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ERG11 mutations associated with azole resistance in *Candida albicans* isolates from vulvovaginal candidosis patients

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ABSTRACT

Objective: To investigate the azole susceptibility of *Candida albicans* (*C. albicans*) from vulvovaginal candidosis patients and to analyze the relationship between *ERG11* gene mutations in these isolates and azole resistance.

Methods: Three hundred and two clinical isolates of *Candida* species were collected. Azole susceptibility was tested *in vitro* in microdilution studies. The *ERG11* genes of 17 isolates of *C. albicans* (2 susceptibles, 5 dose-dependent resistants and 10 resistants) were amplified and sequenced.

Results: Of the 302 isolates collected, 70.2% were *C. albicans*, of which 8.5%, 3.8% and 4.2% were resistant to fluconazole, itraconazole and voriconazole, respectively. In total, 27 missense mutations were detected in *ERG11* genes from resistant/susceptible dose-dependent isolates. Among them, Y132H, A114S, and Y257H substitutions were most prevalent and were known to cause fluconazole resistance. G464S and F72S also have been proved to cause fluconazole resistance. Two novel substitutions (T285A, S457P) in hotspot regions were identified.

Conclusions: Twenty seven mutations in the *ERG11* gene were identified in azole-resistant *C. albicans* isolates, which indicated a possible relation with the increase in resistance to azole drugs and the recurrence of vulvovaginal candidosis. The relationship of two novel substitutions (T285A, S457P) with fluconazole resistance needs to be further verified by site-directed mutagenesis.

1. Introduction

Vulvovaginal candidiasis (VVC) is one of the most frequent ailments in the fields of obstetrics and gynecology and results from over-abundant growth of *Candida* species in the vaginal mucosa. It has been estimated that approximately 75% of all women will experience at least one episode of VVC during their lifetime, among which 40%–50% will experience a further episode [1]. Moreover, 5%–8% of adult women have symptomatic recurrent VVC (RVVC), which is defined as ≥ 4 episodes per year [2]. Patients generally suffer from various symptoms, including burning, itching, soreness, abnormal vaginal discharge and pain during sexual intercourse [3]. *Candida albicans* (*C. albicans*) is the predominant species causing vaginitis and accounts for most VVC cases, followed by *Candida glabrata* (*C. glabrata*), *Candida tropicalis* (*C. tropicalis*), *Candida krusei* (*C. krusei*) and *Candida parapsilosis* (*C. parapsilosis*) [4].

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Several molecular mechanisms have been revealed to cause resistance to azole antifungal agents in *C. albicans* strains, including (i) alteration of the target enzyme, which is encoded by the *ERG11* gene. In *C. albicans*, the target enzyme of azoles is 14-demethylase (Erg11p), a key enzyme in the ergosterol synthesis pathway. Azoles block fungal membrane formation [5]. Mutations in Erg11p can decrease the affinity of the target enzyme for azoles [6,7]. (ii) *ERG11* overexpression also contributes to antifungal resistance. Previous studies have shown that mutations in some transcriptional activators such as upc2p may upregulate *ERG11* [8,9]. (iii) Antifungal resistance may result from decreased intracellular drug accumulation following the upregulation of 2 drug efflux pumps, which are encoded by the ATP-binding cassette transporter genes CDR1, CDR2 and MDR1 [10,11]. (iv) Biofilm formation may also cause resistance to antifungal drugs [12].

The purpose of the present study was to investigate the species distribution and to identify mutations in the *C. albicans* *ERG11* gene associated with azole resistance in women with VVC in Changchun, China.

2. Materials and methods

2.1. Media, drugs and agents

Modified Sabouraud dextrose agar contained 20 g/L dextrose, 10 g/L peptone, 20 g/L agar and 50 mg/L chloromycetin. CHROMagar was prepared as recommended by the manufacturer. Roswell Park Memorial Institute (RPMI) 1640 broth was prepared by dissolving 10.4 g/L RPMI 1640 powder (with glutamine, but without $NaHCO_3$) and 34.53 g/L 3-(*N*-morpholino)-propanesulfonic acid in water, adjusting the pH to 6.9–7.0 and sterilizing the solution by filtration. RPMI 1640 agar medium was prepared by dissolving 2% dextrose and 1.5% agar in RPMI 1640 broth. Standard powders of fluconazole, itraconazole and voriconazole (Sigma, Chemical Co., St Louis, MO, USA) were used.

