Expression of Efflux Pumps and Fatty Acid Activator One Genes in Azole Resistant *Candida Glabrata* Isolated From Immunocompromised Patients

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Abstract- Acquired azole resistance in opportunistic fungi causes severe clinical problems in immunosuppressed individuals. This study investigated the molecular mechanisms of azole resistance in clinical isolates of *Candida glabrata*. Six unmatched strains were obtained from an epidemiological survey of candidiasis in immunocompromised hosts that included azole and amphotericin B susceptible and azole resistant clinical isolates. *Candida glabrata* CBS 138 was used as reference strain. Antifungal susceptibility testing of clinical isolates was evaluated using Clinical and Laboratory Standards Institute (CLSI) methods. Complementary DNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technology, semi-quantitative RT-PCR, and sequencing were employed for identification of potential genes involved in azole resistance. *Candida glabrata Candida* drug resistance 1 (*CgCDR1*) and *Candida glabrata Candida* drug resistance 2 (*CgCDR2*) genes, which encode for multidrug transporters, were found to be upregulated in azole-resistant isolates (≥2-fold). Fatty acid activator 1 (*FAA1*) gene, belonging to Acyl-CoA synthetases, showed expression in resistant isolates ≥2-fold that of the susceptible isolates and the reference strain. This study revealed overexpression of the *CgCDR1*, *CgCDR2*, and *FAA1* genes affecting biological pathways, small hydrophobic compounds transport, and lipid metabolism in the resistant clinical *C.glabrata* isolates.

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Introduction

Candida glabrata, formerly rarely seen in fungal infections, has become an important cause of serious systemic infections in immunosuppressed hosts (1-3). *Candida glabrata* is an agent of bloodstream infection (4-6). Most clinical isolates of this organism show low susceptibility to azole antifungal compounds, and treatment of its infection is problematical. The basis of azole resistance in *Candida* species may be the upregulation or mutation of the *ERG11* gene that encodes for the azole target enzyme, cytochrome p-450 lanosterol 14-alpha demethylase (6-9). Another important mechanism of resistance to azole may be overexpression of the efflux pump genes *Candida glabrata Candida* drug resistance 1and 2 (*CgCDR1* and *CgCDR2*) belonging to the ATP-binding cassette (ABC) transporter family (10-12). The major facilitator superfamily is another class of drug transporters involved in drug efflux pumps (12). In addition, the *C.glabrata* sensitivity to 4 Nitroquinoline N-oxide gene (*CgSNQ2*), encodes a protein belonging to the ABC transporter superfamily and is controlled by the *C.glabrata* pleiotropic drug response1 gene (*CgPDR1*).

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The overexpression of *CgPDR1*could is associated with the development of azole resistance in *C.glabrata* (13). Several studies have demonstrated the central role of the *CgPDR1* gene in acquired azole resistance (14-18). *CgPDR1* acquired gain-of-function (GOF) mutations are responsible for upregulation of many genes, especially ABC transporters, lipid biological pathway genes, and the aldo-ketoreductase gene (14,17,19,20). Since the molecular pathways in the resistant Iranian strains of *C.glabrata* are unclear, the present study investigated the transcript profile of the genes involved in antifungal resistance mechanisms using cDNA-AFLP method. This technology is a PCR-based genome-wide expression analysis method (21). cDNA-AFLP has been successfully applied to assess expression of several genes in various organisms (22,23).

Materials and Methods

Yeast isolates and mycological criteria

Six unmatched *Candida glabrata* isolates used in this study were obtained from the culture collection of an epidemiological survey of candidiasis in immunosuppressed patients at the Department of Medical Mycology and Parasitology in Tehran University of Medical Sciences (Table 1) (20). The *C.glabrata* strain CBS 138 was used as a reference. All clinical isolates were subcultured on CHROMagar *Candida* medium. The strains obtained were confirmed by standard biological criteria containing the assimilation patterns using the API 20C AUX system (bioMérieux, France). The isolates for chlamydospores and pseudohyphal production were subcultured on cornmeal agar with 1% Tween 80. All isolates and the reference strain were grown on yeast extract, peptone, glucose (YEPD) agar plates and incubated for 48 h at 37°C.

