Original Article

Overexpression of *MDR-1* and *CDR-2* genes in fluconazole resistance of *Candida albicans* isolated from patients with vulvovaginal candidiasis

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(Received: 9 May 2017; Revised: 15 July 2017; Accepted: 24 July 2017)

Abstract

Background and Purpose: *Candida albicans* (*C. albicans*) is an opportunistic fungus that can colonize women's mucosal epithelial cell surfaces, causing vulvovaginitis in specific circumstances. The major genes contributing to drug resistance in *C. albicans* are the candida drug resistance (*CDR*) and multi drug resistance (*MDR*) genes. The purpose of this study was to evaluate the *CDR-2* and *MDR-1* gene expression patterns in *C. albicans* strains isolated from patients with recurrent vulvovaginal candidiasis.

Materials and Methods: In this study, 40 isolates of fluconazole-resistant *C. albicans* were cultured on Sabouraud dextrose agar. These isolates were collected from women with vulvovaginitis who were referred to a clinic in Tehran, Iran, and transferred to a mycology laboratory. Then, RNA was extracted from the isolates using phenol-chloroform and glass beads, and the complementary DNA (cDNA) was synthetized. To detect the semi-quantitative expression of *CDR-2* and *MDR-1* genes, the reverse transcriptase-PCR (RT-PCR) technique was performed using specific primers.

Results: Our findings indicated that of the 40 *C. albicans* isolates, 35 (87.5%) strains were positive for mRNA of the *CDR-2* gene, 32 (80%) strains expressed mRNA of the *MDR-1* gene, and 30 (75%) strains were confirmed to express mRNA of both the *CDR-2* and *MDR-1* genes simultaneously using the RT-PCR assay.

Conclusion: According to the obtained results, the expression rates of *CDR-2* and *MDR-1* genes were high in fluconazole-resistant *C. albicans* isolates, which can cause treatments to fail and result in chronic infections. Inhibiting these important genes using novel or natural agents can help with the treatment of chronic and recurrent vaginitis.

Keywords: *C. albicans*, *CDR-2*, Gene expression, *MDR-1*, RT-PCR, Vulvovaginal candidiasis

➢ How to cite this paper:
Khosravi Rad K, Falahati M, Roudbary M, Farahyar S, Nami S. Overexpression of *MDR-1* and *CDR-2* genes in fluconazole resistance of *Candida albicans* isolated from patients with vulvovaginal candidiasis. Curr Med Mycol. 2016; 2(4): 24-29. DOI: 10.18869/acadpub.cmm.2.4.24.

Introduction

Women of reproductive age, consumers of contraceptive steroidal drugs or any of the widespread anti-bacterial agents, diabetic or pregnant women, and patients with an immunological deficiency have the predisposing factors for vulvovaginal candidiasis (VVC) [1]. The rising prevalence of fluconazole-resistant *C. albicans* strains is a major problem after long-term treatment of recurrent VVC (RVVC). Fluconazole resistance can occur through different mechanisms involving mutations in the drug target enzyme and sterol 14a-demethylase (14DM), alterations in sterol biosynthesis, increased expression of the *ERG11* gene, as well as overexpression of genes coding membrane transport proteins of the ABC transporter (*CDR-1*/*CDR-2*) or the major facilitator (*MDR1*) superfamilies [2, 3]. In addition, drug resistance can emerge by environmental factors, leading to fungal colonization or substituting a resistant species such as *Candida glabrata* or *Candida krusei* with a sensitive one [4-6].

