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THE INTERNATIONAL ARABIC JOURNAL OF ANTIMICROBIAL AGENTS ISSN 2174-9094

2014
Vol. 4 No. 3:3
doi: 10.3823/755

Association of ADH1 and DDR48 expression with azole resistance in *Candida albicans*

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Abstract

Background: *Candida albicans* is a frequent opportunistic pathogenic fungus that causes mucosal and systemic infections. The alcohol dehydrogenase 1 (*ADH1*) and *DDR48* genes were found to be upregulated in fluconazole resistant *Candida albicans.* Therefore, understanding the function of drug resistance of genes will help in the development of new antifungal agents that can reverse drug resistance. This study aimed to investigate the role of *ADH1* and *DDR48* genes in development of fluconazole resistant *C. albicans.*

Subjects and Methods: This study involved 19 fluconazole susceptible and 6 fluconazole resistant *C. albicans* isolates from clinical specimens. The MICs of fluconazole were determined by the E-test. Quantitative expressions of Candida Drug Resistance (*ADH1, CDR1*, *DDR48* and *FLU1*) genes were assessed by real time PCR.

Results: There was a statistically significant higher expression levels of *CDR1, FLU1, ADH1* and *DDR48* in resistant and susceptible dose dependent isolates than in susceptible isolates (P = 0.009, 0.008, 0.01, 0.014 respectively). Strong positive correlations were observed between the expression levels of each of *ADH1*and *DDR4* with azole resistance genes *CDR1* and *FLU1* [(rs) = 0.945, 0.815, respectively; $P < 0.001$; Spearman's (rs) = 0.852 and 0.76, respectively; $P < 0.001$].

Conclusion: This is the first study that showed positive correlation between *DDR48* and azole resistance genes. It has also indicated that *ADH1* and *DDR48* are associated with the resistance mechanisms of *C. albicans* to fluconazole. Identification of new drugs that target the

proteins encoded by these genes will help in eradication of fluconazole resistant *C. albicans* in infection.

Keywords: fluconazole resistance, *C. albicans*, *ADH1, DDR48*, *CDR1*, *FLU1*genes.

Introduction

Candida albicans is a human fungal pathogen that causes serious infections in immunocompromised population [1]. In hospitalized patients, this organism can disseminate hematogenously and infect virtually all organs [2]. Fluconazole and other azoles enter *C. albicans* cells by facilitated diffusion [3]. These antifungal drugs inhibit cytochrome P450 enzymes: lanosterol demethylase, a key enzyme in ergosterol biosynthesis [4], and C-22 sterol desaturase [5].

C. albicans is becoming increasingly resistant to azole antifungal agents, particularly fluconazole, a problem of growing importance due to the widespread use of a limited number of antifungal agents, particularly azoles [6, 7]. Various mechanisms can lead to the acquired resistance of *Candida* spp. to azole drugs, and most involve the induction of the efflux pumps encoded by *CDR* (Candida Drug Resistance) or *MDR* (Multiple Drug Resistance) genes, or the over-expression or acquisition of point mutations in the gene encoding the target enzyme lanosterol demethylase [8]. Over-expression of drug-efflux pumps Cdr1, Cdr2 and Mdr1 has been linked to fluconazole resistance [9].

Multidrug efflux transporters of the ABC (ATP-binding cassette) superfamily and of the Major Facilitator Superfamily (MFS) play a key role in the low level of accumulation of azoles in the yeast cell [10]. The ABC transporter superfamily, including Cdr1 and Cdr2, are membrane proteins that have two membrane-spanning domains and two nucleotide-binding domains that utilize ATP to drive substrates across the membrane [11]. *CDR1* and *CDR2* genes have been shown to be upregulated in resistant strains, leading to an enhanced efflux of the drug [10] and hence leading to resistance to multiple azoles [12, 13]. Cross-resistance between fluconazole and other azoles has been reported [14-16], the mechanistic basis for this cross-resistance most often involves the upregulation of genes encoding the CDR pumps that act as ATP-binding cassette efflux transporters [13, 17, 18].