Table 1. List of clinical *C.glabrata* isolates used in this study with their fluconazole, itraconazole, and amphotericin B susceptibility

Identification No.[a]	Age of the patient (years)	MIC[b](µg/ml)		
		Fluconazole	Itraconazole	Amphotericin B
94	62	0.25	0.5	0.25
45	59	0.5	0.25	0.25
51	53	64	2	0.25
153	35	64	4	0.25
137	35	64	2	0.25
219	38	64	2	0.25

a: All strains have been isolated from the oropharynx
b: MIC, Minimum Inhibitory Concentration

Antifungal susceptibility assays

The susceptibility of *C.glabrata* isolates to fluconazole, itraconazole, and amphotericin B was tested by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards, M27-A3 broth microdilution method (24). Briefly, final inocula of 0.5×103 to 2.5×103 organisms per ml were distributed in wells of a microtiter plate in RPMI 1640 medium buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid and incubated at 37°C. The MICs were determined as the lowest azole concentration that reduced yeast growth by 50% compared to that of a non-treated control.

RNA extraction

Total RNA extraction was prepared from logarithmic growth phase cultures in YEPD broth with an RNeasy protect mini kit (Qiagen, Germany). For mechanical disruption, the yeast cell pellet was sonicated with 600 µl volume of acid-washed glass beads (0.45-0.52 mm diameter). Total RNA was treated through an RNase-free DNase treatment step (Qiagen, Germany). The purity of RNA template was determined with the NanoDrop 1000 spectrophotometer (Thermo scientific, USA).

Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP)

cDNA-AFLP method was conducted as described previously with minor modifications (20). For complementary DNA (cDNA) synthesis, an equivalent amount (6 µg) of total RNA from each sample was incubated at 65°C for 10 min followed by cooling on ice. The master mixture included a 5x reverse transcriptase (RT) buffer (Fermentas, Canada), 1 µl of Oligo d (T) (20 pmol/µl), 2 µl of dNTPs (10 mM), 2 µl of Ribolock (20 U) (Fermentas, Canada), and DEPC treated water. Two hundred units of Moloney Murine Leukemia Virus (M-MuLV) RT enzyme (Fermentas, Germany) were added. cDNA was controlled with the

reference gene *URA3* (orotidine-5′-phosphate decarboxylase). Primers were designed by using GenBank sequences of *C.glabrata* genes (as templates) with the Primer3 program (http://primer3.wi.mit.edu/) (Table 2).

Table 2. Primers used for semi-quantitative RT-PCR and internal control

Gene	Primer	Sequence	Gene location (5´-3´)	Product length
URA3	URA3 F	GGGCTCTTTAGCTCATGGTG	432-451	173bp
	URA3 R	CAAGTGCATCGCCTTTATCA	604-585	
CgCDR1	CgCDR1 F	AAGTTGGTTTCCCCTCGTCT	3518-3537	198bp
	CgCDR1R	CTGCTGTAGCAATGGGTTGA	3715-3696	
CgCDR2	CgCDR2 F	CACATCGCTAAGCAATCGAA	467-486	239 bp
	CgCDR2 R	AAGAACATGGCTGCACCTCT	705-686	
FAA1	FAA1 F	TCGTTCCTAACCACACACCA	1793-1812	196bp
	FAA1 R	TCGTCATCGAAGAACACAGC	1988-1969	

The second strand was synthesized using DNA polymerase I (Fermentas, Germany) at 16°C for 3 h and precipitated with ethanol. The integrity of dscDNA was confirmed with the Nanodrop 1000 spectrophotometer. Two micrograms of dscDNA were digested with *Mbo*I restriction enzyme (25) (Fermentas) for 4 h at 37°C, and the enzyme was inactivated at 80°C for 20 min. Eight µg of ADMboI and 4 µg of abMboI, AFLP adaptors (Table 3) (20), were ligated to digested dscDNA fragments by T4 DNA Ligase (Takara Bio Inc. Japan).The following protocol was used: 1 min at 65°C, decreasing to 10°C over 1 h. T4 DNA Ligase was added to the mixture incubated in advance at 16°C for 16 h. The pre-amplification was conducted with the PreAmp adaptor as primer with the touchdown PCR program: 5 min at 94°C; 20 cycles of 30 s at 94°C, 45 s initially at 65°C reduced 0.5°C per cycle; 20 cycles of 30 s at 94°C, 45 s at 55°C, and 2 min 72°C; and final extension 5 min at 72°C. In the sensitive amplification steps, ten PCRs were conducted, including all sensitive adaptors combinations. The PCR products were visualized on 8% non-denaturated polyacrylamide gel electrophoresis (PAGE), and using silver nitrate staining. Differentiated transcription-derived fragments (TDFs) were generated. The cDNA AFLP gels were covered with a plastic seal and scanned.

