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ORIGINAL RESEARCH ARTICLE

Regulatory Role of *ERG3* and Efg1 in Azoles-Resistant Strains of *Candida albicans* Isolated from Patients Diagnosed with Vulvovaginal Candidiasis

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Abstract Vulvovaginal candidiasis (VVC), caused by *Candida albicans*, affects women's health and life. We aimed to explore the correlation between *ERG3* as well as Efg1 mutation/overexpression and azoles-resistance, and the correlation between *ERG3* and Efg1 mRNA expression in *C. albicans*. First, *C. albicans* was isolated from clinical VVC patients. *ERG3* and Efg1 mutations were detected by polymerase chain reaction (PCR) and sequencing, and the expression levels of these two genes were also identified by qRT-PCR. Correlations between mutation/overexpression of *ERG3*/Efg1 and azoles-resistance as well as *ERG3* and Efg1 mRNA expression were analyzed. Based on the *ERG3* sequencing, the results showed that there were 2 missense mutation sites, 1 nonsense mutation site, and 4 silent mutation sites, while 1 missense mutation sites, 1 nonsense mutation site, and 12 silent mutation sites were found in Efg1. Furthermore, the mRNA levels of *ERG3* gene in the strains sensitive to FCA, ITR or VRC were higher than those in the strains resistant to FCA, ITR, VRC ($P < 0.05$). While for the mRNA levels of Efg1, susceptible strains were lower than resistant strains. Besides, there was a significant linear negative correlation between *ERG3* and Efg1 mRNA expression ($r = -0.614$, $P < 0.001$).

Keywords *Candida albicans* · Azoles · Drug resistance · ERG3 · Efg1

Introduction

Vulvovaginal candidiasis (VVC), a common disorder in women, is one of the most common infections of the female genital tract [1]. The symptoms of VVC include vaginal itching, burning with urination, white and thick vaginal discharge, pain with sex, and redness around the vagina [2]. It is estimated that 75% of women have had VVC at least once in their lifetime [3]. A primary pathological factor for VVC is *Candida albicans* strain [4]. At present, azole antifungal drugs, such as voriconazole (VRC), itraconazole (ITR) and fluconazole (FCA), are widely used for the treatment of VVC patients [5]. Unfortunately, the wide use of azole antifungal drugs in clinical leads to azoles-resistant to *C. albicans* strain, which increases the difficulty of VVC treatment [6]. Therefore, it is needed to find the molecular mechanisms underlying azole resistance, which is significant for the treatment of VVC patients.

Currently, studies for the molecular mechanisms underlying azole resistance of *C. albicans* are focused on the following four aspects: changes of drug target enzymes, overexpression of efflux pump gene, regulation of zinc-cluster transcription factors, and biofilm formation [7]. Ergosterol, which is an essential component of membrane of *C. albicans*, influences the activity of membrane-bound enzyme membrane and the membrane permeability [8]. The growth of fungi can be inhibited by anti-fungal azoles via preventing ergosterol synthetic pathway [9]. Erythroblast transformation-specific (ETS) related genes (ERG genes) are targeting enzyme genes in ergosterol synthetic

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pathway, and over expressions and/or mutations of ERG genes are regarded as the main mechanism of azoles-resistance in *C. albicans* [10]. Cytochrome P450 lanosterol 14α-demethylase, coded by *ERG11* gene, is a target enzyme of azoles [11]. Our research group has confirmed that mutation and/or overexpression of *ERG11* leads to azoles-resistance in *C. albicans* [12, 13]. Furthermore, we have found that overexpression of *ERG4* or *ERG5* is associated with azoles-resistance in *C. albicans* [12, 14]. Besides that, there are few studies on other ERG genes in ergosterol pathway at home and abroad. *ERG3* (1163 bp), locating in the upstream of *ERG11*, codes sterol Δ5,6-desaturase, which is a key enzyme in the late stage of ergosterol synthesis [7, 15]. Some studies suggest that mutation of *ERG3* gene induces azoles-resistance in *C. albicans*, and the possible mechanism may be that mutation of this gene prevents the formation of toxic sterol [16, 17]. Akins et al. [18] indicated that overexpression of *ERG3* gene increase drug sensitivity of *C. albicans*. At present, the correlation between *ERG3* expression levels and azoles-resistance is not clear, and there is no study on the effects of *ERG3* in clinical VVC isolates.

Furthermore, *ERG3* is negatively regulated by morphological regulators Efg1 [19]. Efg1, a member of the APSES family, is a major transcription factor in the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway [20]. Efg1 mutation can inhibit the formation of mycelium and decrease the expression of mycelium specific gene, thus reducing the virulence of *C. albicans* [21]. Efg1 was involved in azoles-resistance of *C. albicans* and the susceptibility to antifungal drugs increased when lacking this gene [19]. Thus, Efg1 may be associated with azoles-resistance in *C. albicans*, but the correlation between Efg1 and azoles-resistance is not clear.

C. albicans isolated from VVC patients were used for the present study. *ERG3* and Efg1 mutations were detected, and the expression levels of these two genes were also identified. Furthermore, the correlations between mutation/overexpression of *ERG3* as well as Efg1and azoles-resistance were analyzed. Besides, the correlation analysis between *ERG3* and Efg1 mRNA expression was conducted. We aimed to explore the correlation between *ERG3* as well as Efg1 mutation/overexpression and azoles-resistance, and the correlation between *ERG3* and Efg1 mRNA expression in *C. albicans*.

