Investigation of *ERG11* gene expression among fluconazole-resistant *Candida albicans*: first report from an Iranian referral paediatric hospital

M. TEYMURI*†, S. MAMISHI†‡, B. POURAKBARI†, S. MAHMOUDI†, M. T. ASHTIANI§, R. H. SADEGHI† and M. H. YADEGARI*

Department of Medical Mycology, School of Medical Sciences, Tarbiat Modares University; †Pediatrics Infectious Diseases Research Center; ‡Department of Infectious Diseases; and §Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

The multiplicity of mechanisms of resistance to azole antifungal agents has been described. As fluconazole-resistant clinical *Candida albicans* isolates that constitutively over-express *ERG11* have been identified in previous studies, the aim of this study is to investigate this molecular mechanism involved in fluconazole resistance of *C. albicans* clinical isolates. Fluconazole susceptibility testing was carried out on clinical isolates of *Candida* spp. obtained from hospitalised children in an Iranian referral children's hospital. A polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique was used to differentiate *Candida* spp. The resistant *C. albicans* isolates were subjected to RT-qPCR using primers that identify *ERG11* gene expression. Of the 142 *Candida* spp. isolates studied, *C. albicans* was the most predominant isolate, occurring in 68.3% (97/142) of the patients. According to the CLSI method, the majority of the *C. albicans* isolates (91.7%, 89/97), categorised as susceptible (minimum inhibitory concentration [MIC] ≤8 μg/mL), five isolates were considered resistant (MIC ≥64 μg/mL) and three had dose-dependent susceptibility (MIC=8.16-32 μg/mL). The *ERG11* gene in the five fluconazole-resistant *C. albicans* isolates was upregulated 4.15–5.84-fold relative to the ATCC 10231 control strain. In this study, the expression of *ERG11* was upregulated in all the fluconazole-resistant *C. albicans* isolates. There are limited data on the antifungal susceptibility of *Candida* spp. as well as the molecular mechanism of azole resistance in Iran, especially for isolates causing infections in children. Therefore, the surveillance of antifungal resistance patterns and investigation of other mechanisms of azole resistance in all *Candida* spp. isolates is recommended.

KEY WORDS: Candida albicans.
Children.
ERG11 gene.
Fluconazole resistance.

Introduction

Infections caused by *Candida* spp. have been characterised as the most common cause of fungal infections in hospitalised patients and are associated with high morbidity and mortality. Among the azole antifungals, fluconazole is used commonly for treatment or prophylaxis in most *Candida albicans* infections.[1] However, treatment failures and infection recurrences might occur due to increasing resistance to the antifungal azoles developed in *C. albicans*.[2]

The multiplicity of mechanisms of resistance to azole antifungal agents, including alterations in the target enzyme in the sterol biosynthetic pathway and increased efflux of the drug,[2] have been mediated through multiple underlying mechanisms.[3]

Resistance to azole antifungal drugs has been associated with *ERG11* gene over-expression and/or point mutations as well as alterations in the ergosterol biosynthetic pathway.[4] Lanosterol 14α-demethylase enzyme, the target of fluconazole, is an essential component in the synthesis of ergosterol,[5] and production is encoded by the *ERG11* gene. Over-expression of *ERG11* results in production of a large amount of lanosterol 14α-demethylase and continuous synthesis of ergosterol, which enables *Candida* to resist fluconazole.[3,5,6]

Data on the distribution and susceptibility of *Candida* spp. from children's hospitals are limited.[7] As fluconazole-resistant clinical *C. albicans* isolates that constitutively over-express *ERG11* have been identified in previous studies,[8,9] this study aims to investigate this molecular mechanism involved in fluconazole resistance of *C. albicans* clinical isolates.

