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Full Length Research Paper

Proteomic analysis of fluconazole resistance in *Candida albicans*

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The pattern of azoles resistance-related proteins expression in *Candida albicans* may clarify the mechanism of drug resistance and identify new targets for treatment of *Candida* infection. We identified proteins differentially expressed in strains between fluconazole-sensitive (CA-3) and resistant (CA-16) *C. albicans*. Both strains were from the same parent. Proteins were analyzed using two-dimensional polyacrylamide gel electrophoresis, and differentially expressed proteins were identified through matrix assisted laser desorption/ionization time-of-flight mass spectrometry. The resulting data were searched against a *C. albicans* protein database. Fifteen proteins were differentially expressed in the fluconazole-resistant *C. albicans* strain CA-16. Five *C. albicans* strain (Adh1p, Ynk1p, Cqr1p, Pst3p, and Rdi1p) were up-regulated and ten *C. albicans* strain (Aco1p, Acs2p, Asn1p, Fum11p, Cdc19p, Bat21p, Srb1p, Ifr2p, Dut1p, and Rps21p) were down-regulated. The fluconazole resistance-related proteins of *C. albicans* are mainly involved in energy metabolism and amino acid synthesis.

Key word: *Candida albicans*, fluconazole, resistance, proteomics.

INTRODUCTION

Candida albicans is a common human opportunistic pathogen and a cause of disseminated infection in immunocompromised patients. In the United States, 8 to 10% of blood infections in hospitals are due to *Candida* (Wisplinghoff et al, 2004), and 65.3% of all nosocomial fungal infections are due to *C. albicans* (Pfaller et al., 2010). With the widespread application of fluconazole and other azole antifungal agents, infections caused by drug-resistant *C. albicans* have increased rapidly. A multi-center study demonstrated that during the period from 2000 to 2007, the prevalence of clinical cases of fluconazole-resistant *C. albicans* increased from 0.9 to 1.4% (Pfaller et al., 2010), indicating the ever increasing clinical importance of infections caused by drug-resistant *C. albicans*.

The main focus of studies on the mechanisms of drug resistance in *C. albicans* has been at the gene and mRNA transcription levels (Wang et al., 2009; Feng et al., 2010; Calabrese et al., 2000; Holmes et al., 2008; Lamping et al., 2007; Zhang et al., 2009). However, proteins are the executors of cellular processes, and therefore proteomic patterns should more accurately reflect reality *in vivo*. A variety of pathways are involved in the mechanisms of drug resistance in *C. albicans*. Therefore, to identify resistance-related proteins involved in these mechanisms, we used a proteomics approach to search for differentially expressed proteins in a drug-resistant *C. albicans* strain.

MARERIALS AND METHODS

Strains and chemicals agents

The experimental strains were fluconazole-sensitive CA-3 (fluconazole MIC≤ 8 μg ml^{-1}) and fluconazole-resistant CA-16 (fluconazole MIC≥ 64 μg ml^{-1}) (White et al., 1997), which were from

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a common parent and generously provided by Theodore C. White (University of Washington, and the Seattle Biomedical Research Institute, USA). YEPD agar slant, YEPD broth and RPMI 1640 medium (GIBCO) were used in this research.

Experimental group and protein sample preparation

CA-3 and CA-16 were separately inoculated into YEPD medium. Cultures were grown at 37°C in a shaker set at 200 rpm until OD of the culture at 600 nm reached a value of 1.0. Then, cells of both groups were harvested by centrifugation at 10 000 *g* and at 4°C, and washed with PBS buffer and ice-cold deionized water. Finally cultures were centrifugated again, then wet cells were weighed and transferred to clean 1.5 ml Eppendorf tubes, stored at -80°C until analysed. To the *C. albicans* cell pellet (100 mg wet weight) were added 500 µl of lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHARPS , 1% (v/v) DTT, 2% (v/v) Immobilized pH gradient (IPG) buffer, 20 mg ml^{-1} PMSF and 1% (v/v) protease inhibitors) for 30 min, then cells were freezed rapidly with liquid nitrogen and thawed 4 times, then sonicated 100 times for 3 s each (285 W, 20 kHz) with 9 s interval cooling on ice. After cell lysis, cellular debris was removed by centrifugation for 30 min at 13,200 *g* and at 4°C. The supernatants were stored at -80°C until analyzed (Yan et al., 2007; Ge et al., 2009).

