

Erg11 in drug-resistant of c. krusei



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ERG11 mutations and up-regulation in clinical itraconazole-resistant isolates of *Candida krusei*

1. Some *C. krusei* isolates were resistant to the antifungal drugs.
2. Mutations including T939C, T642C and A756T were found in *ERG11* of *C. krusei*.
3. *ERG11* was highly expressed in resistant *C. krusei* strains.

Abstract

Objective We aimed to provide with light for better understanding of *ERG11* gene in drug-resistance mechanisms in *Candida krusei* (*C. krusei*).

Methods *C. krusei* strains were isolated from patients in the Dermatology from Jan 2010 to May 2013. Susceptibility assays, including 5-fluorocytosine (5-FC), amphotericin B (AMB), voriconazole (VCR), fluconazole (FLC) and itraconazole (ITR), was performed by broth microdilution method according to the National Committee for Clinical Laboratory Standards M27-A2. Isolates were divided into susceptible strains and resistant strains based on their susceptibility to ITR. Mutations in the *ERG11* gene sequence were detected using PCR amplification and gene sequencing. Expression levels of *ERG11* were measured by real-time PCR. Differences of *ERG11* expression levels between susceptible strains and resistant strains were compared by two-tailed Student *t* test.

Results A total of 15 *C. krusei* strains were obtained, among which 8.0, 6.0 and 3.0% were resistant to FCA, ITR and 5-FC, respectively, whereas all isolates were found to be susceptible to AMB and VRC. Three synonymous codon substitutions were found in *ERG11* among all the *C. krusei* strains,

including T939C, T642C and A756T. Expression level of *ERG11* was significantly higher in resistant *C. krusei* strains (1.34 ± 0.08) than that in susceptible *C. krusei* strains (0.94 ± 0.14) ($t = 3.74$, $P < 0.05$).

Conclusions Our study demonstrates that point mutations accompanied with the overexpression of *ERG11* might be involved in the molecular mechanisms of drug resistance in *C. krusei*.

Keywords: *ERG11*; drug resistance; *Candida krusei*; mutation; overexpression.

1. Introduction

Candida species are pathogenic microorganisms for systemic and local opportunistic infections and the fourth leading cause of nosocomial bloodstream infections worldwide [1]. In recent years, an increasing number of infections due to *Candida krusei* has been witnessed [2]. *C. krusei* generally causes infections among immunocompromised patients, especially those suffering from Human immunodeficiency virus (HIV)-acquired immune deficiency syndrome and hematological malignancies [3]. Mortality rates among such patients with *C. krusei* fungemia are reported to be as high as 60-80% [4]. Moreover, the increasing use of immunosuppressive drugs has inevitably increased the risk of *C. krusei* infection, and *C. krusei* has ranked in fifth place among all the species of *Candida* [5, 6].

C. krusei has been regarded as a multidrug-resistant (MDR) fungal pathogen by the reason of its intrinsic resistance to fluconazole (FLC) as well as its considerable reduction in susceptibility to flucytosine and amphotericin B (AMB) [2]. Resistance to these antifungal drugs is a major problem among

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patients with acute myelogenous leukemia, neutropenia and/or critically ill, since these drugs are frequently used for prophylaxis of *C. krusei* infection [7]. Therefore, a better understanding of resistance mechanisms in *C. krusei* was urgently needed to effectively prevent and control infections caused by *C. krusei*.

It has been reported that multiple mechanisms are involved in drug resistance in *C. krusei*, including overexpression of several genes such as multidrug transporters (encoded by CDR1, CDR2, and MDR1), which lead to decreased intracellular accumulation of FLC. Nowadays, an increasing evidence proposed that alterations and overexpression of the *ERG11* gene, which codes the target enzyme cytochrome P450 lanosterol 14 α -demethylase, are major resistance mechanism in *C. krusei* [8, 9]. However, the scarce information available regarding *ERG11* gene mutation and gene expression in the drug-resistant strains makes *C. krusei* resistance to azoles poorly understood [10].

In the present study, we evaluated the susceptibility profiles of 15 *C. krusei* isolates, investigated the potential alterations in the *ERG11* gene sequence of *C. krusei* strains, and further detected the differentially expressed levels of *ERG11* between susceptible and resistant isolates of *C. krusei*. We aimed to provide with light for better understanding of molecular mechanisms in drug resistance of *C. krusei*.

