAGE was conducted according to the methods described in the references (*6,7*). After electrophoresis, the gel was ripped off, silver nitrate or Coomassie blue-stained, and imaged with the Image Scanner.

2.4. *SDS-PAGE separation*

In order to identify less soluble proteins, the insoluble precipitate after extraction with 2D-PAGE lysis buffer was re-extracted with SDS-containing lysis buffer (100 mM Tris-HCl (pH = 6.8), 20% glycerol, 4% SDS, 100 mM DTT). After determination of the protein concentration with the DC-RC method, the precipitate was split into 100 μg/tube and boiled in water for 5 min. It was then loaded into the comb holes for SDS-PAGE. Discontinuous SDS-PAGE electrophoresis vertical plate with gel concentration of 11.5% and stacking gel concentration of 4.8% was used for SDS-PAGE.

2.5. *Image master software analysis*

After repeated 2D-PAGE, the gel was scanned with an Image Scanner, and three pairs of parallel samples were

prepared for analysis with GE Image Master software (version 5.0) in order to identify protein spots with differences greater than 2-fold.

2.6. *In-gel digestion*

Protein digestion was conducted according to the method described by Hellmann *et al.* (*8*) with minor modifications.

2.7. *Liquid chromatography ion trap tandem mass spectrometry (LC-MS/MS)*

The mass spectrometer used was a Bruker ESI ion trap mass spectrometer equipped with a nano-liquid chromatography spray source. All measurements were carried out under positive ion mode. Five peptides with maximum intensity (beyond the threshold) were selected at each time for MS/MS analysis. The acquired data was entered into the database (NCBInr), and compared by the MS/MS Ions Search function in the Mascot software (Matrix Science Ltd., UK) (*http:// www.matrixscience.com*). Peptide with Mowse scores higher than the threshold would be regarded as an identified peptide, and its corresponding proteins would be regarded as identified proteins. For proteins with only one matching peptide, its ion score should be more than 40, and inspected manually to check whether the map had good quality (whether the sample peak was apparent, and signal to noise ratio was acceptable) and whether there were four or more consecutive y ions or b ions (*e.g.*, y2, y3, y4, y5) as well as whether the protein molecular weight and isoelectric point were consistent with the position on the gel. The identification result should have the highest score and should be from the species of *C. glabrata*. Amino acid sequence of the identification result was entered into Génolevures database for Blast (*http://cbi.labri.fr/Genolevures/blast. php#*) to search for homologous proteins corresponding to *Saccharomyces cerevisiae*.

3. Results

3.1. *SDS-PAGE*

Figure 1 shows the insoluble proteins separated by SDS-PAGE. It could be concluded that the SDS-PAGE maps of fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata* are basically the same, with no differing bands.

3.2. *2D-PAGE separation*

Soluble proteins were separated by 2D-PAGE, with sample volume of 250 μg for silver staining and 500 μg for Coomassie blue staining, and the strips were pH 3-10L. A pair of representative gel diagrams is
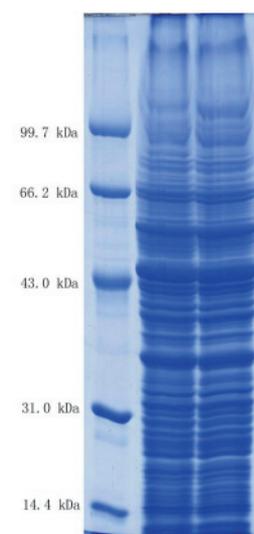

Figure 1. SDS-PAGE map of less soluble proteins of *C. glabrata* (left: Marker; middle: resistant isolates; right: susceptible isolates).

presented in Figure 2. Both of the up-regulated protein spots in the susceptible strains (marked in Figure 2A, 2D-PAGE map of susceptible isolates) and the up-regulated protein spots in the resistant strains (marked in Figure 2B, 2D-PAGE map of resistant isolates) were shown in Figure 2. It was demonstrated that protein spots were well separated, gel diagrams were consistent, and the different points were evident.