On the other hand, MFS drug pumps, including Mdr1 and Flu1, have no nucleotide-binding domain but instead use the proton motive force of the membrane as an energy source [11]. Both *MDR1* and *FLU1* genes were over-expressed specifically in fluconazole-resistant *C. albicans* isolates. Disruption of *FLU1* gene in *C. albicans* mutants with deletions in multidrug efflux transporter genes, including *CDR1*, *CDR2* and *MDR1*, resulted in enhanced susceptibility to several azole derivatives, indicating the contribution of FLU1 in azole resistance [19].

The Adh1p (alcohol dehydrogenase protein) which is encoded by the *ADH1* gene, plays an important role in intracellular energy metabolism [20], as the most critical mechanism governing drug resistance

in *C. albicans* entails the Cdr1p and Cdr2p efflux pump proteins, the functionality of which depends on energy metabolism [19, 21].

Another gene *(DDR48)* acts as cell stress gene that is involved in combating the effects of nitric oxide and in DNA repair [22]. A study reported that *DDR48* is upregulated in azole resistant *C. albicans* than in the sensitive isolates [23]. The relationship between the expression of *ADH1* and *DDR48* with drug resistance in *C. albicans* requires further research. Understanding the resistance mechanisms and the associated drug resistance genes will help in the development of new antifungal agents that can reverse drug resistance.

The aim of this study is to investigate the expression of *ADH1* and *DDR48* genes in fluconazole resistant *C. albicans,* and to assess the correlation between the expression levels of these genes and the expression of the azole resistance genes *CDR1* and *FLU1*.

Materials and Methods

Sample collection

This study involved 25 *C. albicans* clinical isolates. Twenty strains were isolated from patients with vulvovaginal candidiasis and five strains were isolated from patients with respiratory infections. The isolates were collected from patients attending the outpatient clinics of Gynecology and Obstetrics department and patients admitted at Chest department in Kasr Al-Aini Hospitals, Cairo University, Egypt. All laboratory tests were carried out in the department of Medical Microbiology and Immunology, and the department of Biochemistry, Faculty of Medicine, Cairo University during the period from June 2013 to August 2014. The study protocol was approved by the Scientific Ethical Committee at Cairo University Hospitals and informed consent was obtained from each patient. All *C. albicans* isolates were identified by germ tube test, culture on ChromID Candida (Biomerieux, France) and by API C20 identification kit (Biomerieux, France).

Fluconazole disk diffusion susceptibility testing

Fresh subcultures of *C. albicans* isolates were prepared on SDA (Sabouraud dextrose agar). Fluconazole susceptibility was tested by using 25-ug fluconazole disks (Biorad, USA) on Mueller-Hinton agar supplemented with 2% glucose and 0.5mg/L methylene blue inoculated with 0.5 McFarland standard of an inoculum prepared in sterile 0.9% NaCl [24]. The zone diameter was measured after 24 hours. Isolates with zone diameter of ≥19 mm were considered susceptible, those with zone diameter of 15 to 18mm were considered susceptible dose-dependent, and those with zone diameter ≤14 mm were considered resistant [24, 25].

Minimum inhibitory concentration (MIC) of fluconazole

C. albicans isolates were subcultured on SDA and an inoculum was prepared in sterile 0.9% NaCl, adjusting the cell density to 0.5 McFarland standard. The MIC of fluconazole was determined by E-test (AB BIODISK,Solna, Sweden) using Muller Hinton agar supplemented with 2% glucose and 0.5 mg/L methylene blue [24]. The MICs were read after 24 hours. Susceptible, susceptible dose-dependent and resistant isolates were defined by MICs of ≤8 mg/L, 16 to 32 mg/L and ≥64 mg/L respectively [24, 25].

Total RNA isolation

C. albicans isolates were grown overnight at 37°C on SDA. Three to four colonies were suspended in phosphate buffer saline. Total RNA was isolated with RNeasy Mini Kit (Qiagen, USA) according to the man-

ufacturer's protocol and further analyzed for quantity and quality using Beckman dual spectrophotometer (USA). The OD260 and OD280 values were measured, and the ratios were found to be 1.8–2.0. The RNA integrity was assessed by RT-PCR measurement of CaYST1- mRNA (*C. albicans* housekeeping gene) gene expression as the quality control.

