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Characterization of ERG11 Gene in Drug-Resistant *Candida Albicans* Isolated from Iranian Cases of Recurrent Vulvovaginal Candidiasis

Kambiz Diba[1*], Shahram Khademvatan[2], Soudabe Abharian[3], Kowsar Jaafari[4]

[1] *Associate Professor in Medical Mycology, Molecular and Cellular Research Center, Urmia University of Medical Sciences, Urmia, Iran*

[2] *Professor in Medical Parasitology, Molecular and Cellular Research Center, Urmia University of Medical Sciences, Urmia, Iran*

[3] *Medical Mycologist, Esfahan University of Medical Sciences*

[4] *Medical Microbiologist, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran*

***Corresponding authors:** Kambiz Diba, **Address**: Molecular and Cellular Research Center, Urmia University of Medical Sciences, Urmia, Iran, **Email**: diba.k@umsu.ac.ir

Tel: +984432780803

Abstract

***Background & Aims*:** *Candida albicans* is the most common fungal pathogen of human infections. *C. albicans* is responsible for significant mucosal infections such as vulvovaginitis in women. Azoles inhibit the cytochrome P_{450} 14α-lanosterol demethylase, as a part of the ergosterol biosynthetic pathway is encoded by the *ERG11* gene. Some mutations in *ERG 11* could cause resistance to azole drugs. Detection of the mutations of the gene in the present study helped us to explain drug resistances in some vaginal isolates of *C. albicans* and other Candida species.

Materials & Methods: A multicenter, experimental study was conducted at Cellular and Molecular Research Center and Kowsar Gynecology Center affiliated to UMSU from October 2016 to July 2017. Women with symptomatic vaginitis (20-45 years old) were asked to take part in the study. 192 women allowed vaginal swabs to be obtained. For the identifications, culture on SGA4% and CHROM agar Candida were conducted followed by PCR-RFLP. A disc diffusion method was performed based on the standard guideline of the National Committee for Clinical Laboratory Standards (NCCLS) to determine level of susceptibility against fluconazole and clotrimazole (most current use for the treatment of VVC). DNA extraction and PCR amplification of the *ERG11* gene were performed.

***Results*:** As we showed in the Table (1), 69.1% of all *Candida* isolates carried the *ERG11* gene. It was detected in 49(68.1%), 5 (55.6%), and 7(77.8%) cases of *C. albicans, C. krusei,* and *C. glabrata*, respectively. Among the *C. albicans* isolates resistant to Clotrimazole, 8(53.3%) had *ERG11* gene while 7(46.7%) did not. Among all the *C. glabrata* isolates resistant to Clotrimazole, 40% carried *ERG11* while 60% did not show the gene. Also, ERG11 gene was detected in 50% of the isolated *C. glabrata*. *ERG11* gene was observed in 53.3% of *C.krusei* isolates resistant to Clotrimazole and 52% of those of resistant to Fluconazole.

***Conclusion*:** As an approximate finding, Azole resistance in the present study could be attributed to mutations in ERG11 gene