Materials and Methods

Strains

From November 2015 to May 2016, 184 samples of vaginal secretions from patients diagnosed with VVC in department of dermatovenereology, the second hospital of Shanxi Medical University were collected. Among these samples, 50 *C. albicans* strains were obtained and used for the present study. Approval was obtained from the Ethics Committee of Shanxi Medical University and informed consent was provided by all patients. The reference *C. albicans* strain, ATCC11006, was purchased from the Fungi and Fungal Disease Research Center of Peking University (Beijing, China).

Drug Susceptibility Testing

The minimal inhibitory concentrations (MICs) of FCA, ITR and VRC was determined by the broth microdilution method supported by the Clinical and Laboratory Standards Institute (CLSI) standard M27–A3. The specific method was carried out by the study of Feng et al. [22]. Results were analyzed according to the CLSI standard: FCA(sensitive, $\leq$ 8 mg/ml; susceptible dose dependent, 16–32 mg/l; resistant, $\geq$ 64 mg/l), ITR (sensitive, $\leq$ 0.125 mg/ml; susceptible dose dependent, 0.25–0.5 mg/l; resistant, $\geq$ 1 mg/l) and VRC (sensitive, $\leq$ 1 mg/ml; susceptible dose dependent, 2 mg/l; resistant, $\geq$ 4 mg/l) [22].

Isolation of Genomic DNA from C. albicans Strains

Genomic DNA was isolated from all *C. albicans strains* using a Yeast DNAiso kit (Takara Bio, Inc., Otsu, Japan). Briefly, fresh colonies identified as *C. albicans* were selected and inoculated into liquid nutrient medium and cultured with a speed of 220 rpm at 37 °C overnight. Afterwards, 1 mL fungi suspension was used to extract DNA according to the manufacture's instruction. The collected DNA was sealed with a sealing membrane and stored at −20 °C for subsequent use.

Polymerase Chain Reaction (PCR) Amplification

According to the gene sequence in GenBank, specific primers of *ERG3* and Efg1 (*ERG3*-F, 5′-ATGGATATCG TACTAGAAATTTGTGA-3′; *ERG3*-R, 5′-TCATTGTTC AACATATTCTCTATCG-3′; Efg1-F, 5′-ATGTCAACGT ATTCTATACCCTATTACAA-3′.

Efg1-R, 5′-TTACTTTTCTTCTTTGGCAACAG-3′) were designed and synthesized by Shanghai Sangon Bioengineering Co. Ltd. (Shanghai, China).

The PCR amplification was performed using 12.5 μL MasterMix (Sangon Bioengineering Ltd, Shanghai, China), 1 μL specific forward primers (10 μmol/L), 1 μL specific reverse primers (10 μmol/L), 40 ng DNA (after dilution), and 9.5 μL ddH_2O. The PCR condition was set as denaturation for 5 min at 94 °C, followed by 30 cycles of 94 °C

for 30 s, 50 °C (ERG3) or 52 °C (Efg1) for 30 s, and 72 °C for 30 s, and elongation at 72 °C for 8 min and 4 °C for 1 min. PCR products were then separated and sized on a 1.5% agarose (Tianjin No. 3 Chemical Reagent Factory) gel by electrophoresis, and the results were recorded using gel image analyzer to determine whether the amplification was successful or not.

Sequencing and Analysis

The PCR products were purified and sequenced by Shanghai Sangon Bioengineering Co. Ltd. (Shanghai, China). The obtained sequences were compared with the sequence of *ERG3* and Efg1 in GenBank using DNASTAR software (Version 7.1), and sequencing map was observed using Chromas (Version 2.6.5) to find point mutations.

Isolation of RNA from C. albicans Strains

Total RNA was isolated as follows: First of all, 1 mL overnight cultured bacterial solution was added to RNAse-free centrifuge tube (1.5 mL), centrifuging at 10,000 rpm for 1 min at room temperature, culture medium was discarded, and thalli (wet weight: 20 mg) was collected. Then, it was washed with 500 μL DEPC-treated ddH2O, centrifuging at 10,000 rpm for 1 min, and supernatant liquid was discarded. A total of 600 μL Snailase Reaction Buffer and 50 μL Snailase (prepared before the experiment) were added to the above tube, water bathing at 37 °C for 5 min, centrifuging at 10,000 rpm for 2 min at 4 °C, and the supernatant liquid was discarded. Then, 400 μL Buffer Rlysis-YS were added immediately, blending by oscillation and water bathing at 65 °C for 5 min. After that, 200 μL Buffer YK were added after ice-bathing for 5 min, centrifuging at 12,000 rpm for 5 min at 4 °C. The supernatant liquid (about 600 μL) was obtained and was added to a new 1.5 mL Rnase-free tube. Equal volume (about 600 μL) of phenol: chloroform (25:24, pH4.5) were added to the supernatant liquid, blending, centrifuging at 12,000 rpm for 5 min at 4 °C. The supernatant liquid was obtained and was added to a new tube, and absolute ethyl alcohol (1/2 volume) was added to the tube, fully blending. The adsorbing column was put into the collection tube, and all the solution was added to the adsorbing column, resting for 1 min, centrifuging at 10,000 rpm for 1 min at room temperature, and the waste liquid in collection tube was poured out. Then, 500 μL RPE Solution was added, resting for 1 min, centrifuging at 10,000 rpm for 1 min at room temperature, and the waste liquid in collection tube was poured out, repeating one time for this operation. Centrifugation was conducted at 12,000 rpm for 2 min at room temperature. The cover of adsorbing column was opened, resting for a few minutes. The adsorbing column was put into a new centrifuge tube, and 30 μL DEPC-treated ddH2O were added to the center of adsorbing membrane, resting for 2 min. Subsequently, it was centrifuged at 12,000 rpm for 2 min, and the obtained RNA was stored at − 70 °C for spare.