2.2. Isolate collection and identification

Clinical *Candida* samples (n = 302) were collected from patients with VVC in the Second Clinical Hospital of Jilin University during February to October of 2013. All study participants provided informed consent and the study design was approved by the Ethical Committee of Institute of Zoonosis, Jilin University, China. Among these isolates, 210 (69.5%) were obtained from patients with sporadic or infrequent episodes of vaginitis, a condition commonly referred to as acute VVC (AVVC) (Group 1). In addition, 92 isolates (30.5%) were from patients with RVVC episodes (Group 2). The identity of all isolates was confirmed at the species level using CHROMagar *Candida* (CHROMagar; Paris, France) and the API 20C AUX yeast identification kit (bioMérieux SA, Marcy l'E toile, France).

2.3. Drug susceptibility testing

As recommended in the Clinical Laboratory Standards Institute M27-A3 reference document [13], *C. albicans* susceptibility and resistance to fluconazole, itraconazole, and voriconazole were measured using the M27-A3 broth dilution method. The ATCC 6258 and ATCC 22019 strains were used as controls.

Strains showing minimum inhibitory concentrations (MICs) of $\leq$ 8 μg/mL, $\leq$ 0.125 μg/mL and $\leq$ 1 μg/mL with fluconazole, itraconazole, and voriconazole, respectively were considered as susceptible (S). Strains with MIC values of $\geq$ 64 μg/mL, $\geq$ 2 μg/mL and $\geq$ 4 μg/mL with fluconazole, itraconazole and voriconazole, respectively were considered as resistant (R). Strains showing MIC values of 16–32 μg/mL, 0.25–0.5 μg/mL and 1–4 μg/mL with fluconazole, itraconazole and voriconazole, respectively were considered to have sensitivity that was dose-dependent (SDD).

2.4. DNA extraction

Total genomic DNA from each resistant *C. albicans* isolate and 2 randomly selected susceptible isolates were extracted using the yeast DNA kit (Tiangen, China), according to the manufacturer's instructions. DNA concentrations were measured with a GeneQuant spectrophotometer (Amersham Biosciences, USA).

2.5. PCR amplification and sequencing of the ERG11 gene

ERG11 genes from 17 isolates were amplified by PCR using primers with the following sequences: *ERG11*-F:5′-CAAGAAGATCATAACTCAAT-3′and *ERG11*-R:5′-AGAACACTGAATCGAAAG-3′. PCR reactions were performed in a 25 μL mixture containing 2.5 μL of 10 × PCR buffer (with Mg^{2+}), 2 μL of genomic DNA, 10 ng of each deoxynucleotide triphosphates, 1 μL of each primer (10 μmol/L) and 0.25 μL *Taq* DNA polymerase (1 IU/μL). PCR was performed using the following thermocycling conditions: denaturation at 94 °C for 5 min; 35 cycles of denaturation (94 °C, 30 s), annealing (53 °C, 30 s) and extension (72 °C, 2 min) and one final extension cycle at 72 °C for 10 min. The size of each PCR product (1641 bp) was analyzed preliminarily by electrophoresis in 0.8% agarose gels and visualized under an ultraviolet spotlight, after staining with ethidium bromide. PCR fragments of the 17 *C. albicans* isolates were purified with the BioDev DNA purified kit (China) and sequenced with an automated DNA sequencer ABI prism 3700 DNAMAN analyzer (Applied Biosystems) using standard protocols and previously designed primers [14]. For each strain, the entire *ERG11* open reading frame sequence was compared with a previously described *ERG11* sequence (accession number X13296) obtained from a fluconazole-susceptible strain [15].

2.6. Statistical analysis

Chi-square (χ^2) test was used for statistical analysis with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was used to assign statistical significance.

2.7. Nucleotide sequence accession numbers

The *ERG11* gene sequences of 17 *C. albicans* clinical isolates determined in this study were deposited in the GenBank database under accession numbers KM609916–KM609932.

3. Results

3.1. Species identification

In total, 302 *Candida* isolates were collected from non-pregnant outpatients (aged 21–49 years) suspected of having VVC. Among these, 212 *C. albicans*, 28 *C. glabrata*, 22 *C. tropicalis*, 14 *C. krusei*, and 13 *C. parapsilosis* isolates in addition to 13 *Candida* spp. were identified. In total, 75% of women (159/212) positive for *C. albicans* had AVVC, whereas the remaining 25% (53/212) had RVVC. The distribution of the isolates identified was shown in Table 1. There were 51 strains of non-*C. albicans* spp. found among the 210 strains of AVVC group (24.3%) and 39 strains of non-*C. albicans* spp. among the 92 strains of RVVC group (42.4%). The ratio of non-*C. albicans* in the RVVC group was significantly higher than that in the AVVC group ($P < 0.05$).