***Key words*:** ERG11 Gene, Candida Albicans, Vulvovaginal Candidiasis

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Introduction

Candida albicans is the most common fungal pathogen of human infections. It is opportunistic yeast for the immunocompromised people. *C. albicans* is responsible for significant mucosal infections such as vulvovaginitis in women and oropharyngeal disease in AIDS patients. In certain groups of high-risk patients, it causes life-threatening bloodstream infections and subsequent infections in the internal organs. At present, the drugs used for the treatment of *Candida* infections include the polyenes and azoles targeting ergosterol or its biosynthesis. The mechanisms of resistance to azole antifungal agents in *Candida* species are poorly studied. Some mechanisms include a change in the drug target, alteration in the synthesis of ergosterol, decrease in the intercellular concentration of target enzyme, over expression of the antifungal drug target, change in cell permeability, over production or alteration of cytochrome P-450, and modification of other enzyme involved in sterol synthesis (2, 3). Azole-resistant *C. albicans* and other *Candida* species such as *C. glabrata* and *C. krusei* have emerged as serious problems in patients receiving antifungal therapy (2). Azoles inhibit the cytochrome P_{450} related 14α-lanosterol demethylase, as a part of the ergosterol biosynthetic pathway is encoded by the ERG11 gene. Inhibition of Erg11 gene reduces the ergosterol content of membranes and results in the accumulation of toxic intermediates of the sterol pathway and inhibits growth (4). Another enzyme, the $\Delta^{5,6}$ sterol desaturase, encoded by the **ERG11** is associated with the development of resistance in medically important yeast species, *Saccharomyces cerevisiae* and *C. albicans*, which are closely related to *C. glabrata*. Mutations of **ERG11** in fungi result in formation of ergosta-7,22-dien-3 ol as the major sterol of ergostrol pathway and are associated with increased resistance to azole and polyene antifungal agents (3). Another mutation potentially linked to azole resistance is the dysfunction of ERG11. Unfortunately, yeasts with the ERG11 mutation are not viable under aerobic conditions (5).

Materials & Methods

Subjects:

A multicenter, experimental study was conducted at Urmia University of Medical Sciences (UMSU) and related services including Medical Mycology Center, Cellular and Molecular Research Center, and Kowsar Gynecology Center from October 2014 to July 2015. Our subjects were 20-45 years old women with symptomatic vaginitis who were asked to take in part in the study. From all cases approached in the gynecology clinic, 192 women allowed vaginal swabs to be obtained. The administered cases were taken a brief history for the symptoms and previous involvements.

Direct examination and Culture:

The specimens including vaginal discharge were taken by gynecologists or other clinicians using speculum and sterile cotton swabs. Sample swabs were put inside the micro tubes containing 500 µl of sterile distilled water, cut the tails and transported to Medical Mycology lab. A wet preparation and basic culture was performed on Sabouraud dextrose agar (4% [w/v] dextrose, 1% [w/v] peptone, 1.5% [w/v] agar; pH 5.6) and Sabouraud dextrose agar, 0.05% Chloramphenicol, 0.5% Cyclohexamide (Micro Media, Hungary, EU). Yeast positive cultures were tested for *Candida* species identification on CHROM agar Candida (HiMedia Laboratories pvt.ltd, M1297) and Corn Meal agar (Schsrlou Chemie, SA.Barcelona, Europian Union, 01-137) and were grown at 37°C and 30°C, respectively. To confirm the identification of *C. albicans*, a germ tube test was performed (using 0.5 ml of human serum with a full loop of a yeast single colony after a 3 hour incubation at 37°C).

Setting up a PCR-RFLP for the identification:

Susceptibility test:

A disc diffusion method was performed based on the standard guideline of the National Committee for Clinical Laboratory Standards (NCCLS) to determine level of susceptibility against fluconazole and clotrimazole (most current use for the treatment of VVC) (21).

DNA extraction and PCR amplification of the ERG11 gene:

The ERG 11 gene was amplified with primers; primer 1 (5' ATGGGCACCGAAGAAGCA 3', Tm 55°C) and primer 2 (5' CGCGCAGACACGATA 3'). Each reaction included a negative control which was 50 µl of double deionized water (DDW-MERCK) and also employed *C. albicans* (ATCC 10261, DNA extracted (boiling, phenol-chloroform method) as the positive control. The DNA fragments were subjected to electrophoresis system (including 1.5% agarose gel in Tris Borate EDTA buffer and post staining with ethidium bromide 0.50 mg/ml). The PCR assay was performed using 5 µl of the DNA template in a total reaction volume of 50 µl consisting of PCR buffer (20 mM Tris- HCL at pH 8.0), 50 mM KCL, 0.1 mM each and 1.5U of Taq DNA polymerase. All PCRs were performed in a thermo cycler (Bioer XL, Fargene Poyesh, Iran). Thirty amplification cycles were performed after initial DNA denaturation at 95 °C for 5 min. Each cycle consisted of a denaturation step at 95 °C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72 °C for 5 min following the last cycle. PCR products were visualized under UV illumination to verify amplicon as the sharp band.