2. Methods

2.1 Fungal strains and media

The strains used in this study were isolated from patients in the Dermatology of the Second Hospital of Shanxi Medical University from Jan 2010 to May 2013. The standard strain, *Candida krusei* ATCC 6258, was purchased from fungi and fungal disease research center of Peking University and included in each test run for quality control. *C. Krusei* were maintained on agar YPD medium (2% peptone, 1% yeast extract, 2% dextrose) and stored in our Fungi Laboratory. RPMI 1640 medium with L-glutamate (Sigma, St. Louis, Mo.) was used as recommended for susceptibility assays and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS).

2.2 Susceptibility assays

The susceptibility assays of the isolates was performed in 96-well polystyrene microtiter plates by broth microdilution method described in the National Committee for Clinical Laboratory Standards M27-A2 (NCCLS) [11]. The antifungal drugs including 5-fluorocytosine (5-FC), amphotericin B (AMB), voriconazole (VCR), fluconazole (FLC) and itraconazole (ITR), were obtained from their respective manufacturers and used for susceptibility assays. MIC was defined as the concentration of the drug that reduced the fungus growth by 80% compared to that grown in the absence of the drug. The interpretive criteria for susceptibility to the above drugs were published by the NCCLS and listed in Table 1. Samples were divided into susceptible strains and resistant strains based on their susceptibility to ITR.

2.3 PCR amplification and sequence alignment of *ERG11* gene

To amplify *ERG11* gene, genomic DNA was firstly isolated from *C. krusei* cells using UNIQ-10 Column Genomic DNA Isolation Kit (Sangon Biotech,

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Shanghai, China) according to the manufacturer's instruction and used as a template for amplification of the *ERG11* genes. Specific primers of *ERG11* (Table 2) were designed by Primer 3 [12], based on the available sequence information of *C. Krusei ERG11* gene (GI: 163311561) at the National Center for Biotechnology Information (NCBI). The PCR amplification of *ERG11* gene was conducted using 2 µl of genomic DNA, 2 µl specific forward and reverse primers (50 µmol/L) and Taq PCR Master Mix (TIANGEN, Beijing, China). The PCR condition was set as denaturation for 5 min at 94 °C, followed by 35 cycles: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final step of elongation (72 °C for 8 min). PCR products were then separated and sized on a 1.5% agarose gel by electrophoresis, and visualized under UV light after staining with ethidium bromide. Successfully amplified PCR products were sent for sequencing (Invitrogen, Shanghai, China). To verify the point mutations, sequencing results were aligned with the reference sequence of *C. Krusei ERG11* gene (GI: 163311561) using BLAST (Basic Local Alignment Search Tool) program in NCBI.

2.4 Real-time PCR analysis

For quantitative real-time PCR analysis, total RNA was extracted from *C. krusei* cultures with an Yeast RNAiso Kit (TaKaRa, Dalian, China) and reversely transcribed to cDNA with PrimeScript RT Master Mix (TaKaRa, Dalian, China) according to the instructions of the manufacturer. For the *ERG11* target genes and *GAPDH* reference gene, a primer pair were designed with Primer 5.0 program (Table 2). Real-time PCR was processed with a 25-µl volume containing the following reagents: 12.5 µl of SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China), 2 µl of total RNA sample, 1 µl of each primer

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pair at a concentration of 10 µM and distilled water up to the final volume. Samples were subjected to an initial step at 95 °C for 5 min, followed by 40 cycles each of which consisted of 10 s at 95 °C and 30 s at 60 °C. Melting curves were recorded every 5 s during the 65-95 °C by PCR amplifier. Fluorescence data (Ct) in each reaction were collected and were analyzed with the Rotor-Gene Q Series Software 2.0.2 software. A $2^{-\Delta\Delta Ct}$ algorithm was applied to analyze relative expression levels of *ERG11* at susceptible strains and resistant strains.

2.5 Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc. Chicago, IL, USA). All data were presented as mean ± standard deviation (SD). The two-tailed Student *t* test was conducted to compare the differences of *ERG11* expression levels between susceptible strains and resistant strains. A *p*-value < 0.05 was regarded as statistically significant.

3. Results

1. Antifungal susceptibilities of *C. krusei* isolates

A total of 15 *C. krusei* strains were isolated from clinical samples during Jan 2010 to May 2013 in our laboratory, among which 14 isolates were from urine and 1 from hydrothorax (Table 3). Table 4 shows the rates of azole resistance for these *C. krusei* isolates. Among 15 *C. krusei* isolates, 8.0, 6.0 and 3.0% were resistant to FCA, ITR and 5-FC, respectively, whereas all isolates were found to be susceptible to AMB and VRC. Ultimately, by using an MIC ≥ 1 µg/ml to define resistance to the investigational ITR, the 15 isolates included 6 that were resistant and 9 that were susceptible.