Table 1

Distribution of *Candida* species among patients with AVVC and RVVC. *n* (%).

Species	AVVC	RVVC	Total
C. albicans	159 (75.7)	53 (57.6)	212 (70.2)
C. glabrata	18 (8.6)	10 (10.9)	28 (9.3)
C. tropicalis	14 (6.7)	8 (8.7)	22 (7.3)
C. krusei	8 (3.8)	6 (6.5)	14 (4.6)
C. parapsilosis	5 (2.4)	8 (8.7)	13 (4.3)
Other *Candida* spp.	6 (2.9)	7 (7.6)	13 (4.3)

3.2. Antifungal susceptibility tests

The results of *in vitro* susceptibility testing for the 212 clinical isolates of *C. albicans* were shown in Table 2. According to the Clinical and Laboratory Standards Institute definitions, 30 isolates (14.2%) had reduced susceptibility to fluconazole (18 R and 12 SDD), 12 (5.7%) isolates had reduced susceptibility to itraconazole (8 R and 4 SDD) and 13 isolates (6.1%) had reduced susceptibility to voriconazole (9 R and 4 SDD). A significantly higher percentage of isolates had reduced susceptibility to fluconazole than to itraconazole or voriconazole ($P < 0.05$).

Among isolates identified in the AVVC outpatient group, 16/159 (10.1%) had reduced susceptibility to fluconazole (10 R and 6 SDD), 5 (3.1%) isolates had reduced susceptibility to itraconazole (3 R and 2 SDD) and 4 isolates (2.5%) had reduced susceptibility to voriconazole (3 R and 1 SDD). However, in the RVVC group, 14/53 (26.4%) had reduced susceptibility to fluconazole (8 R and 6 SDD), 7 (13.2%) isolates had reduced susceptibility to itraconazole (5 R and 2 SDD) and 9 isolates (17.0%) had reduced susceptibility to voriconazole (6 R and 3 SDD). The percentage of isolates with reduced susceptibility to

Table 2

Antifungal susceptibility of vaginal *C. albicans* isolates.

Antifungal agent	AVVC			RVVC			Total		
	R	SDD	S	R	SDD	S	R	SDD	S
Fluconazole	10	6	143	8	6	39	18	12	182
Itraconazole	3	2	154	5	2	46	8	4	200
Voriconazole	3	1	155	6	3	44	9	4	199

S, SDD, and R were defined as a MIC value of $\leq$ 8, 16–32, or $\geq$ 64 μg/mL of fluconazole for *C. albicans*. S, SDD, and R were defined as a MIC value of $\leq$ 0.125, 0.25–0.5, or $\geq$ 1 μg/mL of itraconazole for *C. albicans*. S, SDD, and R were defined as a MIC value of $\leq$ 1, 1–4, or $\geq$ 4 μg/mL of voriconazole for *C. albicans*.

Table 3

Results of *in vitro* antifungal susceptibility testing and *ERG11* sequence analysis for 17 clinical isolates of *C. albicans*.