Two-dimensional gel electrophoresis and In-gel tryptic digest

Protein concentrations were determined using Bradford assay prior to electrophoresis. Sample of 150 µg protein were solubilized and diluted to 250 µl hydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS), and were then applied onto immobiline pH 3 to 10 non-linear DryStrips (13 cm, GE Heathcare). Isoelectric focusing was performed on the Amersham Biosciences IPG-phor IEF System at 20°C using the following program: 30 V for 2 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V to 64000 vh, finally 2000 V for 10 h (Yan et al., 2007; Ge et al., 2009). After this, IPG strips were reduced (2% w/w dithioerythritol) for 13 min and then alkylated (2.5% w/w iodoacetamide) for 13 min. SDS-PAGE gel electrophoresis was carried out on homogeneous 12.5% (w/w) T, 1.6% (w/w) C polyacrylamide gels (1.0 mm thick) at 12 mA per gel for15 min and then 30 mA for 4 h using a Hoefer SE600 (GE healthcare, Uppsala, Sweden). Proteins were detected by a silver nitrate staining protocol (Wang et al., 2006). Gels were scanned on an Image Scanner II (GE Healthcare, Uppsala, Sweden), and images were analyzed using the Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden). The significant protein spots were excised from the gels for in-gel tryptic digest according to Wang et al. (2006).

Mass spectrometry and database search

Peptide mass spectra were obtained on an ABI 4800 plus MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Both the MS and MS/MS data were interpreted and processed by using the GPS Explorer software (V3.6, Applied Biosystems), then the obtained MS and MS/MS spectra per spot were combined and submitted to MASCOT Search engine (V2.1,MatrixScience,London,U.K.) by GPS Explorer software. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 65 were considered statistically significant ($p < 0.05$) (Ge at al., 2009).

Functional categories of identified protein

Differentially expressed proteins were named according to the CandidaDB database (http://www.candidagenome.org/), and were classified based on their biological functions defined by the *Candida* Genome Database and *Saccharomyces* Genome Database.

Statistical analysis

All experiments were done at least three times to ensure reproducibility of the results. Data are presented as mean ± standard deviations. MASCOT protein scores of greater than 65 were considered statistically significant ($P < 0.05$).

RESULTS

Methods for preparing *C. albicans* proteins

Concentrations of proteins obtained using the aforementioned lysis methods was 9.9 ± 0.7 mg ml^{-1}. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) separated sample proteins ranging from 25 to 250 kDa component.

Two-dimensional gel electrophoresis

About 1250 protein spots were detected in each experimental *C. albicans* strain. Seventeen differentially expressed proteins with at least 2-fold expression changes (5 up-regulated proteins and 12 down-regulated proteins) were found in the resistant strain (Figure 1).

Mass spectrographic analysis and protein function classification

The 17 differentially expressed protein spots (representing 15 proteins) were identified using mass spectrometry (Table 1).

The differentially expressed proteins were divided into six groups based on biological function. Protein database similarity searches found that most proteins are involved in energy metabolism, including fermentation (Adh1p), glycolysis (Cdc19p), glyoxylate cycle (Aco1p), Krebs cycle (Aco1p, Fum11p, and Acs2p), and respiration (Fum11p and Acs2p). The other functions included amino acid biosynthesis and degradation (Asn1p and Bat21p), synthesis of biological macromolecules (Dut1p and Ynk1p), cell wall mannose protein synthesis (Srb1p), cell stress (Ifr2p), and unidentified functions (Pst3p, Cqr1p, Rps 21p, and Rdi1p).

DISCUSSION

We studied proteins differentially expressed in fluconazole-sensitive and resistant *C. albicans* strains using comparative proteomics technology. Analysis of total proteins by 2-D gel electrophoresis revealed electrophoretic patterns of high resolution and reproducibility.