Reverse Transcription

Total RNA was reverse-transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Shanghai). Firstly, the concentration of total RNA was determined using nucleic acid and protein analyzer. Secondly, PCR reaction was performed as follows: 1 μg RNA and 2 μL OR Random Hexamer Primer were added, and then DEPC-treated water was added until the total volume reached to 13 μL. Denaturation was conducted in PCR instrument at 65 °C for 10 min. Then, it was placed on ice, and 4 μL Transcriptor Reverse Transciptase Reaction Buffer, 0.5 μL Protector RNase Inhibitor, 2 μL Deoxynucleotide Mix, and 0.5 μL Transcriptor Reverse Transciptase were added until the total volume reached to 20 μL. Subsequently, it was put into PCR instrument. Finally, it was taken out from PCR instrument and was stored at − 20 °C for spare.

Design and Synthesis of Primers

According to the cDNA sequence in GenBank, specific primers of *ERG3* and Efg1 (*ERG3*-F, 5′-CGCTTGTCACA CTGTCCATC-3′; *ERG3*-R, 5′- TTCTTCTTCTGCCTTT GCATC -3′; Efg1-F, 5′-GCCACAACCTCAGCATTACA-3′; Efg1-R, 5′-GACCTGGTAGTGGTGGCTGT-3′) were designed and synthesized by Shanghai Sangon Bioengineering Co. Ltd. (Shanghai, China).

Real-Time Quantitative PCR (qRT-PCR) Amplification

The qRT-PCR was conducted using FastStart Essential DNA Green Master (Roche Diagnostics, Shanghai). The reaction system included 10 μL FastStart Essential DNA Green Master, 1 μL specific forward primers, 1 μL specific reverse primers, 1 μL cDNA, and 7 μL ddH_2O. The reaction conditions were set as denaturation for 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s, and melting at 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s.

ATCC11006 was regarded as the control. The comparative threshold (Ct) cycle method ($2^{-\Delta\Delta}$Ct) was used to calculate relative expression levels of ERG3 and Efg1.

Statistical analysis was performed using SPSS 23.0 software (SPSS Inc., Chicago, Illinois, USA). The results were expressed as X ± SD (means ± standard deviations),

and *t* test was performed. A *P* value of < 0.05 was regarded as statistically significant difference.

Results

Drug Susceptibility Testing

Drug sensitivity test showed that (Table 1), a total of the 26 strains were sensitive to FCA, 22 strains were FCA-resistant, with a fluconazole resistance rate of 44%. Of the 50 strains isolated, 21 were sensitive to ITR, 1 was susceptible dose dependent and 28 were resistant to ITR, which showed an itraconazole resistance rate of 56%. In addition, the present study identified 23 VRC-sensitive strains, 2 susceptible-dose-dependent strains and 25 VRC-resistant strains, with a voriconazole resistance rate of 50%. Notably, a total of 10 strains were resistant to FCA and ITR, 4 strains were resistant to ITR and VRC, and 12 strains were resistant to all these three drugs. The cross-resistance rates between the three drugs were 20%, 8% and 24%, respectively. The reference strain (ATCC11006) was sensitive to all three drugs [22].

Correlation Between Mutations of ERG3 as Well as Efg1 and Drug Resistance

As presented in Fig. 1, *ERG3* and Efg1 were successfully amplified. The *ERG3* sequencing results showed that among 50 *C. albicans* strains, 46 strains were sequenced successfully and 40 strains had base mutations. There were 2 missense mutation sites [C657G (W219C) and C1055T (R352H)], 1 nonsense mutation site [C309T (W103Stop)], 4 silent mutation sites (T342G, T435C, C441T, and T1047C), and 1 termination codon mutated to codon encoding an amino acid [T384C (Stop128 W)]. There was no mutation for standard strain ATCC11006. A total of 3 strains showed missense mutation (2 strains was W219C and 1 strain was R352H), and these 3 strains were FCA/

Table 1 The drug sensitivity test of 50 *Candida albicans* strains

Strains (ZY)	MIC (μg/mL)			Strains (ZY)	MIC (μg/mL)		
	FCA	ITR	VRC		FCA	ITR	VRC
4	R (64)	R (1)	R (4)	60	S (8)	S (0.125)	S (1)
6	R (64)	R (4)	R (8)	61	S (2)	S (0.125)	R (16)
7	S (2)	R (4)	S (0.5)	62	S (4)	S (0.125)	R (8)
8	R (64)	R (16)	R (8)	63	R (64)	R (8)	R (4)
12	S (1)	S (0.125)	S (0.0125)	64	S (0.25)	SDD (0.5)	R (4)
15	S (0.125)	S (0.0625)	S (0.25)	66	S (0.25)	S (0.0625)	S (0.0313)
16	SDD (16)	R (2)	S (0.5)	72	S (0.125)	S (0.0313)	S (0.0313)
18	R (64)	R (16)	S (0.125)	77	R (64)	R (8)	R (16)
19	R (64)	R (16)	S (1)	78	R (64)	R (16)	S (1)
22	R (64)	R (16)	S (1)	79	R (64)	R (2)	S (0.5)
25	S (8)	S (0.125)	S (0.5)	80	SDD (32)	S (0.125)	R (4)
27	S (8)	S (0.125)	S (0.25)	88	S (1)	S (0.0625)	R (8)
28	R (64)	R (16)	R (8)	89	R (64)	R (1)	R (16)
32	S (0.5)	S (0.0625)	S (0.125)	90	S (1)	S (0.125)	S (0.5)
33	S (4)	S (0.125)	S (0.25)	91	R (64)	R (16)	S (1)
41	S (2)	S (0.0625)	S (0.0313)	92	S (0.25)	S (0.0313)	R (4)
44	R (64)	R (8)	R (4)	94	R (64)	R (2)	R (8)
47	S (0.25)	S (0.0313)	S (0.0313)	95	S (8)	R (1)	R (4)
49	R (64)	R (4)	S (1)	98	S (4)	R (2)	R (8)
51	R (64)	R (2)	S (1)	99	R (64)	R (8)	R (16)
53	R (64)	R (16)	R (8)	100	R (64)	R (16)	R (16)
54	R (64)	R (16)	SDD (2)	101	S (8)	R (2)	R (8)
55	S (1)	S (0.125)	SDD (2)	105	S (2)	S (0.125)	R (8)
57	S (2)	R (1)	R (4)	163	S (2)	S (0.0625)	R (4)
59	R (64)	R (1)	S (1)	170	S (0.25)	S (0.0313)	R (8)

