Mechanisms of azole resistance in 52 clinical isolates of Candida tropicalis in China

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Objectives: To explore the mechanisms underlying azole resistance in clinical isolates of Candida tropicalis collected in China by focusing on their efflux pumps, respiratory status and azole antifungal target enzyme.

Methods: Fifty-two clinical isolates of C. tropicalis were collected from five hospitals in four provinces of China and antifungal susceptibility tests were performed. Rhodamine 6G and rhodamine 123 were used to investigate the efflux pumps and respiratory status, respectively. Transporter-related genes CDR1 and MDR1, mitochondrial gene CYTb, as well as ERG11, were quantified by real-time RT–PCR. Meanwhile, ergosterol content was analysed using liquid chromatography-mass spectrometry/mass spectrometry. An ERG11-deficient (erg11Δ) Saccharomyces cerevisiae strain was generated to study the function of mutations in ERG11.

Results: MICs showed that 31 isolates were resistant to at least one type of azole antifungal. Flow cytometry using rhodamine 123 revealed increased respiration for the azole-resistant isolates, but CYTb was not overexpressed. No significant difference in the efflux of rhodamine 6G was found, which was consistent with the comparable expression levels of CDR1 and MDR1. In contrast, the azole-resistant isolates overexpressed ERG11 and showed increased ergosterol content. Moreover, the isolates resistant to three azole antifungals expressed higher levels of ERG11 mRNA than those resistant to only fluconazole or itraconazole. Two ERG11 mutations, Y132F and S154F, were found in azole-resistant isolates and could be shown to mediate azole resistance by expression in S. cerevisiae.

Conclusions: The up-regulation and mutations of ERG11 mediate azole resistance of C. tropicalis.

Keywords: antifungals, ERG11, ergosterol

Introduction

Candida species are important opportunistic pathogens that cause several diseases in immunocompromised patients. Although Candida albicans remains the most common Candida species encountered, the morbidity and mortality caused by non-albicans Candida species are increasing. According to a survey of invasive candidiasis in the USA,1 the rate of isolation of C. albicans declined between 1997 and 2003, whereas that of Candida parapsilosis and Candida tropicalis increased from 4.2% to 7.3% and from 4.6% to 7.5%, respectively. C. tropicalis, which ranked second among non-albicans Candida species, tends to cause fungaemia among patients with cancer, neutropenia, malignancy or bone marrow transplantation.2–4 The proportion of C. tropicalis in India, Brazil and Taiwan seems to be higher than in other regions.5,6 Furthermore, C. tropicalis causes invasive disease in neonatal intensive care units (ICUs) through cross-contamination and has a slight tendency to progress from colonization to infection.7

Antifungal resistance is a serious issue for treating infections caused by Candida spp. C. tropicalis is intrinsically resistant to 5-fluorocytosine and has a moderate level of fluconazole resistance,8 but the species will quickly develop resistance to azole antifungals after exposure to these compounds. Azole resistance among Candida spp. caused by mutations, the overexpression of the drug target and the up-regulation of transporters is increasingly common and alternative mechanisms, such as biofilm formation and mitochondrial defects, have also been recently documented. The cytochrome P450 lanosterol 14α-demethylase encoded by the ERG11 gene is the primary target forazole antifungals.9 ERG11 overexpression and mutations have been reported to result in azole antifungal resistance; the amino acid substitutions changing the affinity of the enzyme. Moreover, at least two families of multidrug transporters, the
ABC (ATP-binding cassette) transporter family and the major facilitator superfamily (MFS), are involved in azole resistance. The up-regulation of CDR1 and MDR1 genes, encoding the efflux proteins of these two transporter families, respectively, has been reported to contribute to the active efflux of azole drugs in several Candida spp. Azole resistance caused by respiratory deficiency, which primarily decreases the production of ATP and reactive oxygen species (ROS) in mitochondria, has been reported in both C. albicans and Candida glabrata. However, little is known to date about the mechanisms of azole resistance in C. tropicalis.

In this study, we set out to explore the mechanisms underlying azole resistance in 52 clinical isolates of C. tropicalis collected in China by focusing on their efflux pumps, respiratory status and azole antifungal target enzyme.