Isolates No. (GenBank No.)	MIC (μg/mL)				Missense mutation	Amino acid changes in *ERG11*/**hot pot**
	Type	FLZ	ITZ	VOR		
CHC114 (KM609920)	AVVC	> 64	0.031 3	0.50	C488T	A114V/**I**
CHC117 (KM609921)	RVVC	> 64	4.000 0	0.50	T495A, A530C, T541C, G1540A	D116E/**I**, K128T/**I**, Y132H/**I**, G465S/**III**
CHC119 (KM609922)	RVVC	> 64	0.031 3	0.50	C515T, T541C	T123I/**I**, Y132H/**I**
CHC122 (KM609924)	RVVC	> 64	8.000 0	4.00	G487T, T916C, A1177G, A1714G	A114S/**I**, Y257H/-, **K344E**/-, R523G/-
CHC125 (KM609925)	RVVC	> 64	0.031 3	8.00	C515T, A541C, A1000G, G1096A, T1155C, A1697T	T123I/**I**, Y132H/**I**, **T285A/II**, **A317T**/-, **I333T**/-, **E517V**/-
CHC128 (KM609927)	AVVC	> 64	0.031 3	0.50	G487T, T916C	A114S/**I**, Y257H/-
CHC130 (KM609928)	AVVC	> 64	0.500 0	0.50	G487T, T916C	A114S/**I**, Y257H/-
CHC134 (KM609929)	RVVC	> 64	0.500 0	8.00	A217G, T362C, A416G, T495A, A530C, A856G, A926G, G1537A	**S24G**/-, F72S/-, **K90R**/-, D116E/**I**, K128T/**I**, **N237D**/-, **E260G**/-, G464S/**III**
CHC157 (KM609930)	RVVC	> 64	0.031 3	4.00	G487T, T916C	A114S/**I**, Y257H/-
CHC160 (KM609931)	RVVC	> 64	8.000 0	8.00	T495A, A530C, T541C, G1540A, A1714G	D116E/**I**, K128T/**I**, Y132H/**I**, G465S/**III**, R523G/-
CHC95 (KM609917)	RVVC	16	0.500 0	0.25	T541C, A945C, A1466G, T1516C, G1609A	Y132H/**I**, E266D/**II**, N440S/**III**, S457P/**III**, V488I/**III**
CHC106 (KM609918)	RVVC	16	0.031 3	0.50	T848G, G1456A	**V234G**/-, V437I/**III**
CHC120 (KM609923)	AVVC	32	0.031 3	0.50	A650G	**N168S**/-
CHC126 (KM609926)	AVVC	32	0.500 0	1.00	A945C	E266D/**II**
CHC169 (KM609932)	AVVC	32	0.125 0	1.00	A945C	E266D/**II**
CHC34 (KM609916)	AVVC	8	0.250 0	0.50	A1010G	D288G/-
CHC108 (KM609919)	AVVC	8	0.031 3	0.50	–	–

The newly observed substitutions were shown in bold. The sequence of *C. albicans* isolates was compared with that of GenBank accession No. X13296. FLZ: Fluconazole; ITZ: Itraconazole; VOR: Voriconazole.

fluconazole was significantly higher in the RVVC group than in the AVVC group ($P < 0.05$), but no significant differences were found between the two groups in terms of itraconazole and voriconazole susceptibility ($P > 0.05$).

3.3. Cross resistance among different isolates

Two isolates (CHC122 and CHC160) were resistant to all 3 types of azole antifungal agents; 5 isolates (CHC95, CHC117, CHC125, CHC134 and CHC157) were resistant to 2 azoles and 8 isolates (CHC106, CHC114, CHC119, CHC120, CHC126, CHC128, CHC130 and CHC169) were resistant to fluconazole alone (Table 3).

3.4. Mutations in the ERG11 gene

The size of the *ERG11* coding region amplified by PCR was 1 640 bp. By analyzing *ERG11* sequencing results from 17 *C. albicans* isolates, we found 62 mutations, of which 34 were silent mutations that did not result in amino acid changes (data not shown). Twenty-seven missense mutations were detected in R/SDD isolates and 1 mutation was detected in 1 S isolate (CHC34).

Among the 27 missense mutations identified in R/SDD isolates, 15 mutations identified (A114S, A114V, Y257H, K128T, G465S, F72S, D116E, Y132H, E266D, V437I, G464S, T123I, N440S, R523G and V488I) have been reported previously and 12 were novel (S24G, K90R, N168S, V234G, N237D, E260G, T285A, A317T, I333T, K344E, S457P, E517V) (Table 3).

Among them, T285A and S457P mutations were occurred in hotspot regions.

4. Discussion

Among the isolates studied, nearly two-thirds (210/302) were from AVVC patients and the others were from RVVC patients. The most common species found among the isolates tested was *C. albicans* (70.2% of all isolates), followed by *C. glabrata* (9.3%) and *C. tropicalis* (7.3%). The species distribution of *Candida* isolates was similar to a report in the US and a recent report in Southern China [16,17].

There are limited data regarding the antifungal susceptibility of yeast species causing VVC. In this study, the resistance rate of *C. albicans* to fluconazole in AVVC was 6.2% (10/159), which is significantly higher than the rate in Southern China [17], which indicates differences in the genotype of the circulating isolates or in the frequency of the use of fluconazole between different regions of China. However, the resistance rate of *C. albicans* to itraconazole was 1.9% (3/159), which is similar to that in Southern China (1.1%). The level of fluconazole resistance found in this study was significantly higher than that of itraconazole or voriconazole, possibly because fluconazole is more frequently used than itraconazole or voriconazole. It is known that the activity of fluconazole is weaker than itraconazole and itraconazole is weaker than voriconazole [18]. Therefore resistances against fluconazole are more frequently observed than itraconazole and voriconazole.