MIC minimum inhibitory concentration, *FCA* fluconazole, *ITR* itraconazole, *VRC* voriconazole, *R* resistance, *S* sensitive, *SDD* susceptible dose dependent

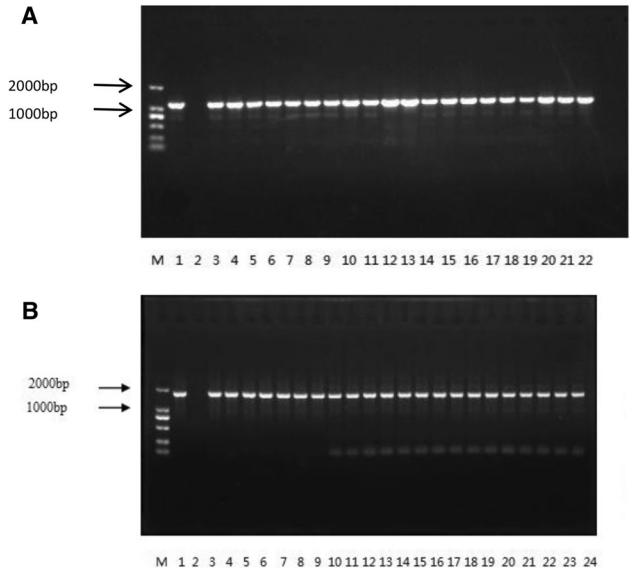

Fig. 1 Electrophoretogram of *ERG3* (**a**) and Efg1 (**b**) in *Candida albicans*. M: DNA marker; lane1: *ERG3* specific amplified fragment of 1161 bp in standard strains; lane 2: blank; lane 3–22: *ERG3* specific amplified fragment of 1161 bp in clinical strains. **b** M: DNA marker; lane1: Efg1 specific amplified fragment of 1653 bp in standard strains; lane 2: blank; lane 3–22: Efg1 specific amplified fragment of 1653 bp in clinical strains

ITR/VRC resistant strains. There was no missense mutation in the susceptible strains (Table 2).

The Efg1 sequencing results showed that among 50 *C. albicans* strains, 40 strains were sequenced successfully and 38 strains had base mutations. There were 1 missense mutation sites [C256T (V86I)], 1 nonsense mutation site [G130A (R44Stop)], 12 silent mutation sites (A150T, A165C, G210A, G267A, G279A, A285T, A744C, A786G, T954A, C1071T, A1055C, and A1317G), and 1 termination codon mutated to codon encoding an amino acid [A1174G (Stop392R)]. There was no mutation for standard strain ATCC11006. A total of 6 strains showed missense mutation, one of which was ITR resistant strain and five of which were FCA/ITR/VRC susceptible strains. There was no V86I mutation for cross resistant strain (Table 3). There were V86I mutations for ITR resistant strain and susceptible strains, and 5 of 16 susceptible strains (mutation rate: 31.3%) and one of 24 resistant strains (mutation rate: 4.2%) showed V86I mutations. Fisher's exact probability test suggested that there was significant difference for mutation rate between ITR resistant strain and susceptible strains ($P < 0.05$).

Correlation Between Expression Levels of ERG3 as Well as Efg1 and Drug Resistance

As shown in Fig. 2, the amplification curves showed a typical S-type fluorescence quantitative kinetic curve, and there was only one a single peak at the melting temperature (Tm) and no impurity peak. Target genes of all strains were amplified.

The results of qRT-PCR (Fig. 3) showed that the mRNA levels of *ERG3* gene in FCA, ITR, VRC, or cross-susceptible strains were higher than those in FCA, ITR, VRC, or cross-resistant strains (2.01 ± 0.60 vs. 0.59 ± 0.36, $P = 0.000$; 2.21 ± 0.52 vs. 0.73 ± 0.43, $P = 0.000$; 2.10 ± 0.57 vs. 0.66 ± 0.40, $P = 0.000$; 2.54 ± 0.39 vs. 0.31 ± 0.21, $P = 0.000$).

In addition, the results of qRT-PCR (Fig. 4) showed that the mRNA levels of Efg1 in FCA, ITR, VRC, or cross-resistant strains were higher than those in FCA, ITR, VRC, or cross- susceptible strains (3.86 ± 4.20 vs. 0.64 ± 0.32, $P = 0.002$; 3.26 ± 3.88 vs. 0.51 ± 0.23, $P = 0.001$; 3.53 ± 4.04 vs. 0.58 ± 0.28, $P = 0.001$; 5.75 ± 5.01 vs. 0.35 ± 0.76, $P = 0.003$).

The results of correlation analysis showed that there was a significant linear negative correlation between *ERG3* and Efg1 mRNA expression ($r = -0.614$, $P < 0.001$).