Materials and methods

Strains and medium

A total of 52 C. tropicalis isolates were collected from five hospitals in Shanghai, Jiangsu, Guangdong and Anhui Provinces in China. The isolates were identified using standard biochemical and microbiological procedures, including assessment of the carbohydrate assimilation pattern (API 21C, Paris, France) and colony colour in chromogenic medium (Chromagar, Paris, France). All of the isolates were maintained by biweekly passages on YPD agar containing 10 g/L yeast extract, 21 g/L peptone and 21 g/L dextrose. Escherichia coli strain DH5α and Luria–Bertani (LB) medium were used for the transformation and plasmid DNA preparation. Saccharomyces cerevisiae W303-1A (MATα SUC2 ade2 can1 his3 leu2 trp1 ura3) was generously provided by Professor Alistair Brown. The transformants were selected on synthetic defined medium with dextrose and the appropriate supplements.

Antifungal susceptibility tests

The MICs of fluconazole, itraconazole and voriconazole (Pfizer Pharmaceuticals, Shanghai, China) for the 52 C. tropicalis strains were determined using the broth microdilution method according to CLSI M27-A3 standard guidelines. Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were used as the quality control isolates. The range of fluconazole, itraconazole and voriconazole was from 0.125 to 64 mg/L, 0.031 to 16 mg/L and 0.007 to 4 mg/L, respectively. MIC breakpoints of fluconazole and voriconazole were determined at 24 h according to reports by Pfaller et al. and those of itraconazole were determined at 48 h according to M27-A3. The MICs of these three azole antifungals for resistant isolates were ≥8, ≥1 and ≥1 mg/L, respectively.

Flow cytometric analysis of the efflux of rhodamine 6G

The activity of efflux pumps was evaluated using flow cytometry by measuring the efflux of rhodamine 6G (Sigma–Aldrich, St Louis, MO, USA), a fluorescent dye that uses the same membrane transporter as azoles in some Candida spp. Yeast cells (10⁸) in YPD were suspended in 2 mL of PBS and incubated at 30°C with constant shaking for 4 h to induce starvation. Rhodamine 6G at 10 μM was then added and incubated for 2 h at 30°C. The uptake of rhodamine 6G was stopped, the cells were washed twice in cold sterile PBS and the fluorescence of the cells was immediately quantified at 535 nm using a Beckman Coulter Epics XL FACScan flow cytometer. Glucose (4 mM) was added and incubated at 30°C for an additional hour. The fluorescence of 20000 cells was then evaluated by FACScan flow cytometry. The fluorescence of the cells incubated without rhodamine 6G served as an unstimulated control. The fluorescence was expressed as geometric mean values and the data presented correspond to fluorescence frequency distribution histograms (relative number of cells versus relative fluorescence intensity, as expressed in arbitrary units on a logarithmic scale). Each experiment was performed in triplicate and a clinical fluconazole-resistant C. glabrata, which has been demonstrated to have an obvious efflux effect in a previous study, was used as a positive control.

Measurement of respiratory capacity

Rhodamine 123 (Sigma–Aldrich) is a fluorochrome that is sensitive to the mitochondrial membrane potential and concentrates specifically in the mitochondria. Therefore we used the method developed by Skowronek et al. to analyse the respiratory status of C. tropicalis, with or without sodium azide (NaN₃). Flow cytometry was performed as described above and each experiment was performed in triplicate.

Quantitative real-time RT–PCR

Total RNA was extracted from isolates grown to the exponential phase in YPD medium using the Yeast RNAiso Reagent Kit (TaKaRa, Tokyo, Japan) according to the instructions of the manufacturer. The total RNA was quantified using a Synergy H1 Microplate Reader (Biotek, Winooski, VT, USA) and reverse transcribed to cDNA using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). The quantitative real-time RT–PCR of CYTb, CDR1, MDR1 and ERG11 was performed using the SYBR Premix Ex Taq Kit (TaKaRa, Tokyo, Japan) and a LightCycler 480 Real-Time PCR System (Roche, Shanghai, China). ACT was used as the internal control. All of the reactions were performed as follows: denaturation at 95°C for 30 s, followed by 40 cycles consisting of 5 s at 95°C and 21 s at 60°C. Each sample was processed in triplicate and a change of 2.5 times was considered to be overexpressed. The primers used in the real-time RT–PCR are described in Table 1.

Ergosterol analysis

Ergosterol was extracted from lyophilized C. tropicalis cells grown to the stationary phase in YPD medium. Methanol (5 mL) was added to 10 mg of dried cells and the cells were sonicated at 60°C for 2 h to dissolve the ergosterol. The sonicated solution was then centrifuged at 10000 × g for an additional hour. The fluorescence of 8 mg/mL ergosterol at 350 nm was used to separate the ergosterol at 35°C using an HPLC System (Shimadzu Inc., Tokyo, Japan). A Venusil XBP Phenyl column (2.1 × 100 mm, 3.5 μm; Agela Technologies, Tianjin, China) was used to separate the ergosterol at 35°C and an elution rate of 0.5 mL/min. A standard curve was constructed for the range 1–20 μg/mL by plotting the signal intensity against the corresponding ergosterol concentration. The concentration of ergosterol in the samples was determined by comparing the peak areas of ergosterol with those of the standard curve. This was accomplished using the Analyst software package (version 1.5.2).