3.3. *Image master software analysis*

Any points of difference were subject to Image Master software analysis and rigorous screening. The gels of the two samples were grouped, and then matched within group to reduce human error in each gel production process. A minimum 2-fold change was considered for up-regulated proteins spots

3.4. *Mass spectrometry of differential protein spots*

All differential protein spots obtained were subject to gel digestion with trypsin, and the sequence of extracted peptides was analyzed by mass spectrometry. Twelve differential protein spots were accurately identified. All identification results are shown in Table 1. Among the 12 proteins, eight [aspartyl-tRNA synthetase, translation elongation factor 3, 3-phosphoglycerate kinase, ribosomal protein L5, coproporphyrinogen III oxidase, pyruvate kinase, G-beta like protein, and F1F0-ATPase alpha subunit] were found to be more abundantly represented, while four [vitaminB12-(cobalamin)-independent isozyme of methionine synthase, microtubule-associated protein, adenylosuccinate synthetase, and aldose reductase] were found to be less abundantly represented in fluconazole-resistant strains versus those with fluconazole-susceptible ones. These proteins are involved in energy metabolism, stress
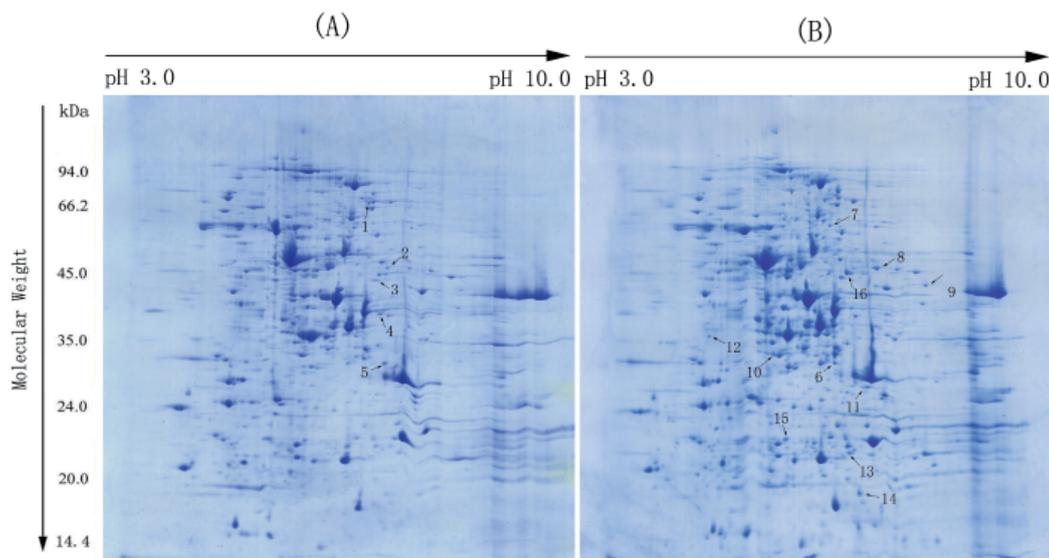
Figure 2. Soluble *C. glabrata* proteins resolved by 2D-PAGE for *C. glabrata* strains (A: susceptible isolates; B: resistant isolates). Spots representing differentially expressed proteins that were identified by mass spectrometry are correspondingly numbered in Table 1. Four proteins (highlighted) were found to be up-regulated in susceptible isolates, 12 protein spots (highlighted) up-regulated in resistant isolates.

Table 1. Differentially expressed proteins identified by mass spectrometry

Spot	Accession No.[a]	Protein (description)[b]	Score	pI	Mw (kDa)	Protein coverage
Up-regulated proteins which exhibited a 2-fold or greater change in susceptible isolates						
1	gi|50290071	YER091c (vitaminB12-(cobalamin)-independent isozyme of methionine synthase)	288	6.07	85809	11%
2	gi|50293659	YOR272w (microtubule-associated protein)	62	6.37	51047	2%
3	gi|50292025	YNL220w (adenylosuccinate synthetase)	789	6.35	48377	42%
4	gi|50289743	YHR104w (aldose reductase)	111	6.4	37460	14%
Up-regulated proteins which exhibited a 2-fold or greater change in resistant isolates						
5	gi|50286523	YLL018c (aspartyl-tRNA synthetase)	520	5.98	63322	28%
6	gi|50285389	YLR249w (translation elongation factor 3(EF-3))	155	5.71	116678	6%
7	gi|50293403	YCR012w (3-phosphoglycerate kinase)	422	6.37	44704	30%
8	gi|50294105	YPL131w (ribosomal protein L5 (L1a)(YL3))	344	6.42	33741	36%
9	gi|50292617	YDR044w (coproporphyrinogen III oxidase)	324	6.08	37720	20%
10	gi|50294908	YAL038w (pyruvate kinase)	301	6.08	54975	20%
11	gi|50286153	YMR116c (G-beta like protein)	213	5.85	30297	36%
12	gi|50294700	YBL099w (F1F0-ATPase alpha subunit)	563	8.99	58599	26%