Discussion

Antifungal azole drugs are widely used to treat *Candida* infections. The molecular mechanisms underlying azole resistance in *C. albicans* are needed for patient management. Mutations or over-expression in target enzyme genes of ergosterol synthetic pathways (e.g. *ERG11*) were significantly associated with azoles-resistance in *C. albicans* [23]. In the present study, the *ERG3* sequencing results showed that there were 2 missense mutation sites [C657G (W219C) and C1055T (R352H)], 1 nonsense mutation site [C309T (W103Stop)], 4 silent mutation sites (T342G, T435C, C441T, and T1047C), and 1 termination codon mutated to codon encoding an amino acid [T384C (Stop128 W)], while 1 missense mutation sites [C256T (V86I)], 1 nonsense mutation site [G130A (R44Stop)], 12 silent mutation sites (A150T, A165C, G210A, G267A, G279A, A285T, A744C, A786G, T954A, C1071T, A1055C, and A1317G), and 1 termination codon mutated to codon encoding an amino acid [A1174G (Stop392R)] were found in Efg1. Furthermore, the mRNA levels of *ERG3* gene in FCA, ITR, VRC, or cross-susceptible strains were higher than those in FCA, ITR, VRC, or cross-resistant strains, while for the mRNA levels of Efg1, susceptible strains were lower than resistant strains. Besides, there was a significant linear negative correlation between *ERG3* and Efg1 mRNA expression.

In the present study, the *ERG3* sequencing results showed that 3 strains were missense mutation, 2 strains were W219C and 1 strain was R352H, and these 3 strains were FCA/ITR/VRC resistant strains. Missense mutation

Table 2 Base mutation sites and amino acid replacement for ERG3 in *Candida albicans* strains

Strains (ZY)	Susceptible to FCA/ITR/VRC	Base mutation sites	Amino acid replacement
4	FCA/ITR/VRC	T384C/T435C/C441T/T1047C	No
6	FCA/ITR/VRC	C309T/T435C/C1055T[a]	R352H
7	ITR	C309T	No
8	FCA/ITR/VRC	C309T	No
12	S	C309T	No
15	S	C309T	No
16	ITR	C309T/T384C	No
18	FCA/ITR	C309T	No
19	FCA/ITR	C309T	No
22	FCA/ITR	C309T	No
25	S	C309T/T384C/T435C/C441T	No
27	S	C309T/T435C	No
28	FCA/ITR/VRC	T342G/T384C/T435C/C441T/C657 G[b]	W219C
32	S	C309T	No
33	S	C309T	No
41	S	C309T/T384C/C441T/T435C/T1047C	No
53	FCA/ITR/VRC	C309T/T435C	No
54	FCA/ITR	C309T	No
55	S	C309T	No
57	ITR/VRC	C309T	No
59	FCA/ITR	C309T	No
60	S	C309T	No
61	VRC	C309T/T384C/T435C/C441T	No
62	VRC	C309T/T435C	No
63	FCA/ITR/VRC	T384C/T435C/C441T/T1047C	No
64	VRC	T384C/T435C/C441T/T1047C	No
66	S	C309T	No
72	S	C309T	No
77	FCA/ITR/VRC	C309T	No
78	FCA/ITR	C309T	No
79	FCA/ITR	C309T	No
80	VRC	C309T	No
89	FCA/ITR/VRC	T342G/T384C/T435C/C441T/C657 G[b]	W219C
90	S	C309T/T384C/T435C/C441T	No
91	FCA/ITR	C309T/T435C	No
92	VRC	C309T/T435C	No
94	FCA/ITR/VRC	C309T	No
95	ITR/VRC	C309T	No
163	VRC	C309T	No
170	VRC	C309T	No

W (tryptophan), C (cysteine), R (arginine), H (histidine); FCA/ITR/VRC: resistant to FCA, ITR, and VRC; FCA/ITR: resistant to FCA and ITR; ITR/VRC: resistant to ITR and VRC; S: susceptible to FCA, ITR, and VRC

[a,b]Missense mutation sites. a: C1055T; b: C657G

was found in FCA/ITR/VRC resistant strains but not in susceptible strains, suggesting that W219C and R352H might be associated with azole resistance. However, the sample size was small and only 3 strains had missense mutation, which might affect the accuracy of the results. One study found that some single mutation, such as D19E,

Table 3 Base mutation sites and amino acid replacement for Efg1 in *Candida albicans* strains

Strains (ZY)	Susceptible to FCA/ITR/VRC	Base mutation sites	Amino acid replacement
4	FCA/ITR/VRC	A165C/G210A/A1055C	No
6	FCA/ITR/VRC	G130A/A150T/G210A/G267A/A786G/A1174G	No
7	ITR	G210A/C256T[a]	V86I
12	S	G210A/C256T[a]	V86I
16	ITR	G130A/A150T/G267A/A1174G	No
18	FCA/ITR	G210A	No
19	FCA/ITR	T878G/T954A/C1071T	No
22	FCA/ITR	G210A/A1317G	No
27	S	G210A/A786G/T954A	No
28	FCA/ITR/VRC	G210A/G279A/A285T/A786G/A1174G	No
32	S	G210A/C256T[a]	V86I
33	S	T878G/T954A/C1071T	No
41	S	C256T[a]/A744C	V86I
44	FCA/ITR/VRC	G210A	No
47	S	G130A/A150T	No
49	FCA/ITR	A150T/G267A/A1174G	No
51	FCA/ITR	A1174G	No
53	FCA/ITR/VRC	T878G/T954A/C1071T	No
57	ITR/VRC	G210A/A1317G	No
61	VRC	G267A/A1174G	No
62	VRC	G130A/A150T/G267A/A1174G	No
63	FCA/ITR/VRC	A1174G	No
64	VRC	A285T/A786G	No
66	S	G210A/C256T[a]	V86I
72	S	G210A/C256T[a]	V86I
78	FCA/ITR	C1071T	No
80	VRC	G210A/A1317G	No
88	VRC	G210A/A786G/T954A	No
91	FCA/ITR	A150T/A1174G	No
94	FCA/ITR/VRC	G210A/A786G/T954A	No
95	ITR/VRC	G210A/T878G/T954A/C1071T	No
98	ITR/VRC	T878G/T954A	No
99	FCA/ITR/VRC	A150T/G267A/A1174G	No
100	FCA/ITR/VRC	G210A/A1317G	No
101	ITR/VRC	A150T/A1174G	No
105	VRC	G210A/A786G/T954A	No
163	VRC	G210A/A1317G	No
170	VRC	G210A/A1317G	No