Previous studies illustrated that developing efflux pumps is the most frequent mechanism for azole resistance in *Candida* species. Efflux pumps coded by two carrier gene families include *CDR-1* and *CDR-2* genes belonging to the ATP-binding cassette superfamily, as well as *MDR-1* genes from the major facilitator superfamily [7, 8]. It was confirmed that enhancing the expression levels of *CDR-1*, *CDR-2*, and *MDR-1* in *C. albicans* causes fluconazole resistance [9, 10]. Activating efflux pumps coded by *CDR-1* can affect all azole drugs, while efflux pumps coded by *MDR* are selective for

fluconazole [11]. However, overexpression of several different genes contributes to fluconazole resistance in *Candida* species. For instance, mutations in *ERG11* reduced binding of the drug target enzyme, lanosterol C14-alpha demethylase (14DM), to fluconazole and conferred higher resistance compared to the identical genes without mutation [12-14]. Moreover, fluconazole resistance protein (FLU1) is responsible for fluconazole resistance in *C. albicans* strain; thus, with inactivation of FLU1, fluconazole susceptibility can be increased. However, overexpression of FLU1 has not yet been approved as a cause of fluconazole resistance in clinical *C. albicans* isolates [15].

In recent years, by increasing the growth of azole resistant *C. albicans* affecting the proper treatment of VVC and regarding the major role of *MDR* and *CDR* genes as the major culprit for azole resistance in *C. albicans*, the present study was designed to determine the pattern of *MDR-1* and *CDR-2* genes in clinical samples of *Candida* isolated from Iranian women with VVC.

Materials and Methods

C. albicans strains and culture conditions

Fourteen fluconazole-resistant *C. albicans* isolates were obtained from patients with VVC, who were admitted to gynecology centers in Tehran, Iran. The isolates were identified using the conventional method based on colony color on CHROM agar *Candida*, and molecular methods included polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) [16]. The isolates were stored in sterilized distilled water until the time of the experiment.

Moreover, resistance to fluconazole was shown by disk diffusion assay performed according to the Clinical Laboratory Standards Institute recommend-dations in our previous study [16]. The standard strain of *C. albicans* (ATCC10231) was used as a fluconazole sensitive species. This study was carried out in Medical Mycology and Parasitology laboratory in Iran University of Medical Sciences, 2016.

Total RNA extraction in Candida isolates

For this study, isolates were cultured on Sabouraud dextrose agar medium (SDA, Merck, Germany) and incubated at 37ºC for 24 h. The cell wall of *C. albicans* was disrupted using an RNA lysis buffer and glass beads. Then, RNA was extracted using RNx-plus (Cinnagen, Tehran, Iran) and a chloroform/isoamyl alcohol solution [17]. After centrifugation, the sediment was dissolved in distilled water and stored at -20ºC until use.

Elimination of genomic DNA from the total RNA

To eliminate DNA contamination from the RNA, all the samples were treated by deoxyribonuclease (DNase) enzyme (Fermentas, Paisley, England) according to the following steps: 1 µg of RNA was added to a sterilized, nuclease-free microtube; 1 µl of DNase 10X reaction buffer and 1 µl of DNase-1 were added to microtubes; 2 µl of ethylenedia-minetetraacetic acid (25 mM) was added to each microtube, and the microtubes were stored at 65ºC for 10 min. The purified RNA was used for complementary DNA (cDNA) synthesis.

cDNA synthesis and reverse transcriptase-PCR (RT-PCR) assay

After DNase enzyme treatment, RNA was converted to cDNA according to the manufacturer's recommendations using a cDNA synthesis kit (Fermentas, USA). RT-PCR was performed with the reactions containing 2 µl of template cDNA, 0.6 µl of each specific primer for the *MDR-1* and *CDR-2* genes, 10 µl of Taq DNA polymerase, $MgCl_2$, dNTP, and vivantis buffer), as well as 6.8 µl of diethylpyrocarbonate (DEPC) water in a final volume of 20 µl. The RT-PCR protocol was begun with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min), and extension (72°C for 1 min); the protocol was terminated with a final extension step at 72°C for 3 min. The appropriate negative and positive controls were included in each test.

The primers for *MDR-1* and *CDR-2* were designed by Gene Runner software, and their sequence is presented in Table 1. *ACT1* was used as the house-keeping gene and for confirmation of the PCR process in all the molecular tests. Ultimately, the PCR products were visualized by gel electrophoresis.