The statistic analysis including Fisher and χ^2 tests were used for the comparison of differences between subjects with ERG11-positive PCR and those with ERG11-negative PCR. For the sequencing, 20 µl of 10 clinical sample PCR products were submitted to Takapoozist molecular services company, Karaj, Iran.

The highest number of VVC cases caused by *C. albicans* included onetime laboring (26 cases) and the lowest number included 6 times laboring. Most of the cases with *C. krusei* had 3 times of laboring while *C. glabrata* had one labor. Presence of *ERG11* gene is another parameter that we studied among VVC isolates of *Candida* species. Totally, 69.1% of all *Candida* isolates carried ERG11 gene. It was detected in 49(68.1%), 5 (55.6%), 7(77.8%) cases of *C. albicans, C. krusei* and *C. glabrata*, respectively. Among the *C. albicans* isolates resistant to Clotrimazole, 8 cases (53.3%) had ERG11 gene while 7 cases (46.7%) did not have the gene. Among all *C. glabrata* isolates resistant to Clotrimazole, 40% carried ERG11 while 60% did not show the gene. Also in 50% of the isolated *C. glabrata* ERG11 gene was detected. ERG11 gene was detected in 53.3% of *C.krusei* isolates resistant to Clotrimazole and 52% of those resistant to Fluconazole.

Results

Totally 216 subjects took part in our study, 205 with clinical symptoms and 11 without clinical symptoms (within the age of 22 to 46 years old). Nearly 55.2% of all cases (106 of 205) were identified as symptomatic infection. Among all vaginal infections with similar symptoms, 97 (98.1%) yeast and 7 (8.9%) bacterial infections were detected. Among the yeast isolates, 76.3% were identified as *C. albicans* followed by *C. glabrata* and *C. krusei* (9.3% each) and other non albicans *Candida* species (4.1%). . No matter what the *Candida* species causing, the total sensitivity to tested antifungal drugs include 45.4% of cases were resistant and 30.9% sensitive. Among all *C. albicans* isolates, 37(52.1%), 27(38%) and 7(9.9%) were sensitive, resistant, and intermediate to antifungal drug Fluconazole, respectively. Also, among all C. *albicans* isolates 46(64.8%), 15(21.1%) and 10(14.1%) were sensitive, resistant, and intermediate to clotrimazole, respectively. Among the 9 *C. glabrata* isolates recovered from the total sample, only 2(22.2%) expressed dose dependent susceptibility to Clotrimazole (Table 1), while 55.6% were resistant to it. The sensitivity and resistance percentage to Fluconazole were 33.3% and 66.7%, respectively. Susceptibility and resistance to Clotrimazole were exhibited in 44.4% of *Candida krusei* isolates and the isolates revealed 11.1% susceptibility vs. 66.7% resistance to Fluconazole. Among 71 *C. albicans* isolates, susceptibility to Clotrimazole is more than that of Fluconazole (64.8 to 52.1). For the *C. glabrata* clinical isolates, susceptibility to intermediate dose of Clotrimazole is higher than Fluconazole as well as *C. krusei* isolates. Laboring rates in the proved VVC cases were 29, 16, 13, 12, 6, 1 for cases with 1, 2, 0, 3, 4 and 6 labors, respectively. The highest number of VVC cases caused by *C. albicans* included onetime laboring (26 cases) and the lowest

number included 6 times laboring. Most of the cases with *C. krusei* had 3 times of laboring while cases with *C. glabrata* had one time. Presence of ERG11 gene is another parameter that we studied among VVC isolates of *Candida* species. As we showed in the Table (1), totally, 69.1% of all *Candida* isolates carried ERG11 gene. It was detected in 49(68.1%), 5 (55.6%), 7(77.8%) cases of *C. albicans, C. krusei* and *C. glabrata*, respectively. Among the *C. albicans* isolates resistant to Clotrimazole, 8(53.3%) had ERG11 gene and 7(46.7%) did not. Among all *C. glabrata* isolates resistant to Clotrimazole, 40% carried ERG11 while 60% did not show the gene. Also in 50% of the isolated *C. glabrata*, ERG11 gene was detected. ERG11 gene was detected in 53.3% of *C.krusei* isolates resistant to Clotrimazole and 52% of those of resistant to Fluconazole.