Table 3. cDNA-AFLP adaptors used in current study

Adaptors	Sequence (5´- 3´)
ADMbo1	AGCACTCTCCAGCCTCTCACCGCA
adMBO1	GATCTGCGGTGA
Pre Amp	AGCACTCTCCAGCCTCTCACCGCAGATC
S1Mbo1	AGCACTCTCCAGCCTCTCACCGCAGATCC
S2Mbo1	AGCACTCTCCAGCCTCTCACCGCAGATCG
S3Mbo1	AGCACTCTCCAGCCTCTCACCGCAGATCA
S4Mbo1	AGCACTCTCCAGCCTCTCACCGCAGATCT

cDNA-AFLP fragments

The TDFs displaying differential expression were extracted from silver stained PAGE and re-amplified using the suitable sensitive adaptors (Table 3). The amplified fragments were cloned using a TA-cloning kit (Invitrogen-USA), and the recombinant plasmids were confirmed by using M13 universal primers with the following PCR conditions: 30 cycles of 94°C for 1min, 55°C for 1 min, and 72°C for 1 min with extension of the final cycle at 72°C for 7 min. PCR products were visualized by 2% agarose gel electrophoresis. The recombinant plasmids including unknown DNA were sequenced using M13 forward (-20) and M13 reverse universal primers. Some TDFs were determined by direct sequencing (Bioneer, Korea). Sequence data were verified in non-redundant nucleic and protein databases BLAST (http://www.ncbi.nim.nih.gov/BLAST/).

Semi-quantitative RT-PCR technique

Semi-quantitative RT-PCR analysis was used to confirm the mRNA specific expression pattern visualized in the cDNA-AFLP profile (23). An equivalent amount (6 µg) of total RNA from clinical isolates and CBS standard strain was conducted for the

first strand cDNA synthesis as described above, and the evaluation pattern in cDNA-AFLP was performed using specific primers (Table 2) on RNA of clinical isolates and the reference strain. The *URA3* gene was applied as a reference gene and internal control. The PAGE image was captured digitally with a Sony XC-ST50CE camera (Sony, Japan). The intensity of band was analyzed with gel analysis software UVI (Roche, Germany).

Sequencing

For reconfirming the genes which analyzed in semi-quantitative RT-PCR technique were sequenced with the same primers (Bioneer, Korea). Sequence results were analyzed using the BLAST program.

Results

Standard mycological study

The clinical isolates and reference strain produced pink-purple colonies, in contrast to the white colonies exhibited by *C.bracarensis* and *C.nivariensis*, on CHROMagar *Candida* medium (26). The isolates could not produce chlamydospore and pseudohyphae on cornmeal agar with 1% Tween 80. The isolates of *Candida glabrata* were able to assimilate glucose and trehalose and could not assimilate L-lysine and glycerol (27).

Antifungal sensitivity

Susceptibility to fluconazole, itraconazole, and amphotericin B was examined by the CLSI method (document M27-A3). MICs for fluconazole, itraconazole, and amphotericin B are shown in Table 1.

cDNA-AFLP profile

The TDFs of cDNA-AFLP were separated on 8% non-denaturing PAGE by silver staining (Figure 1). Several TDFs were visualized using ten primer combinations. The TDFs that showed differential expression were selected from the silver stained PAGE and identified using cloning and DNA sequencing with the sequences analyzed by BLAST (Table 4). Three differentially expressed TDFs were produced at approximately 200-600 bp length when using S2Mbo1, S4Mbo1 and S1Mbo1, S3Mbo1 and S3Mbo1, and S4Mbo1 as sensitive primers (Figure 1).