Correspondence to: Mohammad Hossein Yadegari
Department of Mycology, Faculty of Medical Sciences,
Tarbiat Modares University, Tehran, Iran
Email: yadegarm@modares.ac.ir

Materials and methods

Study design

Fluconazole susceptibility testing was carried out on clinical isolates of *Candida* spp. obtained from hospitalised children in an Iranian referral children's hospital. The samples were collected and cultured on Sabouraud dextrose agar at 37°C for 24 h. The pure isolates were stored in 10% glycerol in brain-heart infusion broth at –80°C. The initial identities of the clinical isolates were confirmed by germ tube formation

in serum-containing medium, and a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique was used to differentiate *Candida* spp.[10,11]

PCR- RFLP

Genomic DNA was extracted using glass bead disruption. The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification of ITS1-5.8S-ITS2 ribosomal DNA in all *Candida* spp. PCR amplification was carried out according to the method outlined by Mirhendi *et al*.[11]

Differentiation of *Candida* spp. was performed using M*sp*I (Takara Bio) for differentiation of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. guilliermondii*; and B*ln*I (Takara Bio) for differentiation of *C. albicans* and *C. dubliniensis*. Digestion was performed by incubating 21.5 μL PCR products obtained using the ITS1 and ITS4 primers with 10 units enzyme in a final reaction volume of 25 μL at 37˚C for 2.5 h. Restriction fragments were separated by 2% agarose gel in TBE buffer and the gel was stained with ethidium bromide (0.5 μg/mL). The size of DNA fragments was determined directly by comparison of molecular size marker and distinct banding patterns.[10,11]

Antifungal drug susceptibility testing

Fluconazole susceptibility testing of *Candida* spp. was performed according to the guidelines in document M27-A by a broth microdilution method[12] in flat-bottomed, 96-well polystyrene microtitre plates. Briefly, a 100 μL volume of RPMI 1640 medium was added to a series of wells, except for the initial well, to which 195 μL was added. Fluconazole (Sigma, Germany) was added to the initial well at twice the maximum concentration to be tested, and two-fold serial dilutions were made by transferring 100 μL of this solution to subsequent wells. The final well in each series contained RPMI 1640 medium alone and served as a growth control. Logarithmic-phase cultures of yeast were diluted in RPMI 1640 medium, and a final density of 10^4 cells/mL was added to each well. Plates were incubated at 35˚C, and the absorbance at 630 nm was read at 24 h and 48 h with a microplate reader. Isolates for which fluconazole minimum inhibitory concentration (MIC) was above 64 μg/mL were considered resistant (R), whereas those inhibited at a lower concentration of 8 μg/mL were labelled susceptible (S).

RNA isolation and reverse transcription

Total RNA was isolated from *Candida* spp. using the GeneJET RNA Purification Kit (Thermo Scientific, USA). Reverse transcription was performed on 5 μg total RNA using Moloney murine leukaemia virus (M-MuLV) reverse transcriptase with random primers, according to the manufacturer's instructions. The concentration and purity of the RNA was determined using an ultraviolet (UV) spectrophotometer, measuring the absorbance (*A*) at 260 nm and 280 nm. The *A*260nm/*A*280nm of the samples, reflecting the average purity, ranged from 1.80 to 2.05.

RT-qPCR

Synthetic complementary DNA (cDNA) was used for qPCR analysis, which was performed on an ABI StepOne real-time PCR machine (Applied Biosystems, USA), using EvaGreen qPCR Mix Plus (ROX) (Biotium, USA).

The resistant isolates were subjected to RT-qPCR using primers that identify expression of *ERG11*, with the housekeeping gene 18S rRNA as a control. The primers used to amplify and identify the *C. albicans ERG11* and 18S rRNA were as follows: ERG11-F: 5 -TTTGGTGGTGGTAGACATA-3 , ERG11-R: 5 GAACTATAATCAGGGTCAGG-3 and 18S rRNA-F: 5 -TCTTTCTTGATTTTGTGGGTGG-3 , 18S rRNA-R:5′ TCGATAGTCCCTCTAAGAAGTG-3 .[13] The control strain ATCC 10231 was used for relative expression comparison.