Table 1. Nucleotide sequences of *MDR-1* and *CDR-2* primers

Primer sequence	Tm (°C)	Primer name	Accession number
5´-TGGCAAACAATCCAACAATAC A-3´	56.6	*CDR-2* Forward(F)	U63812
5´-AATCAAGGGAATAGATGGGTC A-3´	58.4	*CDR-2* Revers(R)	
5´-TACGCGGGTTCTTTGTTGTAT G-3´	60.3	*MDR-1* Forward (F)	Y14703
5´-GATAATGTTTAGCAAGCCGAGGA-3´	61.1	*MDR-1* Revers (R)	

Results

Fluconazole susceptibility testing against C. albicans isolates

All the 40 *C. albicans* isolates were resistant to 25 µg of fluconazole in disk diffusion method. The inhibition zone was determined < 14 mm against fluconazole.

Patients' age ranged between 18 and 50 years, and 57% of the patients consumed several antibiotics, 28% cases used contraceptives, and 15% of the women had diabetes.

RNA extraction

The quality of RNA was evaluated by gel electrophoresis. Figure 1 illustrates the total RNA of the isolates before and after the DNase enzyme treatment. The quantity of RNA was determined using BioPhotometer plus (Eppendorf AG, Germany), and the RNA concentration for all the samples was adjusted to 1.5 ng.

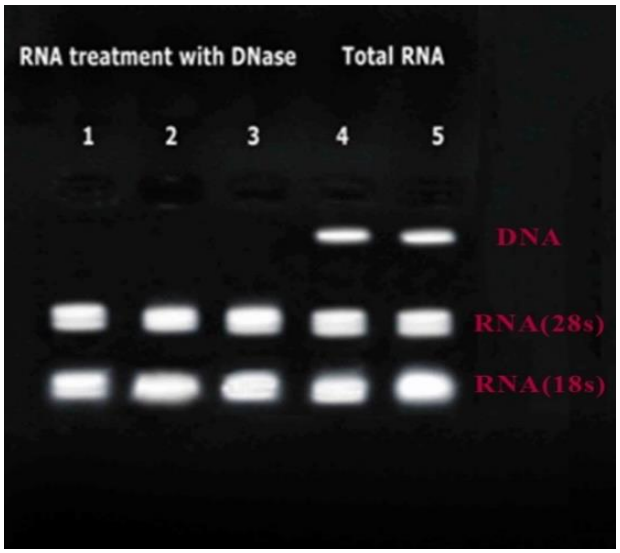

Figure 1. Lines 1-3: Total RNA after treatment with DNase; Lines 4 and 5: RNA before treatment

MDR-1 and CDR-2 gene expression

An RT-PCR reaction was carried out using special primers for *MDR-1* and *CDR-2* for 40 *C. albicans* specimens. The semi-quantitative expression of both *MDR-1* and *CDR-2* was assessed in *C. albicans* clinical isolates (figures 2, 3) using RT-PCR. The PCR product sizes were 125 bp and 148 bp for *CDR-2* and *MDR-1*, respectively, as explained previously [18].

Table 2 indicates the semi-quantitative expression levels of *CDR-2* and *MDR-1*.

The results of the semi-quantitative expression of *CDR-2* and *MDR-1* genes showed that out of the 40 clinical isolates of *Candida albicans*, 35 (87.5%) samples expressed *CDR-2*, leaving only 5 (12.5%) specimens that did not express *CDR-2*. Further, 32 (80%) isolates expressed *MDR-1*, while only 8 (20%) samples did not show expression of the *MDR-1* gene. Finally, 3 (7.5%) samples expressed neither the *CDR-2* nor the *MDR-1*, whereas 30 (75%) isolates expressed both genes simultaneously.