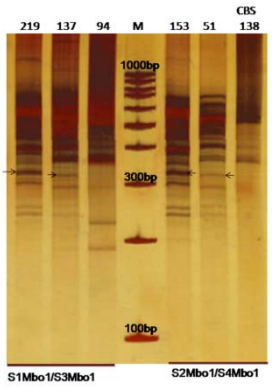

Figure 1. cDNA-AFLP expression profile on PAGE with silver nitrate staining. Sensitive amplification of cDNA-AFLP using four sensitive adaptor combinations, S1Mbo1, S2Mbo1, S3Mbo1, and S4Mbo1, as primers. The lane numbers correspond to the clinical isolates presented in Table 1. The arrows show differentially expressed TDFs. M=Marker 100 bp.

The sequencing results showed matches the TDFs with *CgCDR1*, *CgCDR2*, and Fatty acid activator 1 (*FAA1*) genes. An additional clone contained an unnamed protein sequence (Table 4).

Table 4. Sequences of differentiated transcription-derived fragments (TDFs) determined by cDNA-AFLP profiles

Accession No.	Product length (bp)	Explanation
XM_ 447677.1	375	Fatty acid activator 1
AF109723	330	*Candia glabrata* candida drug resistance 1
XM_ 446088	330	*Candia glabrata* candida drug resistance 2
XM_445802.1	300	unnamed protein product

Semi-quantitative RT-PCR assay

In order to verify the genes found in the cDNA-AFLP profile, a semi-quantitative RT-PCR assay was carried out using cDNAs from resistant and sensitive isolates and the CBS 138 reference strain. Figure 2 shows the upregulation of *CgCDR1*, *CgCDR2*, and

FAA1 mRNA expression in resistant isolates. The semi-quantitative RT-PCR showed *CgCDR1* mRNA expression levels to be ≥2-fold those of the sensitive isolates (Figures 2,3). In addition, *CgCDR2* was upregulated in four resistant isolates to levels 2-3-fold those of the susceptible isolates and the CBS 138 reference strain (Figures 2, 3).

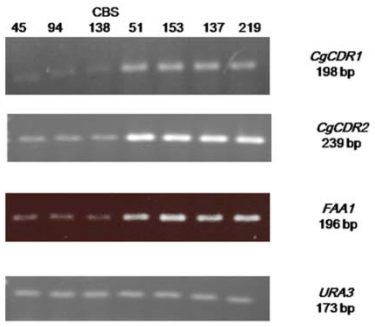

Figure 2. Semi-quantitative RT-PCR assay. Comparison of expression patterns of *CgCDR1*, *CgCDR2*, and *FAA1* genes in azole resistant and azole susceptible *C.glabrata* isolates. *URA3* gene was used as internal control. The lane numbers correspond to the clinical isolates presented in Table 1.

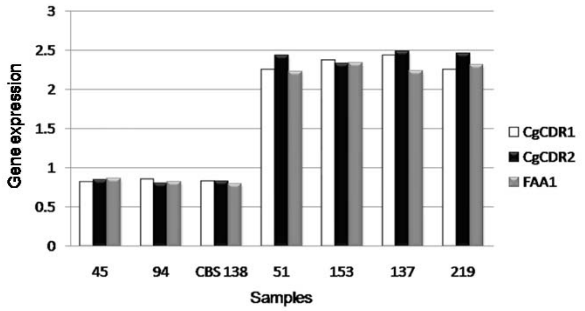

Figure 3. Expression levels of *CgCDR1*, *CgCDR2*, and *FAA1* genes in azole resistant *C.glabrata* isolates compared to azole sensitive isolates. Expression levels are shown after normalization with the *URA3* gene. The sample numbers correspond to the clinical isolates presented in Table 1.

The semi-quantitative RT-PCR results of resistant strains showed that mRNA expression levels of the *FAA1* gene, which encodes Faa1p, a principal long chain acyl-CoA synthetase (ACSL), were ≥2 fold those observed in the sensitive isolates and reference strain (Figures 2,3). The *URA3* gene was used as internal control (Figures 2,3).

Sequencing results

The sequencing results showed highly matched with *CgCDR1*, *CgCDR2*, and Fatty acid activator 1 (*FAA1*) genes of *C.glabrata* CBS 138 genome.

Discussion

Among non-*C.albicans* species, *C.glabrata,* has emerged as an important cause of mucosal and systemic candidiasis in immunocompromised patients. Due to the extensive use of antimycotic drugs in the prophylaxis of candidiasis, azole resistant clinical isolates of *C.glabrata* have been increasingly reported (28).