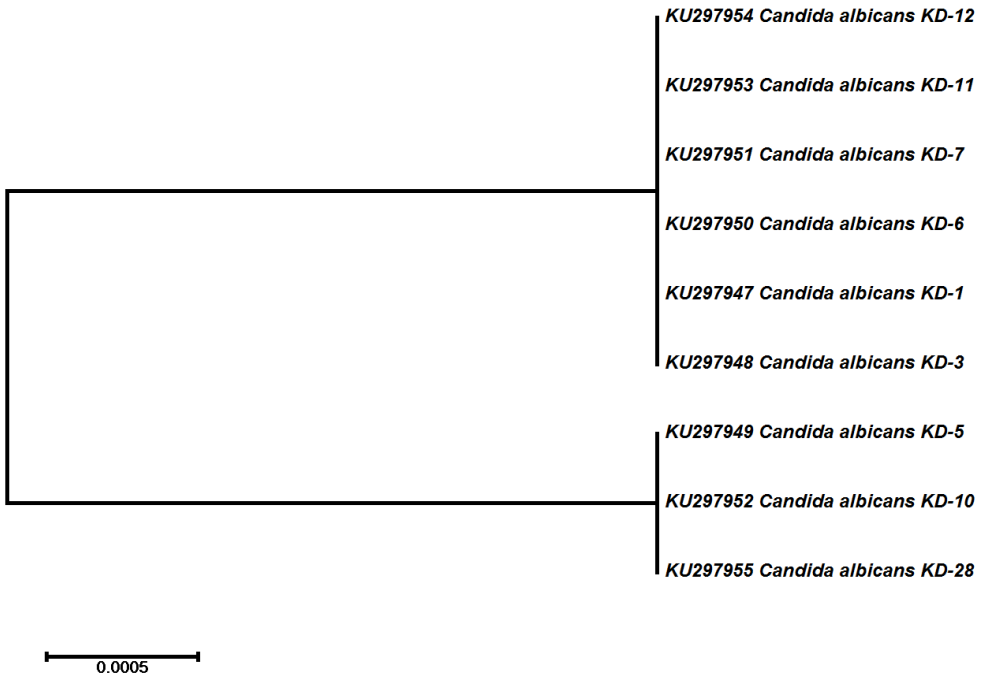

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method

Table-1. Susceptibility to drug azoles based on the CLSI standards

species	Clotrimazole						Fluconazole						Susc / number	
	Susceptible		Resistant		Intermed		Susceptible		Resistant		Intermed			
	No	%	No	%	No	%	No	%	No	%	No	%		
C. albicans	46	64.8	15	21.1	10	14.1	37	52.1	27	38.0	7	9.9%	0.64.7	0.52.1
C. glabrata	2	22.2	5	2	2	22.2	-	-	6	66.7	3	33.3	0.55.5	0.33.3
C. krusei	-	-	4	50	4	50	1	12.5	6	75.0	1	12.5	0.5	0.12.5

Discussion

The role of *ERG11* in azole resistance originates from the observation that treatment of yeasts with azoles results in the accumulation of 14α-methylated sterols and 14α-methylergosta-8, 24(28)-dien-3,6-diol. Formation of the latter sterol metabolite is thought to be catalyzed by sterol $\Delta^{5,6}$-desaturase; thus, inactivation of *ERG11* can suppress toxicity and therefore causes azole resistance. In this study, it seems that there is no correlation between the prevention methods of