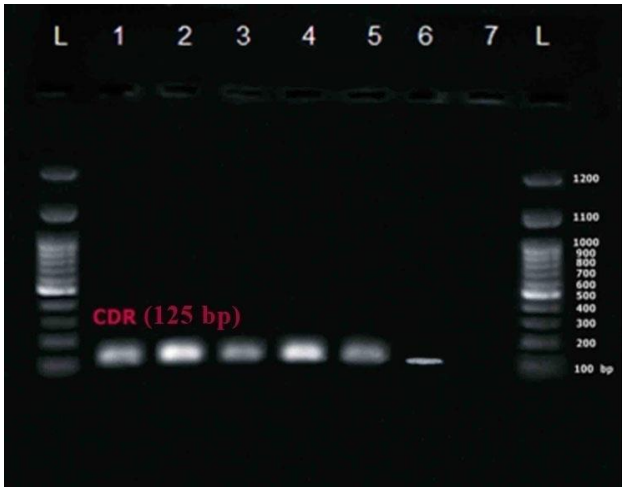

Figure 2. Lines 1-5: *CDR-2* gene expression (125 bp); Line 6: *CDR-2* gene expression in *Candida albicans* (ATCC10231) standard strain; Line 7: Negative control; L: 100 bp ladder

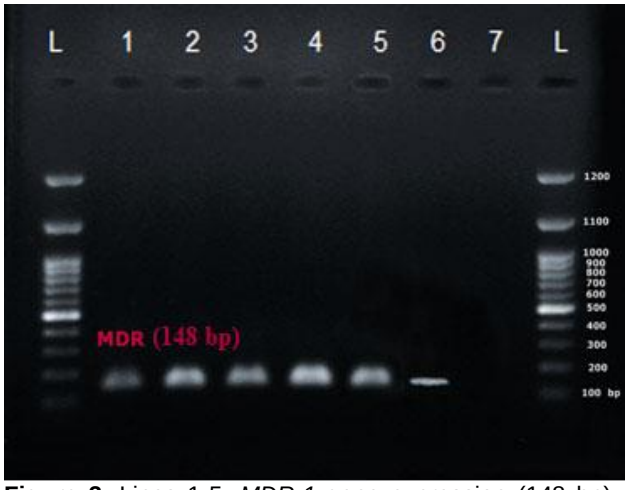

Figure 3. Lines 1-5: *MDR-1* gene expression (148 bp); Line 6: *MDR-1* gene expression in *Candida albicans* (ATCC10231) standard strain; Line 7: Negative control

Table 2. Semi-quantitative expression of *MDR-1* and *CDR-2* genes in the isolates

Evaluated genes	*CDR-2* gene expression	*MDR-1* gene expression	Expression of *CDR-2* and *MDR-1*	No expression of *CDR-2* or *MDR-1* genes
Number of isolates	35	32	30	3
Percent	87.5	80	75	7.5

Discussion

Recent headways in our understanding of the molecular mechanisms causing azole resistance in *C. albicans* revealed that increased efflux of drug, mediated mostly by the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters, leads to resistance to azole anti-fungal agents [19, 20].

Our findings indicated that the expression rates of *MDR* and *CDR* genes were high in fluconazole-resistant *C. albicans.* In our study, the high expression rates of genes in the isolates may be due to taking high doses of fluconazole, as the patients had RVVC. The expression level of *CDR-2* was higher than that of *MDR-1* in the isolates, indicating that the role of *CDR* in forming fluconazole resistance in *C. albicans* is more pronounced than that of *MDR-1*. Regarding the assumption that *CDR* is specific to *C. albicans*, these results were favorable for our isolates.

Emerging fluconazole resistant *C. albicans* isolates leads to a wide range of complications in RVVC treatment, the most important of which is biofilm formation, that is, aggregate of a rigid network by *Candida.* The expression of *MDR* and *CDR* genes during the early phase of biofilm formation and alterations in membrane sterol composition are responsible for resistance of these biofilms against azole agents.