ERG11 amplification and sequencing

C. tropicalis genomic DNA was extracted as previously described and was used as the template for the amplification of the full-length ERG11 gene. The primers used for the amplification and sequencing were designed as previously described.
Disruption, replacement and functional verification of ERG11 in S. cerevisiae

Cloning and expression of ERG11

The ERG11 ORFs of a pair of fluconazole-susceptible and fluconazole-resistant matched C. tropicalis strains, 1a and 1b, were amplified from isolates using primers containing the restriction enzyme sites KpnI and XhoI (Table 1). The amplified KpnI-XhoI fragment was then ligated into the KpnI-XhoI sites of pYES2/CT, which contains the GAL1 promoter and CYC1 terminator. Because S. cerevisiae cannot survive without a functional lanosterol 14α-demethylase, S. cerevisiae W303-1A was first transformed with the cloned plasmid using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA) and grown on selective synthetic dropout-uracil plates (SD-URA) containing galactose and raffinose to induce the expression of ERG11.

Disruption and replacement of ERG11

The promoter-dependent disruption of genomic ERG11 was performed as previously described, with a few modifications. Briefly, a linear TRP1 DNA fragment was amplified from the pY24GAL plasmid using ERG-TRP primers (Table 1) and transformed into the transformants mentioned above. After culturing on selective synthetic dropout-tryptophan plates (SD-TRP) at 30°C for 3–5 days, the isolated clones were selected and cultured overnight in liquid SD-TRP for further screening. The genomic DNA was extracted and amplified using ERGuF-ERGiR and ERGuF-TRPiR primers (Table 1). Information about all of the S. cerevisiae strains used in this study is listed in Table 2.

Functional verification of the ERG11 mutations

The MICs of fluconazole, itraconazole and voriconazole for S. cerevisiae W303-1A, SC1, SC4 and SC5 were determined using the broth microdilution method according to CLSI M27-A3.

Statistical analysis

Data were analysed using SPSS 17.0. Statistical significance was determined using Student’s t-test. Statistical significance was determined as P<0.05.

Results

Azole susceptibility of C. tropicalis

Of the 52 isolates of C. tropicalis, 18 (34.6%) were resistant to fluconazole, 21 (40.4%) were resistant to itraconazole and 4 (7.7%) were resistant to voriconazole. Among these resistant strains,
four isolates were resistant to both fluconazole and itraconazole and another four isolates were resistant to all three azole antifungals (Table S1, available as Supplementary data at JAC Online). Thus 31 isolates were resistant to at least one type of azole antifungal and 21 isolates were susceptible to all three of the azole antifungals.

**Uptake and efflux of rhodamine 6G**

The accumulation and efflux of rhodamine 6G were quantified using flow cytometry to evaluate the function of the yeast transporters. A pair of fluconazole-susceptible and fluconazole-resistant matched *C. tropicalis* (1a and 1b) were isolated from the same patient and their homology was demonstrated by multilocus sequence typing (MLST) and repetitive sequence-based PCR (Rep-PCR) (data not shown). The visual variation of rhodamine 6G for the pair is presented in Figure 1. After a 4 h starvation and 2 h incubation in PBS with fluorochrome, the mean fluorescence intensity of the cells did not change significantly (black line). Moreover, the efflux of rhodamine 6G in the resistant isolate, after the removal of the free dye and an additional incubation of 1 h in PBS with 4 mM glucose, was comparable to that of the susceptible isolate (grey line). The activity of efflux pumps was similar in 31 azole-resistant and 21 azole-susceptible *C. tropicalis* strains.

**Respiratory status of *C. tropicalis***

The fluorescent dye rhodamine 123 was used to evaluate the mitochondrial respiratory status of *C. tropicalis* using flow

![Flow cytometric analysis of rhodamine 6G uptake and efflux](image)