Real Time PCR for quantitative expression of ADH1, CDR1, DDR48 and FLU1 genes

The mRNA expression level was measured by qRT–PCR (quantitative reverse transcription polymerase chain reaction). Briefly, 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas, Germany) using an Applied Biosystem with software version 3.1 (StepOne™, USA) as follows: 10 minutes at 95^0C for enzyme activation followed by 40 cycles of 15 seconds at 95^0C, 20 seconds at 55^0C and 30 second at 72^0C for the amplification step. We used 1μM of both primers specific for each target gene. The threshold cycle (C_t) value of CaYST1 was subtracted from that of the gene of interest to obtain a ΔCT value. The strain with the lowest ΔCT value for ADH1 was used as the baseline control strain, and the expression levels of the other strains were quantified relative to that of the control strain. Finally, RQ (relative quantity) expression level for each target gene was assessed relative to the calibrator and was expressed as 2–ΔΔCT. Primers sequences specific for each target gene are demonstrated in **table 1**.

Statistical Analysis

Data were analyzed using SPSS version 16. The results were expressed as means ± standard deviation (SD). The relative mRNA expression levels of *ADH1, CDR1, FLU1* and *DDR48* in the susceptible and resistant isolates were compared using the independent samples t test, Kruskal Wallis H test was used to compare the relative mRNA expression levels of *ADH1, CDR1, FLU1 and DDR48* in the susceptible, susceptible dose dependent and resistant isolates. Spearman's rho (rs) was used for the analysis of correlation between the mRNA expression levels of the four studied genes. Statistical significance was defined at $P \leq 0.05$.

Table 1. Sequences of the gene-specific primers for amplification of the studied genes

Gene	Primer sequence 5'-3'	Gene bank accession number
ADH1	5'- TGT CTG GTT ACA CTC ACG ATG G -3' 5'- GCA TCG AAA ACT GGA GCA GT -3'	XM_002420714.1
CDR1	5'- CTT AGT CAA ACC ACT GGA TCG -3' 5'- CCA AAA GTG ATG AAA AGG C -3'	XM_718116.1
FLU1	5'- TGT TGC CTT TGA TGG TCC CG -3' 5'- ACC GAT AAG GCA GCA AGA CC -3'	XM_715807.1
DDR48	5'- TTT CGG TTT CGG TAA AGA CG -3' 5'- TGA GTC GGT CTT GGA GGA AC -3'	XM_709160.1
CaYST1	5'- AAGTATTTGGGAGAAGGGA-AAGGG -3' 5'- AAAATGGGCATTAAGGA-AAAGAGC -3'	AJ251858.1

Results

Fluconazole susceptibility of the *C. albicans* isolates

Fluconazole susceptibility testing revealed that 19 out 25 isolates were susceptible to fluconazole by both disk diffusion method and E-test (zone diameter ≥19; MIC≤8 mg/L), and one isolate was resistant by both tests (zone diameter ≤14; MICs ≥64 mg/L). The remaining 5 isolates showed discrepancy between disk diffusion and E test results, they were fluconazole resistant by disk diffusion, however by the E test, 2 of them were susceptible and 3 were susceptible dose-dependent (MIC =24 mg/L). Those 5 isolates had high expression levels of *CDR1* and *FLU1* azole resistance genes as well as *ADH1* and *DDR48* genes.

Expression of the studied genes among fluconazole susceptible and resistant *C. albicans* isolates

Both the results of the disk diffusion, fluconazole resistant *C. albicans* isolates had significantly higher expression levels of *ADH1, CDR1, FLU1* and *DDR48* genes than fluconazole susceptible *C albicans* isolates. **Table 2** shows the means, standard deviations and P-values of the expression levels of the four studied genes among fluconazole resistant and susceptible *Candida albicans* isolates.

Comparing the expression levels of the studied genes among *C. albicans* isolates based on the E-test results by Kruskal-Wallis H test revealed statistically significant higher expression levels of *CDR1, FLU1, ADH1* and *DDR48* in susceptible dose dependent and resistant isolates compared to susceptible ones, data are shown in **table 3**.

Correlation between expression of *ADH1* and *DDR48* with azole resistance genes

There were strong positive correlations among all 25 clinical *C. albicans* isolates between the relative mRNA expression levels of *ADH1* and the azole resistance genes *CDR1* and *FLU1* (rs) = 0.945, 0.815, respectively; $P < 0.001$) (**Table 4)**.