In the present study, we used the cDNA-AFLP method to investigate the genes involved the resistance phenotypes of clinical *C.glabrata* isolates. The cDNA-AFLP method is a PCR-based technique widely used to assess gene expression levels and the effects of agents controlling up- and down-regulation of genes (21,23).

Previous studies have indicated that ABC-transporter genes *CgCDR1, CgCDR2*, and *CgSNQ2* are the most important biological pathway in azole resistance of *C.glabrata*, eventually leading to broad cross-resistance to antifungal triazoles (29,30). In this study, differential expression of TDFs of cDNA-AFLP was determined by DNA sequencing. DNA sequencing showed a match with the *CgCDR1* and *CgCDR2* ABC transporter, which are involved in protecting organisms against cytotoxins and xenobiotics. Semi-quantitative analysis showed increased mRNA expression levels of *CgCDR1* and *CgCDR2* genes in resistant clinical *C.glabrata* isolates, whereas these genes were normally regulated in susceptible isolates as well as CBS reference strain.

Upregulation and mutation of genes involved in sterol biosynthesis are important in azole resistance of *Candida* sp. Molecular pathways of azole resistance in *Candida* sp. are also associated with enhanced relative mRNA expression levels or mutation in the *ERG11* gene, which encodes cytochrome p-450 lanosterol 14-α demethylase, the principle step in ergosterol biosynthesis (31-33). The results of cDNA-AFLP profile and semi-quantitative RT-PCR showed *FAA1* gene mRNA expression levels in resistant isolates ≥2-fold that of sensitive isolates (Figures 2,3). As expected, results revealed that the regulation patterns of *FAA1* in susceptible isolates were normal. In *Saccharomyces cerevisiae*, four ACSL genes, *FAA1* to *FAA4*, have been characterized (34,35). This family of enzymes plays important roles in lipid metabolism, fatty acid transport, energy production, and cell wall synthesis (36). Faa1p, which constitutes the major cellular activity located in the endoplasmic reticulum, plasma membrane and vesicles catalyzes acyl-CoA. A study of a fluconazole-resistant mutant of *C.glabrata* reported overexpression of multiple genes that were found to be responsible for small molecules transport and cell wall function, as well

as lipid and fatty acid metabolism (17). In addition, mutation of the *PDR1* gene was found to control the expression of many genes, such as those involved in sterol biological pathways (14,17). Ferrari *et al.,* showed that 626 genes were regulated by at least one GOF mutation in *CgPDR1*. Analysis of the differentially regulated genes showed them to be involved in specific biological pathways. The *POX1* gene CAGL0A03740g (fatty acyl-coenzyme A oxidase), which is involved in the fatty acid beta-oxidation pathway (highly similar to sp P13711 *Saccharomyces cerevisiae*), showed upregulation in *C.glabrata*. Also, the *FAS1* gene CAGL0D00528g (Beta unit of fatty acid synthetase), which catalyzes the synthesis of long-chain saturated fatty acids, was over-expressed in resistant *C.glabrata*. The studies showed fatty acid metabolism to be essential for cellular life (34-36), and upregulation of genes belonging to this pathway in resistant *C.glabrata* provides a protective effect. The results of the present study underscore overexpression of the *FAA1* gene as an important factor in lipid metabolism and suggest that upregulation of the ABC efflux pump genes is a principal mechanism of azole resistance. Many investigations indicate that several molecular pathways are involved in the development of azole resistance in *C.glabrata.*

Candida glabrata often exhibits low susceptibility to fluconazole, and the incidence of all clinical forms of candidiasis caused by this microorganism is on the rise. The results of the cDNA-AFLP assay revealed that overexpression of the ABC efflux pump genes (*CgCDR1*, *CgCDR2*) and *FAA1* (the principal ACSL gene) is associated with azole resistance in clinical isolates. In addition, Faa1p, which is encoded by the *FAA1* gene, is necessary during the exponential growth-phase for fatty acid transport, metabolism, and energy production. This study reports upregulation of *FAA1* in azole resistant clinical *C.glabrata* isolates. Further studies are needed to clarify the role of *FAA1* gene.

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