[a] Accession numbers from NCBInr database; [b] Name and decription of *Saccharomyces cerevisiae* homologous proteins as denoted in the Génolevures database.

response, and biosynthesis of macromolecules in *C. glabrata*.

4. Discussion

In previous studies (*9,10*), sets of matched susceptible and resistant isolates, both derived from the same strain, were used to investigate the molecular mechanisms underlying the development of azole resistance in *Candida* species. These isolates, however, were rarely available in clinical settings. Therefore, those studies were limited either in that only a relatively small number of clinical isolates were investigated or the isolates originated from a single patient (*9*). Resistant isolates used in some studies were formed by laboratory induction (*10*). However, it is currently not clear

whether the mechanisms of resistance identified in resistant isolates formed by laboratory induction are the same as those resistant in arbitrarily selected isolates. In this study, genetically unmatched clinical *C. glabrata* isolates from different patients was used to investigate proteomic differences between the susceptible and resistant isolates of *C. glabrata*.

Proteomic technologies were applied in this study to identify the proteomic expression differences between fluconazole-resistant and fluconazole-susceptible isolates of *C. glabrata*. Twelve differentially expressed proteins were identified, 8 of them were up-regulated in the resistant isolates while 4 were up-regulated in the susceptible isolates. These differential proteins may be implicated in the resistance of *C. glabrata* to fluconazole, however their relationship with

fluconazole resistance needs to be confirmed by further studies. These proteins are mainly involved in energy metabolism, stress response, and biosynthesis of macromolecules in *C. glabrata*. Rogers *et al.* (*10*) conducted proteomic analysis of experimentally induced fluconazole resistance in *C. glabrata* and identified a number of differentially expressed proteins involved in glycolysis and glycerol metabolism, oxidative stress and biosynthesis of macromolecules. The authors also reported two differentially expressed proteins, 3-phosphoglycerate kinase and fecal porphyrin original III oxidase, while 10 differentially expressed proteins identified in our study have never been reported so far. These differential proteins reflect the physiological, biochemical, metabolic and other changes with the development of resistance of *C. glabrata* to fluconazole, but their specific roles in the development of resistance still need to be further studied.

This study failed to identify any differential expression of proteins that have been confirmed to be associated with fluconazole resistance, such as Cdr1p (encoded by *CDR1* gene), Cdr2p (encoded by *CDR2* gene), Erg11p (lanosterol 14α-demethylase, encoded by *ERG11* gene). The lack of changes in the membrane proteins in our study contradicts published results on protein changes as well as our previous results on gene expression proving significant increase in the expression of *ERG11*, *CDR1* and *CDR2* genes linked to azole resistance in *C. glabrata* clinical isolates (*11*). This inconsistency may originate from the protein isolation and preparation. These three proteins are membrane proteins. The former two are ATP-binding transporter proteins, while the last one is the target enzyme for fluconazole. Fungal membrane proteins are highly hydrophobic and fungi have cell walls, so isolation of such proteins is very difficult. In order to improve protein isolation efficiency, grinding in liquid nitrogen was applied in this study to fully release the proteins in cell walls. Meanwhile, the study also adopted step-wise protein extraction methods by referring to methods in the literature which extracted soluble proteins and insoluble proteins respectively, and separated them with 2D-PAGE and SDS-PAGE, respectively, to improve the separation efficiency of insoluble proteins. However, the study ultimately failed to detect the differential expression of insoluble proteins, while mainly soluble proteins were detected. Therefore, the extraction scheme of fungal proteins and two-dimensional gel electrophoresis remain to be further improved and optimized. In addition, the treatment with liquid nitrogen during the extraction procedure and the use of mortar should have had effect on the cell wall.