2. *Mutational analysis in ERG11 of C. krusei isolates*

DNA fragment with consistent length was successfully amplified from *C. krusei* isolates (Figure 1). Sequence alignment showed three synonymous codon substitutions in *ERG11* among all the *C. krusei* strains, including T939C, T642C and A756T (Figure 2). Among the three synonymous mutations, T642C and A756T only presented in ITR-resistant strains, while T939C in susceptible as well as resistant *C. krusei* strains. Nevertheless, no point mutation was observed in the standard strain.

3. *Different expression levels of ERG11 gene in susceptible and resistant C. krusei strains*

Real-time PCR was performed to verify the varied expression levels of *ERG11* in ITR-susceptible and ITR-resistant *C. krusei* strains. Result showed that mRNA transcription level of *ERG11* was significantly higher in resistant *C. krusei* strains (1.34 ± 0.08) than that in susceptible *C. krusei* strains (0.94 ± 0.14) ($t = 3.74, P < 0.05$).

4. Discussion

With the drug-resistance character, *C. krusei* has emerged as one of the leading agents causing candidemia, especially in immunocompromised patients [13]. Previous studies have shown the crucial role of *ERG11* gene in FLC-resistant clinical isolates of *Candida* species, while the molecular mechanism specially in *C. krusei* remains unclear. Herein, we evaluated the susceptibility profiles of 15 *C. krusei* isolates, subsequently searched for mutations in the *ERG11* gene sequence of *C. krusei* using PCR amplification and gene sequencing, and further detected the differentially expressed

levels of *ERG11* between susceptible and resistant isolates by real-time PCR. We found three synonymous codon substitutions in *ERG11* of *C. krusei* which have not yet been described previously. Among the three point mutations, T642C and A756T only presented in ITR-resistant strains, while T939C were also presented in ITR-susceptible strains. Moreover, mRNA transcription level of *ERG11* was significantly higher in ITR-resistant *C. krusei* strains than that in ITR-susceptible *C. krusei* strains.

It is reported that point mutations in the *ERG11* gene can lead to conformational changes which may affect the affinity of the drug with the target, but not influence the enzyme function in ergosterol biosynthesis [14]. Many studies have been focused on the *ERG11* alteration in *Candida* species. Ricardo *et al.* [9] found two different types of mutations by sequencing the *C. krusei* *ERG11* gene, including a heterozygous alteration at 1,389 bp (T→C) presented in all of the susceptible and resistant *C. krusei* strains in their study, and a missense mutation in two strains at position 418 bp (T→C) which yields a Tyr→His amino acid change. Tavakoli *et al.* [15] displayed a heterozygous polymorphism at position 939 (T→C) in *ERG11* coding region, and speculated that this polymorphism might play a key role in the transcriptional regulation of genes and be involved in the processes of ergosterol biosynthesis. Sionov *et al.* [16] have identified a single missense mutation at amino acid 145 in the *ERG11* of *C. neoformans* strain isolated from an FLC-treated patient, and verified that this mutation was sufficient to lead high FLC resistance. In the present study, we discovered three novel synonymous codon substitutions in *ERG11* of *C. krusei*, among which T642C

and A756T only presented in ITR-resistant strains. These alteration in *ERG11* might be involved in the resistance mechanism of *C. krusei*.

Many studies have also been designed to explore the exact molecular mechanism behind the *ERG11* up-regulation in response to azoles and other antifungal drugs [14, 17, 18]. A well-characterized matched pair of FLC-susceptible and FLC-resistant *C. albicans* isolates was analyzed and the resistant strains were found mainly associated with up-regulation of *ERG11* gene [14]. Henry *et al.* [17] demonstrated that treating *C. krusei* with the triazole FLC at a concentration of 2 to 9 mg/ml could result in four- to five-fold increase in *ERG11* RNA levels. Accompanied with previous reports, they hypothesized that the upregulation of *ERG11* gene contributed at least partly to the ability of *C. krusei* to tolerate azole [19-21]. The overexpression of *ERG11* was also directly shown to confer FLC resistance in *S. cerevisiae* [18]. In accordance with previous studies, our results reflected that mRNA transcription level of *ERG11* was found to be significantly higher in ITR-resistant *C. krusei* strains compared with that in ITR-susceptible *C. krusei* strains. The increased production of 14 α -demethylase was reported to exceed the inhibitory capacity of the antifungal drugs. Therefore, we speculated that *ERG11* gene overexpression might be relevant in the drug resistance in *C. krusei*.

In conclusion, there were synonymous codon substitutions observed in *ERG11* of *C. krusei*. These point mutations accompanied with the overexpression of *ERG11* might be involved in the molecular mechanisms of drug resistance in *C. krusei*.