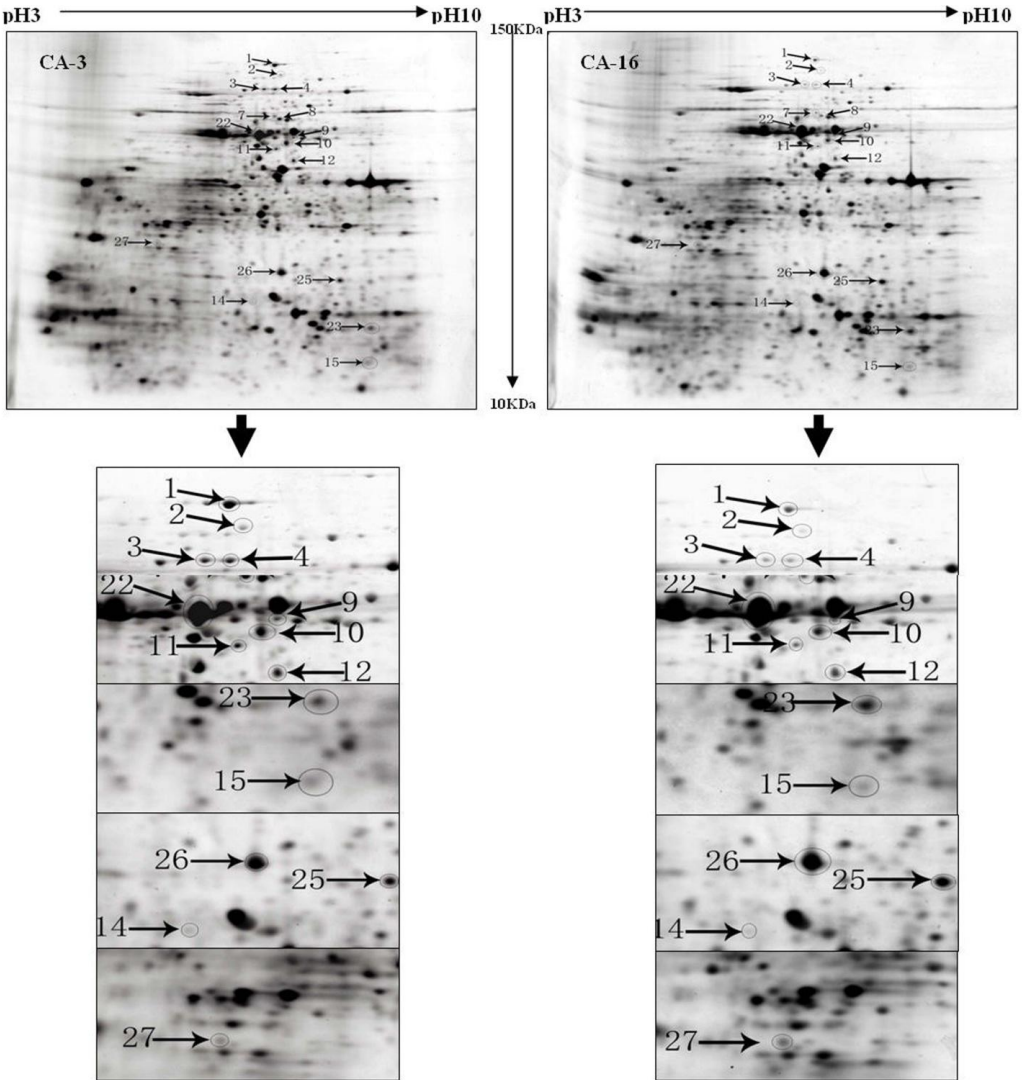

Figure 1. The results of 2D electrophoresis of proteins from the fluconazole-sensitive *C. albicans* CA-3 strain and the fluconazole-resistant *C. albicans* CA-16 strain. Arabic numerals and arrows represent 17 differentially expressed proteins between CA-3 and CA-16 strain.

The 17 differentially expressed protein spots were identified as 15 different proteins by mass spectrometry. The expression of five proteins, including Adh1p, was up-regulated and that of the other ten, including Aco1p, was down-regulated in the resistant strain. Probable functions of these proteins were identified from protein database similarity searches as: Energy metabolism, amino acid synthesis, cell wall synthesis, and other biological processes.

There are some previous studies on the mechanism of fluconazole resistance in *C. albicans* at the protein level in the literature. Yan et al. (2007) found that fluconazole resistance of *C. albicans* was related to the up-regulation of Adh1p, Pgk1p, Fba1p, and other proteins. However, an *in vitro* method was used to obtain the resistant strain used in their study and the stability of the resistant strain was unclear, warranting re-evaluation. Hooshdaran et al. (2004) found that up-regulation of Grp2p, Ifd1p, Ifd4p, Ifd5p, and Erg10p was related to resistance to azoles in *C. albicans*. Their analysis was limited to differences in cytoplasmic protein expression between the sensitive and resistant strains. Hoehamer et al. (2009) studied the proteomic differences between the fluconazole-resistant and sensitive strains induced by *ERG11* up-regulation. Our study had two advantages over some of these earlier studies: (1) The fluconazole-sensitive (CA-3) and resistant (CA-16) *C. albicans* strains were from the same parent and had been cultured for more than 600 generations, the resistance of the both strains was stable. Therefore, we did not compare an ATCC strain with them; (2) Ultrasonic lysis provided higher yields of protein than glass beads lysis method (Okungbowa et al., 2007),

Table 1. Summary of the identified proteins with different expressions between FLC-resistant strain and FLC-susceptible strain.