Progress has been made in some studies by examining drug resistance mechanisms of *C. glabrata* through protein levels. Niimi *et al.* (*12*) found that after exposure to fluconazole, *C. glabrata* can quickly induce the expression of two membrane proteins Cdr1p and 14-DM of *Candida*. These two membrane proteins changed from barely detectable levels to become the main composition of the cell membrane. To further confirm that CDR1 overexpression is associated with drug-resistance, the authors studied the functional expression of the *CDR1* gene in Saccharomyces cerevisiae and found that after the *CDR1* gene was transferred into Saccharomyces cerevisiae that lacked other protein-coding genes, the isolates changed from fluconazole-susceptible to highly fluconazole-resistant. Additionally, the expression of the membrane proteins Cdr1p were also found to be up-regulated. Marichal *et al.* (*9*) found that through a comparative analysis of protein 2D-PAGE maps of fluconazole-resistant *Candida* isolates and susceptible isolates that at least 25 proteins had increased expression, while 76 proteins had decreased expression. It was believed that these differentially expressed proteins may be related to drug-resistance related proteins, however, differential proteins were not identified. As a result, it was not identified which specific proteins may be related to drug-resistance. Rogers *et al.* (*10*) carried out a proteomic study on laboratory-induced fluconazole-resistant isolates of *C. glabrata* and detected a total of 25 differential proteins, in which expressions of Cdr1p and 14-DM in drug-resistant isolates were increased. It was believed that in addition to up-regulated expressions of Cdr1p and 14-DM related to drug-resistance of *C. glabrata*, other differential proteins might also be a cause of the formation of drug-resistance. These studies examined drug-resistance mechanisms of *C. glabrata* from protein levels, and investigated other possible mechanisms of the formation of drug-resistance, but such studies (including ours) did not carry out further studies on the specific functions of differential proteins. Therefore, the specific role of these differential proteins in the formation of drug-resistance of *C. glabrata* remains to be further studied and confirmed.

Proteomics has provided a powerful tool for the study of resistance mechanisms of *C. glabrata* and has enabled its in-depth study. However, proteomics research of *C. glabrata* is still in its initial stages and there are still many issues to be further addressed. One major issue so far is that only a limited number of open reading frames have been identified in *C. glabrata* genome, restricting the identification of the *C. glabrata* proteins. Additionally, techniques for extraction, separation and identification of *C. glabrata* proteins are to be further improved and optimized. Many *C. glabrata* resistance-associated proteins are membrane proteins. Due to their strong hydrophobicity and poor solubility, only a very limited number of membrane proteins can be obtained through the current protein preparation methods and these proteins cannot be effectively extracted. Also, some

of the functional proteins have low expression and cannot be displayed with the current color-developing methods (*13*) as well as some low-abundance proteins that can be shown in 2D-PAGE gel cannot be fully identified by mass spectrometry. With the development and advances of proteomics technology, proteomics research will play a greater role in the studies of drug-resistance mechanisms of *C. glabrata*.

Some limitations to our study should be noted. We found 12 kinds of differential proteins in fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata*, but there were no gene level data about these proteins or comparable function in these pathogens. We have not done the real-time PCR to confirm protein level data. We used pooled isolates for the extraction of the proteins for both susceptible and resistant strains. The clinical strains were not studied individually in terms of proteomics. Such a performance may have flattened the results and we were not able to know whether or not there are specific differences in the single strains. Therefore, the differential proteins identified in this report should be interpreted with caution.

In conclusion, this study used proteomics technologies to isolate and identify 12 proteins that may be associated with the development of resistance of *C. glabrata* to fluconazole, including energy metabolism-related enzymes, stress response proteins and macromolecular synthesis-related proteins. This study has demonstrated from protein levels that energy metabolism-related enzymes, stress response proteins and macromolecular synthesis-related proteins are all likely involved in the formation of drug-resistance of *C. glabrata* and further confirmed that the formation of drug-resistance of *C. glabrata* is the result of multiple mechanisms. We have also provided a theoretical basis for the discovery and study of new drug-resistance related genes and drug-resistance mechanisms.

Acknowledgements

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