*Figure 1.* Flow cytometric analysis of rhodamine 6G uptake and efflux. The uptake of the fluorochrome was quantified by incubating the cells of fluconazole-susceptible (a) and fluconazole-resistant (b) matched *C. tropicalis* with 10 μM rhodamine 6G at 30°C for 2 h after starvation (black lines). The efflux was evaluated by quantifying the residual fluorescence of the cells after the removal of the free dye and an additional incubation of 1 h in PBS with 4 mM glucose (grey lines). A clinical fluconazole-resistant *C. glabrata* that has been demonstrated to have an efflux effect in a previous study was used as a positive control (c).
cytometry. The results showed that the azole-resistant isolates incorporated more rhodamine 123 than the susceptible ones (Figure 2; \( P, 0.05 \)). Additionally, the fluorescence of the azole-resistant isolates decreased sharply when they were pre-treated with NaN3, but the fluorescence of the susceptible isolates did not change to a great extent (Figure 2). Furthermore, quantitative real-time RT–PCR of CYTb, a gene functioning in the respiratory chain in mitochondria, was also performed, but no significant difference was observed with regard to the expression level between the two groups (data not shown).

Expression of the CDR1, MDR1 and ERG11 genes

Quantitative RT–PCR experiments revealed that 31 azole-resistant isolates had higher expression levels of the ERG11 gene than the 21 azole-susceptible isolates (Figure 3). In contrast, the CDR1 and MDR1 expression levels in the 31 azole-resistant isolates were not significantly different from the 21 azole-susceptible isolates (Figure 3). Moreover, 4 isolates resistant to all three of theazole antifungals had a higher level of ERG11 gene expression than 10 isolates that were only resistant to itraconazole or 13 isolates that were only resistant to fluconazole (Figure 4).

Ergosterol analysis

The ergosterol contents of the 31 azole-resistant and 21 azole-susceptible C. tropicalis isolates were analysed using LC-MS/MS. The results did not reveal a significant change in their overall sterol levels, but the mean ergosterol content of the resistant group was higher than that of the susceptible group (4.88±0.13 µg/mg versus 3.84±0.04 µg/mg). Additionally, the fluconazole-resistant isolate 1b showed a 1.2-fold higher ergosterol content than its matched fluconazole-susceptible isolate 1a (4.79 µg/mg versus 3.85 µg/mg). Furthermore, the ergosterol contents of the isolates that were multiresistant to two or three types of azole antifungals (5.01±0.07 µg/mg and 5.11±0.09 µg/mg) were slightly higher than the isolates that were only resistant to fluconazole or itraconazole (4.80±0.05 µg/mg and 4.83±0.06 µg/mg).

Mutations in ERG11

The DNA sequence analysis of the ERG11 ORFs revealed that there were no mutations in the 21 azole-susceptible isolates compared with the corresponding sequence in the GenBank database (GenBank accession number M23673). Two missense mutations Y132F and S154F were discovered in 12 isolates among the 31 azole-resistant isolates; interestingly, these two...
Mutations Y132F and S154F are involved in azole resistance

Lanosterol 14α-demethylase is a prominent intermediate in ergosterol biosynthesis and S. cerevisiae cannot survive without this enzyme. Therefore the expression plasmid pYES2/CT with the full-length ERG11 ORF amplified from C. tropicalis 1a and 1b, with or without the Y132F and S154F mutations, was transformed into S. cerevisiae W303-1A. An empty plasmid was transformed as the control (Table 2). The genomic ERG11 of SC2 and SC3 was replaced by homologous recombination with a linear TRP1 DNA fragment, amplified from pY24GAL using primers designed with the promoter-dependent disruption with a linear TRP1 DNA fragment, amplified from pY24GAL using primers designed with the promoter-dependent disruption method, as previously documented. The principle of the final screen and the electrophoresis results of ERG11 and TRP, respectively. (a) Primers for the screening were designed according to the diagram, in which ERG<sub>IR</sub> indicates the upstream forward primer of ERG11, and ERG<sub>IR</sub> and TRP<sub>IR</sub> indicate the internal reverse primers of ERG11 and TRP, respectively. (b) Lanes 1 and 5 are the PCR products using ERG<sub>IR</sub>-TRP<sub>IR</sub> primers and lanes 2 and 4 are the PCR products using ERG<sub>IR</sub>-ERG<sub>IR</sub> primers. A DL1000 DNA ladder was used as the marker.

Table 3. MICs of azole antifungals for four S. cerevisiae isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>fluconazole</th>
<th>itraconazole</th>
<th>voriconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae W303-1A</td>
<td>0.5</td>
<td>0.031</td>
<td>0.015</td>
</tr>
<tr>
<td>SC1</td>
<td>1</td>
<td>0.031</td>
<td>0.015</td>
</tr>
<tr>
<td>SC4</td>
<td>1</td>
<td>0.062</td>
<td>0.031</td>
</tr>
<tr>
<td>SC5</td>
<td>32</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

MICs of fluconazole, itraconazole and voriconazole for S. cerevisiae W303-1A, SC1, SC4 and SC5 were determined using the broth microdilution method. As illustrated in Table 3, compared with SC4, the MICs of fluconazole and itraconazole in SC5 increased from 1 mg/L to 32 mg/L and from 0.062 mg/L to 1 mg/L, respectively. Although voriconazole resistance was not induced, the MIC of this compound also increased from 0.031 mg/L to 0.25 mg/L.