V (valine), I (isoleucine); FCA/ITR/VRC: resistant to FCA, ITR, and VRC; FCA/ITR: resistant to FCA and ITR; ITR/VRC: resistant to ITR and VRC; S: susceptible to FCA, ITR, and VRC

[a]Missense mutation site C256T

W228Stop, and L266Stop, did not cause azole resistance [24]. Thus, whether single mutations will involve in azole resistance remain to be proved. W219C and R352H were single mutations. To sum up, further studies about the correlation between W219C as well as R352H and azole resistance are needed.

For Efg1 sequencing, 6 strains showed missense mutation [C256T (V86I)], one of which was ITR resistant strain and five of which were FCA/ITR/VRC susceptible strains.

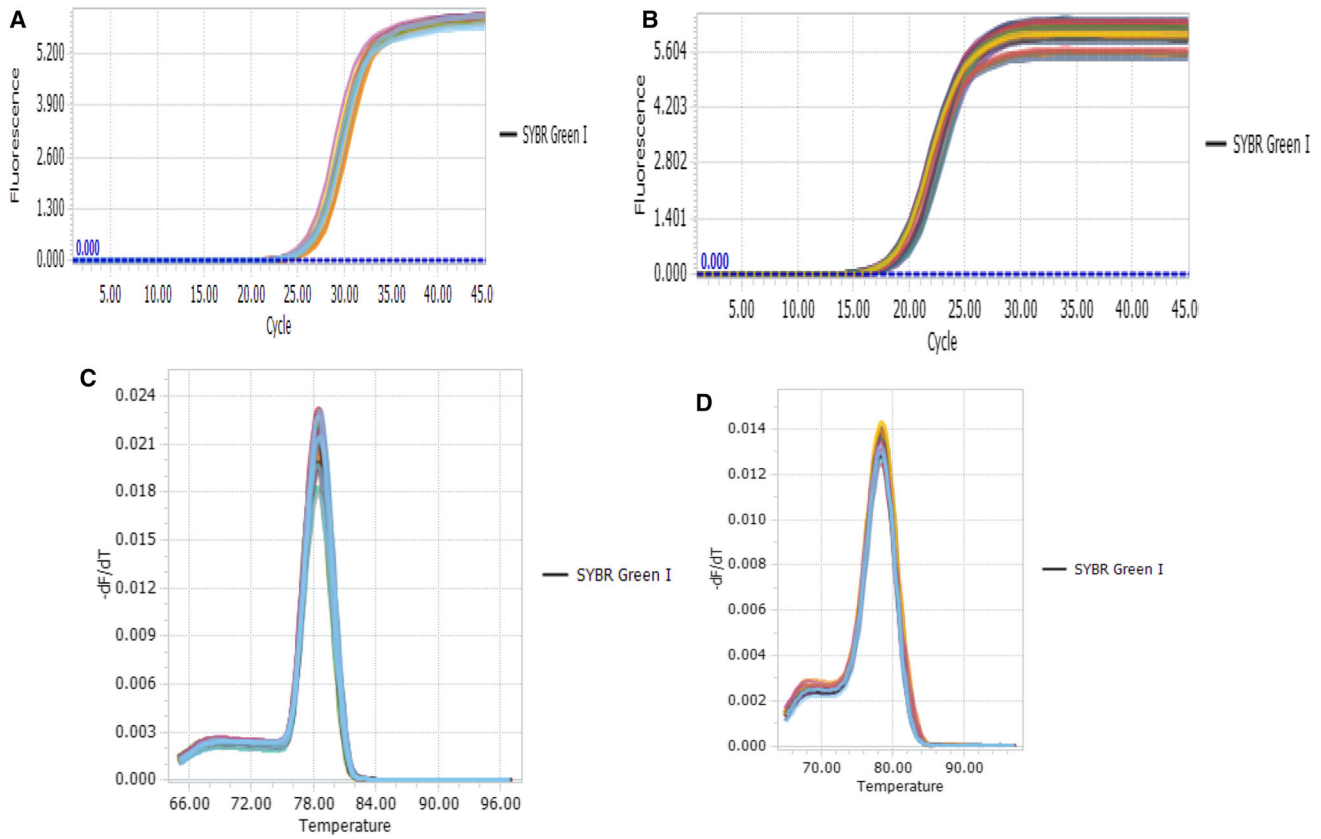

Fig. 2 The amplification curves and dissolution curves of ERG3 and Efg1. **a** The amplification curves of ERG3; **b** The amplification curves of Efg1; **c** The dissolution curves of ERG3; **d** The dissolution curves of Efg1

There were V86I mutations for ITR resistant strain and susceptible strains, and 5 of 16 susceptible strains (mutation rate: 31.3%) and one of 24 resistant strains (mutation rate: 4.2%) showed V86I mutations. The mutation rate of susceptible strains was higher than that of ITR resistant strains. Furthermore, no V86I mutations were found for FCA/VRC resistant strains. We speculated that V86I mutations might increase the susceptibility of azole. The possible mechanisms might be that the substitution of amino acids led to the change of the spatial structure of the protein encoded by Efg1, affecting the function of protein and even inactivating it, or the substitution of amino acids down-regulated the expression levels of Efg1. However, because of small sample size, fewer mutant strains, and factors other than Efg1, we could not get direct conclusion for the correlation between V86I mutations and azole resistance. Thus, further studies are needed to investigate the relationship between V86I mutations and azole resistance.