The fluconazole resistance rate in the RVVC group was significantly higher than that in the AVVC group, which indicates that more frequent use of fluconazole can generate resistance. In contrast, itraconazole and voriconazole are seldom used and the associated resistance rates between the AVVC and RVVC groups are not significant.

In the present study, the Y132H substitution was found in 5 isolates (CHC95, CHC117, CHC119, CHC125 and CHC160) and G465S substitutions also occurred in two of the isolates (CHC117, CHC160). The Y132H mutation is situated in the B–B′ helix cluster, a highly conserved region among lanosterol 14a-demethylases that facilitates substrate binding. Mutations in the B–B′ helix cluster can cause 4-fold increases in fluconazole MIC because of decreased affinity between the target enzyme and fluconazole [5,19–21].

A high frequency of the D116E mutation was also found in fluconazole-resistant isolates (CHC117, CHC134 and CHC160), which is consistent with the findings of Cernicka and Subik, who identified D116E amino acid alterations in Erg11p that are conserved in fluconazole-resistant strains [22].

Combined A114S and Y257H substitutions were found in 4 isolates (CHC122, CHC128, CHC130 and CHC157); these mutations were previously reported to occur simultaneously in fluconazole-resistant isolates [23]. A114S is located near to the substrate channel in Erg11p, and its mutation may interfere with the entry of inhibitor or binding to the active site [23]. Fluconazole MIC values were previously found to be 2- to 4-fold greater in *C. albicans* with the Y257H mutation [24]. We found that the combined A114S and Y257H substitutions were associated with an 8-fold increase in fluconazole resistance. Y257H is located in the G helix, far away from the active center or substrate access channel of the protein and thus does not directly affect the affinity of the Erg11 protein for azoles [25]. The contribution of the Y257H mutation to azole resistance must be further verified by site-directed mutagenesis.

The G464S substitution only occurred in a single isolate (CHC134), which was resistant to fluconazole, itraconazole and voriconazole. The G464S substitution occurs in the heme-binding region and prevents the heme region amino acids from binding to fluconazole [26]. Previously, it was found that the G464S substitution caused a 64-fold increase in the fluconazole MIC. Sanglard *et al.* also demonstrated that Y132H and G464S could increase the resistance to fluconazole and itraconazole [27].

The F72S substitution was found in one azole-resistant strain (CHC134). Changing the amino acid from a polar residue (phenylalanine) to an apolar, neutral residue (serine) alters the hydrophobic channel in terms of substrate access and the F72S substitution prevents the substrate from accessing the active site, resulting in azole resistance [26].

The V488I substitutions were found in one azole-resistant strain (CHC95), combined with Y132H and E266D. Cernicka and Subik also reported the same substitution in fluconazole-resistant strains [22]. The E266D substitution was detected in 3 azole antifungal SDD strains (CHC95, CHC126 and CHC169). E266D probably does not contribute to azole resistance because it is found in both azole-resistant and azole-susceptible strains [5,27,28]. T123I substitution was found in two resistant isolates (CHC119 and CHC125) and it was also observed in two resistant isolates (C97 and C112) from Anhui in China [29]. The location of this substitution in hot pot I highlights the possible relationship with fluconazole-resistance and should be verified in future. The N440S substitution found in SDD stain CHC95 was also found in a fluconazole-resistant clinical isolate CA490 [30]. The location of this substitution in hot pot III indicates that they may play a role

in fluconazole-resistance. The R523G substitution was identified in two resistant isolates (CHC122, CHC160) and the substitution has been identified in a fluconazole-resistant clinical isolate CA490, but the contribution of it to fluconazole-resistant needs further confirmation [30]. V437I substitutions occurred in one SDD isolate (CHC106), accompanied by other point mutations. Some studies have reported the occurrence of the V437I mutations in both azole-susceptible and azole-resistant isolates [12,26,28]. The A114V substitution was found in one fluconazole-resistant strain (CHC114). The mutation occurs near to the substrate channel in Erg11p and may interfere with the entry of inhibitor or binding to the active site. The substitutions of T285A and S457P were the first reported in this study and their contribution to azole resistance must be further verified by site-directed mutagenesis.

There are 38 missense mutations occurred in 9 R/SDD isolates from RVVC patients. But only 8 missense mutations were found in 6 R/SDD isolates from AVVC patients (Table 3). The much higher mutation rate in the RVVC group than in the AVVC group may indicate multiple nucleotide substitutions in the *ERG11* gene fragments that would increase fluconazole resistance and lead to the recurrence of VVC.

Conflict of interest statement

We declare that we have no conflict of interest.

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