pregnancy and labor rate of women with the frequency of VVC. As it was shown in Table 1, *Candida albicans* remains the most common cause of VVC cases (76.3%). This is in agreement with the results of other studies. This finding is confirmed by other studies (32, 33, 34 and 35). *C. glabrata* and *C. krusei* were the next with 9 (9.3%) cases. Also in the study conducted by Eifeky in Egypt (22), *C. glabrata* was more common among non-albicans *Candida* species. Also in an Iranian study *C. glabrata, C. tropicalis* and *C. glabrata* were isolate frequently form VVC cases (23). Another study in Yemen (24) showed the same results; *C. albicans* (65%), followed by and *C. krusei* (24%, 12.7% and 4.3%) respectively). Against it Khan and Baqai from Pakistan had different results about *C. tropicalis, C. parapsollosis* and *C. krusei* (21, 10 and 8% each, respectively) (25). The other study from India showed *C. glabrata* and *C. tropicalis* as the most isolate *Candida* species (26). Although *C . glabrata* is the common isolated non-albicans species, *C. krusei* goes to be fixed as the most frequent species causing VVC. *Candida albicans* and non-albicans species are closely related but differ from each other with respect to epidemiology, virulence characteristics, and fungal susceptibility. Prolonged therapy and increased use of antifungal for recurrent candidiasis are the most common risk factors for azoles resistance among Candida isolates from vulvovaginitis (27). In the present study, among studied *Candida* species, the tested isolates of *C. albicans* showed higher susceptibility (64.7%) to Clotrimazole compared to *C. glabrata* isolates (55.5%) and *C. krusei* (50%). In a study from Yemen also *C. albicans* had the higher susceptibility to Clotrimazole after Miconazole (24). The study conducted in Pakistan reported that in vitro antifungal activity of Clotrimazole on *C. albicans* is more effective as compared to other tested drugs (25). Therefore, with regard to resistance to antifungal drug, Clotrimazole is more common in non-albicans *Candida* species including *C. glabrata* and *C. krusei* than that of *C. albicans.* This conclusion is in agreement with findings of other studies (28, 29 and 30). Antifungal susceptibility pattern in our study showed that *C. albicans, C. krusei,* and *C. glabrata* were sensitive to Fluconazole in 52.1%, 12.5% and 0 respectively, so that susceptibility of three tested Candida species is considerably different. Against it, the effect of intermediate dose of Fluconazole on *C. albicans* isolates was minimum and the resistance to Fluconazole in *C. krusei* was much more than that of *C. albicans*. On the other hand, comparing the amounts Susceptibility/Numbers among three *Candida* spices showed that resistance to Fluconazole is higher not only in non-albicans *Candida* compared to *C. albicans* isolates but also it is higher than Clotrimazole as a whole. In the study of Gandhi from India, 78% of *C. albicans* were sensitive to Fluconazole and 17% were resistant, it is in agreement with our findings (31). Another study from Yemen reported that *C. albicans* was the most common species among the isolates, and it had the highest susceptibility to Ketoconazole and Cloterimazole; while 73 isolates were resistant to Fluconazole (24). *C. albicans* is susceptible to both Clotrimazole and Fluconazole and as we expected, *C. krusei* and *C. glabrata* demonstrated higher resistance among all the isolates. The results suggest that missense mutations in *ERG11* might arise in *C. albicans* more frequently than currently supposed and that the clinical significance of *ERG11* mutants, including those in which additional mechanisms also contribute to resistance, should not be discounted (32).In the present study, *ERG11* gene was detected in 68.1% of *C. albicans* isolated from VVC cases, when we considered the resistant isolates to Clotrimazole and Fluconazole, it declined to 53.3% and 46.7%, respectively. Therefore, the proportion of *ERG11* negative *C. albicans* among the resistant cases is more than those of others. Also *ERG11* gene was detected in 77.8% of all *C. glabrata* isolates compared to the 50% of cases resistant to Clotrimazole and Fluconazole. In other words, some of the azole resistant Candida yeasts do not carry the *ERG11* gene. Our findings are in agreement with Kakeya study on *C. albicans ERG11* gene, they deleted the *ERG11* gene in *C. albicans*, and the deletion resulted in reduced susceptibility of the mutant to azoles. Sterol analysis revealed that ERG11 mutant lost both ergosterol and diol when cultured with Fluconazole (33).

Also, *S. cerevisiae* mutations of the *ERG11* gene can result in azole resistance. However, inactivation of *ERG11* does not always result in azole resistance: in *Candida glabrata*, a null mutation in *ERG11* does not result in azole resistance (34).

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