A study of Sanglard et al. [25] showed that *ERG3* deletion mutations were azole resistant in *C. albicans*. The possible mechanisms might be as follows: over-expression of *ERG3* increased the synthesis of sterol Δ5,6-desaturase, and then more accumulated nontoxic 14α-methylsterol intermediates was transformed into cytotoxic 14α-methylergoster-824 (28)—diene-3 β, 6 α-diol, increasing the synthesis of toxic steroids. As a result, the cell membrane of fungi was destroyed, accelerating their death. Namely, the susceptibility to azole increased. In addition, Akins et al. [18] showed that *ERG3* inactivation would confer azole resistance and indicated that wildtype *C. albicans* strains exposed to azoles typically accumulate the toxic sterol, whereas *ERG3* mutants accumulate mostly 14a-methylfecosterol after azole exposure. Another study has showed that the *ERG3* expression was increased in FCA-resistant *C. parapsilosis* and decreased in ITR-resistant and amphotericin B-resistant *C. parapsilosis* [17]. In our study, the mRNA levels of *ERG3* gene in FCA, ITR, VRC, or cross-susceptible strains were higher than those in FCA, ITR, VRC, or cross-resistant strains. Combined with our results, *ERG3* mutation might be associated with azoles-resistance in *C. albicans*, and overexpression of *ERG3* was speculated to increase the susceptibility of *C. albicans* to azoles. However, the mechanisms of *ERG3* down-regulation in azole-resistant *C. albicans* need to be further explored.

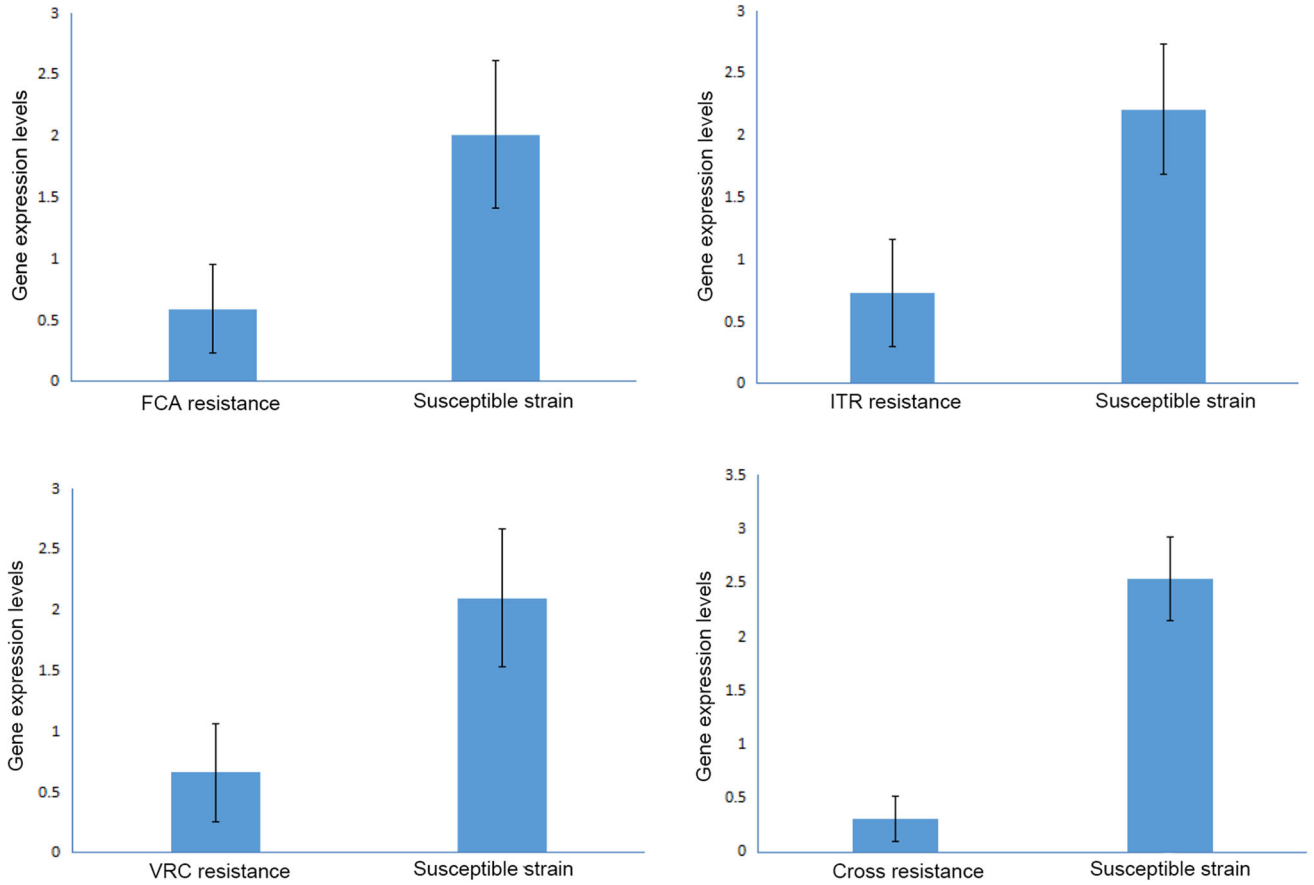

Fig. 3 The mRNA levels of ERG3 gene in FCA, ITR, VRC, and cross-resistant strains and the corresponding susceptible strains