Discussion

The current increasing clinical isolation rate of C. tropicalis, especially among patients with cancer or haematological malignancies, has drawn the attention of epidemiologists. Although azole resistance has been widely investigated in several Candida spp., such as C. albicans and C. glabrata, related studies in C. tropicalis are insufficient to date. In the present study we investigated the azole resistance of 52 clinical isolates of C. tropicalis, primarily with regard to their efflux pumps, respiratory activity and azole antifungal target cytochrome P450 lanosterol 14α-demethylase.

The number of isolates in this study is still not high enough, therefore the rate of azole resistance is higher than clinical reports. Of the 52 isolates of C. tropicalis, most were resistant to fluconazole and itraconazole, and no isolate was only resistant to voriconazole, which indicates that voriconazole has a greater potency against C. tropicalis.

We demonstrated an active role of efflux pumps in azole-resistant C. glabrata (data not shown), however, no similar phenomenon was observed in C. tropicalis (Figure 1). During pre-experiments, the efflux effect was also tested without glucose, but the mean fluorescence intensity of the cells was also comparable (data not shown). Similarly, the real-time RT-PCR quantification of two transporter-related genes, CDR1 and MDR1, showed no overexpression, which was consistent with the results of Vandeputte et al.20 Barchiesi et al.24 reported that CDR1 and MDR1 were overexpressed in a fluconazole-resistant C. tropicalis isolate. However, this was the result of an experimentally induced isolate in vitro and may have represented a different mechanism than our in vivo isolates; alternatively, the difference may be caused by heterogeneity between the isolates. Therefore, according to our study, efflux pumps do not play a vital role in inducing azole resistance in C. tropicalis.

The petite mutant phenotypes of C. albicans and C. glabrata caused by mitochondrial deficiency have been correlated to
their azole resistance. However, in our study, an increased respiratory status rather than respiratory deficiency was observed in azole-resistant C. tropicalis using rhodamine 123 (Figure 2). On the other hand, between these two groups, no significant difference was observed regarding the expression of CYTb, a gene that functions in the mitochondrial respiratory chain (data not shown). Further research should examine the other genes involved in the respiratory chain, including CYTc and CYTaa3.

We then focused on ERG11, which encodes the primary azole target enzyme cytochrome P450 lanosterol 14α-demethylase. Lanosterol is an intermediate produced in the ergosterol biosynthesis pathway. Although there was only a slight increase in the ergosterol content in the azole-resistant isolates, other products in this pathway are suspected to be involved. Our results indicated that ERG11 was overexpressed in azole-resistant C. tropicalis (Figure 3) and, as shown in Figure 4, there is a tendency for C. tropicalis to increase its ERG11 mRNA expression when the isolate is multidrug resistant to azole antifungals. However, as only one pair of susceptible and resistant matched isolates (1a and 1b) was collected in this study, we cannot disregard the possibility that this difference resulted from strain variations. Dunkel et al. reported that a mutation in the transcription factor Upc2 can induce the overexpression of ERG11 in C. albicans, but this has not been investigated in C. tropicalis. In future studies we intend to collect additional matched C. tropicalis isolates and investigate their expression levels of ERG11 and mutations in the function of its promoter and transcription factors.

Sanglard et al. demonstrated in 1998 that mutations of ERG11 in C. albicans primarily lead to azole resistance by affecting the affinity of the target enzyme to azole derivatives. In our study, we discovered two missense mutations, Y132F and S154F, in azole-resistant C. tropicalis. Interestingly, another mutation, Y132H, was reported in C. albicans by Favre et al. and Feng et al., indicating that amino acid substitution at the 132 site of the ERG11 gene encoding this enzyme was meaningful. All of the azole-resistant C. tropicalis isolates in this study showed an increased respiratory status. In comparison with the efflux pumps, the azole antifungal target cytochrome P450 lanosterol 14α-demethylase played a more vital role. The ERG11 gene encoding this enzyme was overexpressed in the azole-resistant isolates and two mutations, Y132F and S154F, were demonstrated to be responsible for azole resistance, particularly to fluconazole and itraconazole.

### Acknowledgements

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### Transparency declarations

None to declare.

### Supplementary data

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).