Strong positive correlations were also observed between the relative mRNA expression levels of *DDR48* and each of *CDR1* and *FLU1* (rs) = 0.852 and 0.76, respectively; $P < 0.001$) (**Table 4**).

Table 2. Expression levels of the four studied genes among fluconazole resistant and fluconazole susceptible *C. albicans* isolates

	Fluconazole susceptible n=19		Fluconazole Resistant n=6		P-value
	Mean	SD	Mean	SD	
ADH1	3.32	1.7	12.11	2.0	<0.001
CDR1	2.28	1.3	10.34	2.09	<0.001
FLU1	2.09	1.14	7.93	0.89	<0.001
DDR48	2.71	1.19	8.4	3.36	0.008

Table 3. Comparing the expression levels of the four studied genes among *C. albicans* isolates based on the E-test results

	Fluconazole E-test	N	Mean Rank	P-value
CDR1	Susceptible	21	11.05	0.009
	Susceptible dose dependent	3	22.67	
	Resistant	1	25.00	
FLU1	Susceptible	21	11.00	0.008
	Susceptible dose dependent	3	23.67	
	Resistant	1	23.00	
ADH1	Susceptible	21	11.05	0.01
	Susceptible dose dependent	3	22.67	
	Resistant	1	25.00	
DDR48	Susceptible	21	11.14	0.014
	Susceptible dose dependent	3	22.00	
	Resistant	1	25.00	

Table 4. Correlation of the mRNA expression levels of *ADH1* and *DDR48* to azole resistance genes *CDR1* and *FLU1*

Gene	Mean±SD	Spearman's rho (r_s) Correlation Coefficient to ADH1	Spearman's rho (r_s) Correlation Coefficient to DDR48
		5.43±4.20	4.07±3.09
CDR1	4.21±3.81	0.945*	0.852*
FLU1	3.5±2.76	0.815*	0.760*

*P-value < 0.001

Discussion

Fluconazole susceptibility of *C. albicans* isolates

Nineteen *C. albicans* isolates were fluconazole susceptible and one isolate was resistant by both disk diffusion and the E-test. The remaining five isolates were found resistant by disk diffusion, susceptible or susceptible dose-dependent by the E-test. These 5 isolates had high expression levels of azole resistance *CDR1, FLU1* genes as well as with *ADH1* and *DDR48* genes.

Previous studies reported discrepancy between fluconazole disk diffusion and E-test, although Torres and colleagues (2009) reported a positive correlation between disk diffusion method and the E-test. They observed that one Candida isolate was resistant to fluconazole by disk diffusion but susceptible

by the E-test [26]. Furthermore, a study by Vandenbossche and colleagues (2002) reported that one *C. tropicalis* strain, with MIC>256 mg/L was observed to be susceptible by the E-test (MIC = 0.5 mg/L) using broth macro-dilution method [27]. The agreement between the E-test and macro-dilution method ranged from 87-100% within two dilutions for *Candida* spp. In general, there was a tendency to read the E-test MICs slightly lower than macro- and micro-dilution methods [27-29]. It is worth mentioning that fluconazole susceptibility testing of *C. albicans* using the E-test technique is difficult to interpret due to the fact that scattered inner colonies can make the inhibition zones difficult to read, a phenomenon called trailing endpoints [28-31]. Presence of trailing in Candida isolates, although susceptible to fluconazole, means express the same molecular mechanisms as SDD and resistant isolates following fluconazole exposure [32]. Therefore, molecular tools should become the gold standard for the identification of Candida drug resistance. Interestingly, the five isolates, that were resistant by disk diffusion and susceptible or susceptible dose-dependent by the E-test, had high expression levels for tested azole resistance genes (*CDR1, FLU1*, *ADH1* and *DDR48*).