Furthermore, the mRNA levels of Efg1 in FCA, ITR, VRC, or cross- resistant strains were higher than those in susceptible strains, and there was a significant linear negative correlation between *ERG3* and Efg1 mRNA expression. Prasad et al. [26] indicated that Efg1 level was associated with drug susceptibility in *C. albicans*. Thus, we speculated that overexpression of Efg1 contributed to azole resistance in *C. albicans*. The possible mechanisms might be as follows: on the one hand, overexpression of Efg1 affected the content of oleic acid and ergosterol in cell membrane, weakening the membrane fluidity and promoting the formation of biofilm, which strengthened the self-defense system of the strain and weakened the passive diffusion of the drug. As a result, drug resistance of strains increased. On the other hand, overexpression of Efg1 inhibited the expression of *ERG3*, and lower expression of Efg1 led to drug resistance. Furthermore, one study found that Efg1 deletion strain had higher susceptibility to drugs that targeting ergosterol and its metabolites [19], which also proved this point.

There are some limitations in this study. The W219C and R352H were newly discovered mutation sites in this experiment, but whether the mutation sites will involve in azole resistance remains to be proved. In addition, the correlation between ERG3 and Efg1 as well as azole-resistance should be further explored by gene knockdown or site-directed mutagenesis.

In conclusion, *ERG3* mutation was associated with azoles-resistance in *C. albicans*, and overexpression of *ERG3* might increase the susceptibility of *C. albicans* to azoles. Efg1 mutation might increase the susceptibility to azoles, while overexpression of Efg1 might increase azoles-resistance in *C. albicans*. Furthermore, there was a significant linear negative correlation between *ERG3* and Efg1 mRNA expression in *C. albicans*. The results in this study may help to improve our understanding of azole-

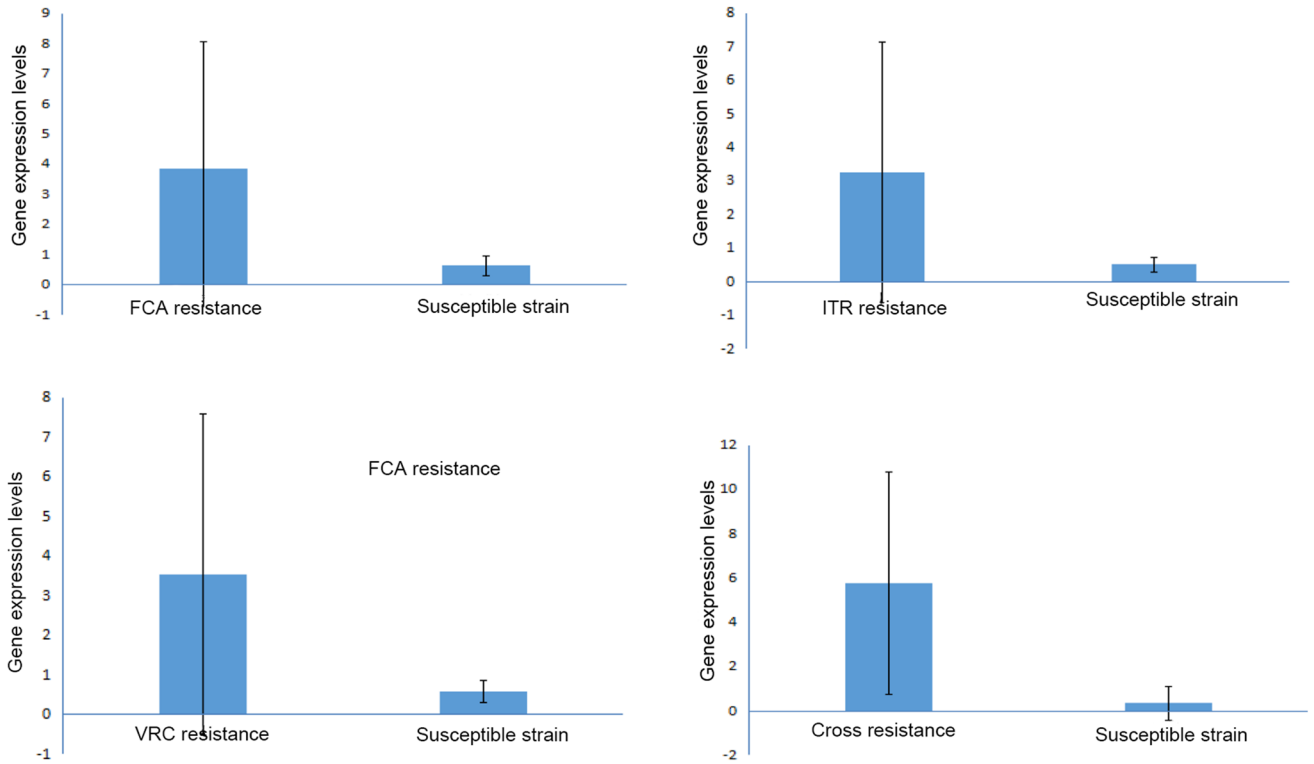

Fig. 4 The mRNA levels of Efg1 gene in FCA, ITR, VRC, or cross- resistant strains and the corresponding susceptible strains

resistant mechanism of *C. albicans* and design new strategies for antifungal therapy in VVC.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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