Although resistance is multifactorial and other molecular mechanisms participate in this phenol-menon, it is worth mentioning that the expression of drug efflux pumps during the early phase of biofilm formation and alterations in membrane sterol composition contribute to resistance of these biofilms against azoles [21, 22].

Consistent with our results, Gulat et al. assessed the expression levels of *CDR-1*, *CDR-2*, and *MDR-1* in fluconazole-resistant *Candida albicans* isolates using real-time PCR. Our findings indicated that the expression levels of *CDR-1*, *CDR-2*, and *MDR-1* genes in sensitive isolates were lower compared to resistant ones, suggesting that high expression levels of efflux genes is a major mechanism for fluconazole resistance in *Candida albicans* [23].

Zhang et al. evaluated the expression levels of *CDR-1*, *CDR-2*, *MDR-1*, and *FLU-1* in 18 fluconazole-resistant isolates of *Candida* strains from VVC patients and reported a significant increase in *CDR-1* expression, while expression levels of *CDR-2*, *MDR-1*, and *FLU-1* did not significantly elevate [24].

In our study, lack of expression of *MDR-1* and *CDR-2* genes in 7.5% of the cases may be explained by the report presented by Lohberger et al. indicating the expression levels of drug-resistance genes (i.e., *CDR-1*, *CDR-2*, *MDR-1*, and *ERG-11*) are controlled by transcription factors such as TAC-1, which are responsible for controlling the expression of *CDR-1* and *CDR-2*. MRR-1 and UPC-2 factors are responsible for controlling the expression levels of *MDR-1* and *ERG-11*, respectively. Moreover, there are some enhancing mutations (GOF) in activated alleles for increasing the expression levels of the target genes [19]. It can be concluded that the lack of *MDR-1* and *CDR-2* in some isolates in our study may be associated with the activation of ultra-genetic factors rather than transcription factors including *ERG11* [25, 26], which can be considered in future studies.

In 2013, Guo et al. assessed the correlation between *alcohol dehydrogenase* (*ADH-1*) gene expression and *CDR-1*, *CDR-2*, and *FLU-1* in *Candida albicans* collected from patients with VVC. Expression of *CDR-1*, *CDR-2*, *MDR-1*, and *ERG-11* showed a positive correlation between the expression levels of *ADH-1* mRNA and *CDR-1*, *CDR-2*, and *FLU-1* [27].

Ariana et al. evaluated the expression of *CDR-1*, *CDR-2*, and *MDR-1* in resistant *Candida albicans* isolates compared to fluconazole susceptible isolates. Their outcomes indicated moderate expression of *CDR-1*, *CDR-2*, and *MDR-1* genes, while resistant isolates showed slight or no expression [28].

Our findings were in line with those of Salari et al. who evaluated the *CDR-1*, *CDR-2*, *MDR-1*, and *ERG11* genes expression in *C. albicans* clinically isolated from HIV-infected patients in Iran by real-time PCR. Their results indicated that the *CDR-1* gene expression in fluconazole-resistant *C. albicans* increased significantly compared to other known genes [29].

This finding was not in congruence with the results of the current study. This discrepancy could be related to the source of infection, the number of isolates, and genetic diversity of isolates in different geographic areas.

Conclusion

The high expression levels of *MDR-1* and *CDR-2* genes in *C. albicans* isolates in RVVC highlights the important role of these genes in developing fluconazole resistance, causing treatment attempts to fail and leading to chronic infections. Therefore, inhibition of the key genes involved in the disease as well as combination therapy using novel synthetic or natural drugs could help patients with chronic and recurrent VVC.

Acknowledgments

The authors wish to thank the Research Admini-

stration of the international campus of Iran University of Medical Sciences, Tehran, Iran.

Author's contribution

K. K. performed the tests. M. F. and M. R. designed and managed the research project. S. F. helped with molecular testing and S. N. analyzed the data.

Conflicts of interest

None declared.

Financial disclosure

This study was financially supported by Iran University of Medical Sciences (Grant No: 25647).

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