Spot no.	Accession no. *	Protein name†	Function description*	Protein MW(Da)/PI	Protein score‡	CA16 vs CA3§
1	gi|68479554	Aco1p	likely mitochondrial aconitate hydratase	84 168.7/ 5.96	681	-
2	gi|68484894	Acs2p	likely acetyl CoA synthetase	73 815.4 /5.73	828	-
3	gi|46433951	Asn1p	Asparagine synthetase 2	64 608.4/ 5.79	483	-
4	gi|46433951	Asn1p	Asparagine synthetase 2	64 608.4/ 5.79	483	-
7	gi|238881138	Fum11p	Fumarate hydratase, mitochondrial precursor	52 498/6.35	287	-
8	gi|238882315	Cdc19p	Pyruvate kinase	55 467.7/6.35	576	-
9	gi|238882204	Bat21p	Branched-chain-amino-acid aminotransferase	43 838.7/6.99	258	-
10	gi|46396146	Srb1p	Mannose-1-phosphate guanyltransferase	39 950.1/5.91	414	-
11	gi|68485595	Ifr2p	Potential zinc-binding dehydrogenase	38 494.1/5.87	213	-
12	gi|68482226	Cdc19p	Pyruvate kinase	55 409.6/ 6.54	416	-
14	gi|1169437	Dut1p	Deoxyuridine 5'-triphosphate nucleotidohydrolase	16 934.8/5.65	295	-
15	gi|15214291	Rps21p	40S ribosomal protein S21	9 632.9/7.85	220	-
22	gi|1168348	Adh1p	Alcohol dehydrogenase1	36 856/6.02	683	+
23	gi|238882685	Ynk1p	Nucleoside diphosphate kinase	16 879.8/6.15	155	+
25	gi|46436428	Cqr1p	Potential reductase, flavodoxin	21 198.8/6.14	141	+
26	gi|46431555	Pst3p	Hypothetical protein CaO19.5285	21 160.9/5.83	546	+
27	gi|68465635	Rdi1p	Potential Rho protein GDP dissociation inhibitor	22 947.9/5.15	354	+

*, Protein accession numbers and description according to NCBI protein database; †, protein named according to the *C. albicans* genomic database (*Candida*DB). ‡, It is based on NCBInr database using the MASCOT searching program as MALDI-TOF data. MASCOT protein scores of greater than 65 were considered statistically significant ($p< 0.05$); §: protein expressed differences between FLC-resistant strain CA-16 and FLC-susceptible strain CA-3. "+", up-regulation in CA-16; "-", down-regulation in CA-16.

which in turn enabled a more complete screening of differentially expressed proteins.

In this study, we found that Adh1p expression was significantly up-regulated in the fluconazole-resistant strain. Alcohol dehydrogenase, located in cell surface and cytoplasm, participates in multiple biological processes such as biofilm formation, fermentation, interaction with host. Previous studies found that the up-regulation of *ADH1* and *CDR1* is related to fluconazole resistance in *C. albicans* (Zhu and Lu, 2005) and that fluconazole induces *ADH1* gene expression (Copping et al., 2005). Therefore, we hypothesized that Adh1p is an important candidate protein of fluconazole-resistance in *C. albicans*. Meanwhile, Ynk1p, Cqr1p, Pst3p, and Rdi1p expression were up-regulated in the resistant strain, suggesting that these proteins may be the new candidate proteins of fluconazole-resistance in *C. albicans*.

In addition, we also found that some proteins involved in the citric acid cycle, aerobic respiration, and amino acid biosynthesis were expressed at a low level in the resistant strain, such as pyruvate kinase (Cdc19p), Fum11p, Acs2p, aminotransferase enzyme (Bat21p), aspartic acid synthase (Asn1p), aconitic acid synthase (Aco1p), and so on. Thus, we hypothesize that the fluconazole resistance of *C. albicans* refers to the shift of energy metabolism and amino acid synthesis. But, in fact, we still have lots of work to clarify how to cause fluconazole resistance by energy metabolism and other biological processes. Additionally, like most proteomic research, two-dimensional gel electrophoresis does not recognize plasma membrane well because of the high molecular weight, low abundance, and hydrophobic properties of membrane proteins. Given that economic factors, we did not identify all the protein spots in 2-D

gels, which may include some membrane proteins. That is why our study could not identify the important efflux pump resistant proteins, such as ABC transporters, Cdr1p, Cdr2p, Flu1p, Mdr1p and target enzyme Erg11p.

In summary, we identified proteins differentially expressed in fluconazole-sensitive and resistant *C. albicans* strains using a proteomics approach and did some preliminary analysis to determine the function of these proteins in biological processes. These findings will contribute to our understanding of the molecular mechanisms of multidrug-resistance in *C. albicans*. In the future, we will study membrane proteins in drug-resistant *C. albicans* using a quantitative proteomic approach involving multidimensional liquid chromatography-tandem mass spectrometry in order to identify more directly resistance-related proteins.

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