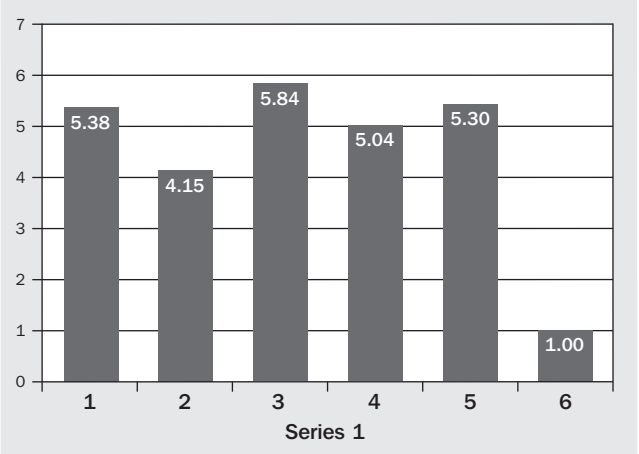

Fig. 1. Differential expression of the *ERG11* gene relative to the reference 18S rRNA gene in five fluconazole-resistant *C. albicans* isolates compared to the control strain ATCC 10231 (lane 6).

Amplification was achieved using the following cycle settings: 2 min at 95˚C followed by 40 cycles of 95˚C for 10 sec, 58˚C for 30 sec, and 72˚C for 20 sec. Negative controls, which were run simultaneously, did not contain either RNA (no template control) or reverse transcriptase (RT negative control), to control for RNA and genomic DNA contamination, respectively. After amplification, a melting curve was analysed to ensure the absence of primer dimers. Expression of *ERG11* was calculated using the $2^{-\Delta\Delta CT}$ method,[14] using 18S RNA as a reference.

Cycle of threshold (C_T) was considered as the average threshold cycle number from three independent experiments. Data were presented as the fold change in gene expression normalised to the 18S rRNA gene as a control.

Real-time PCR efficiencies were acquired by amplification of a standardised dilution series of the template cDNA and were determined for each gene as the slope of a linear regression model. PCR efficiency was determined by measuring the C_T to a specific threshold for a serial dilution of cDNA. The corresponding real-time PCR efficiencies were then calculated according to the equation: $E=(10^{-1/slope}-1) \times 100$.

Results

From December 2012 to January 2013, a total of 142 *Candida* spp. isolates were obtained from 95 boys and 47 girls. Of the 142 isolates, 122 (85.9%) were isolated from urine, eight (5.6%) from wounds, seven (4.9%) from blood and five (3.5%) from the trachea.

The patient admission units included pediatric intensive care (21.1%), urology (15.5%), neonatal intensive care (12.7%), cardiac intensive care (10.6%), surgery (9.9%), gastrointestinal (9.2%), immunology (4.9%), nephrology (4.9%), the infectious ward (4.9%) and others (6.3%).

Of the 142 *Candida* spp. isolates, *C. albicans* was the most predominant, occurring in 68.3% (97/142) of the patients, while *C. dubliniensis* was the second most common species (9.9%, 14 isolates). *C. parapsilosis* was the most frequent non-albicans species isolated in 7.7% (11/142) of the patients, followed by *C. guilliermondii* (4.9%, 7/142), *C. tropicalis* (3.5%, 5/142), *C. glabrata* (3.5%, 5/142) and *C. krusei* (2.1%, 3/142).

Of the 97 patients with positive culture for *C. albicans*, 65 received no treatment and 32 patients received antifungal agents (12 patients received fluconazole, seven patients received clotrimazole, seven patients received nystatin, four patients received amphotericin B). A combination of fluconazole and amphotericin B was used in three patients. Nearly 60% of patients received more than two antibiotics (36% more than two, 23.7% more than three).

According to the Clinical and Laboratory Standards Institute (CLSI) method, the majority of *C. albicans* isolates (91.7%, 89/97) were categorised as susceptible (MIC ≤8 μg/mL), five isolates were considered resistant (MIC ≥64 μg/mL) and three isolates had dose-dependent susceptibility (MIC=8.16–32 μg/mL). The MIC of fluconazole-resistant strains of *C. albicans* was 64 μg/mL in four isolates and 128 μg/mL in one isolate. The demographic characteristics of patients with fluconazole-resistant *C. albicans* are shown in Table 1.