Expression of the studied genes among fluconazole susceptible and resistant *C. albicans* isolates

The present study showed significantly higher expression levels of *CDR1, FLU1, ADH1* and *DDR48* genes in fluconazole resistant than in fluconazole susceptible *C. albicans* isolates. These results are in concordance with a study by Guo et al. (2013) who reported that *ADH1, CDR1* and *FLU1* were highly expressed in the resistant *C. albicans* isolated from patients with vulvovaginal candidiasis than in susceptible dose-dependent isolates. They also reported a significant positive correlation between the expression of *ADH1* and the MICs of fluconazole [33]. On the other hand, Siikala et al. (2011) demonstrated a negative correlation between the relative expressions of *ADH1* and each of *CDR1* and *CDR2* in *C. albicans* isolated from autoimmune polyendocrinopathy–candidosis–ectodermal dystrophy patients. They also observed no correlation between the expression levels of *ADH1* and the MICs of fluconazole. They conferred these results to metabolic changes in the isolates. For example: catabolite repression leading to *ADH* repression which could induce *CDRs* in their patients [34].

Correlation between ADH1 and the azole resistance genes expression

In this study, there were strong positive correlations between the relative mRNA expression levels of *ADH1* and the azole resistance genes *CDR1* and *FLU1* (Spearman's (r_s) = 0.945, 0.815, respectively; $P < 0.001$). Similarly, Guo et al. (2013) reported that the expression of the *ADH1* gene was positively correlated with the expression of the azole resistance-associated genes *CDR1* and *FLU1,* indicating that high *ADH1* mRNA expression is closely correlated to drug resistance in *C. albicans* [33].

Adh1p is involved in glycolysis, which is the major metabolic reaction under anoxic conditions in yeast, and is responsible for most of the alcohol dehydrogenase activity in yeast [35]. The high expression of the *ADH1* gene is likely plays a role in promoting drug resistance in *C. albicans*. Elevated *ADH1* gene expression may signify the activation of the glycolysis pathway, which can result in enhanced ATP generation. Thus, more energy is produced for the drug efflux pump located in the *C. albicans* cell membrane, leading to the export of azole drugs and a decrease in the intracellular accumulation of these drugs, and results in drug resistance [33]. Furthermore, the Adh1p was found to be upregulated at the protein level in fluconazole resistant *C. albicans* [36, 37].

Correlation between DDR48 gene expression and azole-resistance genes

There were also strong positive correlations between the relative mRNA expression levels of *DDR48* and each of *CDR1* and *FLU1* (Spearman's (r_s) = 0.852 and 0.76, respectively; $P < 0.001$). Barker et al. (2004) reported that the expression of the cell stress gene DDR48, a member of a set of genes that displays increased transcription in response to DNA lesions, heat-shock stress [38], and in *C. albicans* biofilms [39], was up-regulated by 7.4 fold in the azole resistant *C. albicans* isolate than in the sensitive one [23]. Cleary et al. (2012) demonstrated a role for *DDR48* in sensing or responding to environmental nutritional conditions, they concluded that this protein has an important influence on pathogenesis [40]. Furthermore, *DDR48* mutant strain generated in a study by Dib et al. (2008) was susceptible in a dose-dependent manner to fluconazole and itraconazole compared with the resistant parent strain that possesses *DDR48* gene [41]. Previous data also showed that exposure to fluconazole and itraconazole led to increased expression of *DDR48* RNA [42]. It is worth pointing out that the importance of *DDR48* for filamentation, stress response and biofilm formation highlights its role in virulence and makes it a prime target for antifungal drug design [41]. Interestingly, our results indicate that the correlation of *ADH1* was higher than that of *DDR48* to azole resistance genes, suggesting that the *ADH1* gene has more crucial role and is more reliable in detecting azole resistance in *C. albicans.*

In conclusion, the mRNA expression levels of *ADH1* and *DDR48* had strong positive correlation with the expression levels of *CDR1* and *FLU1* azole resistance genes. Furthermore, *ADH1* and DDR48 expression levels were significantly higher in fluconazole resistant than in fluconazole susceptible *C. albicans* clinical isolates. These results indicate that these genes have an important role in the resistance mechanisms of *C. albicans* to fluconazole and probably to other azoles. Identification of new drugs that target the proteins encoded by these genes will help in eradication of fluconazole resistant *C. albicans* during infection. To our knowledge, this is the first study that investigates the association between *DDR48* and azole resistance genes. Further research is required to clarify the role of *DDR48* in drug resistance mechanisms. However, the role of *ADH1* seems to be more crucial in azole resistance in *C. albicans.*

There is no conflict of interest

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