Table 1. Demographic and clinical characteristics of patients from whom fluconazole-resistant *C. albicans* was isolated.

		n	%
Gender	Male	3	60
	Female	2	40
Age	Neonates	1	20
	1 month to 1 year	4	80
Specimen	Urine	5	100
Ward	PICU	2	40
	Neonatal intensive care	1	20
	Urology	1	20
	Immunology	1	20
Length of hospitalisation	More than three days	5	100
Antimicrobial therapy	More than two antibiotics	3	60
	More than three antibiotics	2	40
Underlying disease	Congenital heart disease	1	20
	Renal disease	3	60
	Prematurity	1	20
Urinary catheterisation		3	60
Fluconazole prophylaxis		1	20

RT-qPCR analysis

RT-qPCR was performed for fluconazole-resistant strains of *C. albicans* and the increase in fluorescence of the EvaGreen dye was monitored using a StepOne real-time PCR system. All samples gave a single peak, indicating a single pure product and no primer/dimer formation. All PCR reactions displayed efficiencies between 95% and 100%. The expression of *ERG11* was upregulated in all five fluconazole-resistant *C. albicans* isolates 4.15–5.84-fold relative to the ATCC 10231 control strain (Fig. 1).

Discussion

In the present study, a relative quantification RT-qPCR method was used to investigate *ERG11* expression in fluconazole-resistant clinical isolates of *C. albicans*. According to previous studies conducted in children's hospitals, a few *C. albicans* isolates were found to be resistant to fluconazole.[7,15,16]

In the present study, the frequency of fluconazole-resistant *C. albicans* was 5.1% (5/97), which is higher than in other studies conducted in children settings.[7,15,16] In the Belet *et al*. study,[15] fluconazole susceptibility, dose-dependent susceptibility and resistance in *C. albicans* were 86.6% (20/23), 8.6% (2/23) and 4.3% (1/23), respectively. In the Zaoutis *et al*. study,[7] susceptibility in *C. albicans* isolates was 92%, dose-dependent susceptibility was 6% and only 2% were resistant to fluconazole, while in the Gualco *et al*. study, resistance to fluconazole was reported in only 0.9% of *C. albicans* isolates.[16]

In many clinical *C. albicans* isolates, azole resistance has often been associated with over-expression of the *ERG11* gene.[17] In the present study, all fluconazole-resistant *C. albicans* isolates showed upregulation of *ERG11*, compared with the control isolate ATCC10231, while over-expression of this gene was seen in only 37% (5/14) of the isolates in the study by Chen *et al*.[13]

C. albicans is the most common *Candida* species in paediatric patients and is reported to be responsible for 30–76% of invasive *Candida* spp. infections. In this study, which is similar to others that included paediatric patients, *C. parapsilosis* is reported to be the most common non-albicans *Candida* spp., in contrast to adult patients in whom *C. glabrata* is more common.[7,15,16,18–20]

Prior or concomitant exposure to antibiotics is a major risk factor for candidiasis.[21] In a study by Fraser *et al*., 94% of patients with candidiasis had prior exposure to antibiotics.[22] In the present study, nearly 60% of patients with positive culture of *C. albicans* had received more than two antibiotics. In addition, all fluconazole-resistant *C. albicans*, which showed upregulation of *ERG11*, received more than two antibiotics (three patients) or three antibiotics (two patients). Therefore, the strategic goals to optimise antimicrobial use, including optimising choice and duration of empiric therapy as well as monitoring and providing feedback regarding antibiotic resistance, are recommended.

Limited data are available on the antifungal susceptibility of *Candida* spp. in Iran, especially for isolates causing infections in children. Therefore, the surveillance of antifungal resistance patterns and the spectrum of *Candida* spp. in children, which can provide important information about differences in susceptibility patterns and molecular mechanisms of azole resistant, are recommended.

As demonstrated in this study, resistance to fluconazole among clinical *C. albicans* isolates in Iranian children suggests the need for regular investigation of antifungal resistance in children's hospitals in order to manage candidiasis more efficiently. In addition, further investigation of other mechanisms of azole resistance in all *Candida* spp. isolates in different populations is recommended. □

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