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Original article

Mechanisms of azole resistance in *Candida albicans* clinical isolates from Shanghai, China

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Abstract

This study was undertaken to characterize the mechanism(s) of azole resistance in clinical isolates of *Candida albicans* collected in Shanghai, China, focusing on the role of efflux pumps, target enzymes of fluconazole (Erg11), respiratory status and the ergosterol biosynthetic pathway. Clinical isolates of *C. albicans* ($n = 30$) were collected from 30 different non-HIV-infected patients in four hospitals in Shanghai. All 30 *C. albicans* isolates were susceptible to amphotericin B and 5-fluorocytosine. Twelve *C. albicans* isolates showed resistance to at least one type of triazole antifungal. Flow cytometry analysis of rhodamine 6G efflux showed that azole-resistant isolates had greater efflux pump activity, which was consistent with elevated levels of *CDR1* and *CDR2* genes that code for ABC efflux pumps. However, we did not observe increased expression of *ERG11* and *MDR1* or respiratory deficiency. Several mutations of *ERG11* and *TAC1* genes were detected. The F964Y mutation in the *TAC1* gene was identified for the first time. Two main sterols, ergosterol and lanosterol, were identified by GC–MS chromatogram, and no missense mutations were found in *ERG3*. Furthermore, seven amino acid substitutions in *ERG11*, A114S, Y132H, Y132F, K143Q, K143R, Y257H and G448E were found, by Type II spectral quantitative analysis, to contribute to low affinity binding between Erg11 and fluconazole.

Keywords: Triazole resistance; *ERG11*; Efflux pumps; Transcription factor; Ergosterol; Biosynthetic pathway

1. Introduction

Candida sp. are common opportunistic pathogens. In immunocompetent patients, *Candida* sp. infects the skin, nails and oral-pharyngeal, gastrointestinal and vaginal mucosae. In immunocompromised or immunodeficient patients, *Candida* sp. can cause invasive life-threatening infections such as disseminated candidiasis [1,2]. Although the number of infections with other *Candida* sp. has been increasing lately, *Candida albicans* remains the most prevalent. According to the SENTRY Antimicrobial Surveillance Program of 2008–2009 in the United States, among 2085 cases of *Candida*-associated bloodstream infections in five different regions, 48.4% were attributable to *C. albicans* [3]. Recently, epidemiological studies on nosocomial *Candida* sp. bloodstream infections in China have been reported [4–7]. These studies showed that *C. albicans* was the *Candida* species that led to invasive candidiasis in 37.2–57.4% of cases.

Due to its broad spectrum, high efficiency, good bioavailability and safety profile, fluconazole is recommended as the first choice for treating *Candida* infections by the Infectious Disease Society of America (2004) *Candida* treatment guidelines [8]. However, long-term or repeated treatment with fluconazole can lead to resistance, possibly leading to azole cross-resistance, which could seriously hamper the clinical

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treatment of candidiasis. In one multicenter China-SCAN study, the rate of fluconazole resistance in *C. albicans* was almost 14.1% [6]. Thus, understanding the molecular mechanism(s) of resistance to azoles in *C. albicans* has become an active area of research.

Currently, several mechanism(s) are thought to lead to azole resistance in *C. albicans* [9–12]. The primary hypotheses include: (1) upregulation of transporters, either in the ATP binding cassette (ABC) family, such as Cdr1p or Cdr2p, or in the major facilitator superfamily (MFS), such as Mdr1p; (2) alterations in expression of the structure of the azole target enzyme, lanosterol 14α-demethylase (Erg11p); (3) mitochondrial defects leading to decreased ATP and ROS; and (4) a defect in sterol $\triangle^{5,6}$ desaturase (encoded by *ERG3*), leading to alterations in the ergosterol biosynthetic pathway. Interestingly, multiple mechanisms of resistance usually contribute to azole resistance in a single isolate [13–15].

Here we evaluated the resistance mechanisms of azole-resistant strains of *C. albicans* isolated from non-HIV-infected patients from four different medical hospitals in Shanghai. We focused on characterizing alterations in the azole target enzyme, the activity of the efflux pumps and their associated transcription factors, respiratory status and mutations to *ERG3*.

2. Materials and methods

2.1. Strains and medium

C. albicans isolates ($n = 30$) were collected from non-HIV infected patients ($n = 30$) with vulvovaginal candidiasis, mucocutaneous candidiasis and candida infections of the skin, respiratory tract and digestive tract at four hospitals in Shanghai over a two-year period. The isolates were identified as *C. albicans* using germ tube formation in serum-containing medium, morphology analysis on CHROMagar *Candida* medium (CHROMagar, Paris, France) and carbohydrate assimilation tests (API 20C AUX BioMerieux, Marcy I'Etoile, France). All isolates were stored in YPD (yeast extract peptone dextrose) liquid medium (1 g/L yeast extract, 2 g/L peptone, 2 g/L glucose) with 25% glycerol at −20 °C. The *Escherichia coli* strain DH5α and LB (lysogeny broth) medium were used for transformation and plasmid DNA preparation.

Saccharomyces cerevisiae strain INVsc-1 (MATα his3△1/his3△1 leu2/leu2trp1-289/trp-289ura3-52/ura3-52, His-, Leu-, Trp-, Ura-) and plasmid pYES2 were purchased from Invitrogen (Carlsbad CA, USA).

SD-URA3-Minus-2% glucose medium (Genmed, Minneapolis, MN, USA) was used to propagate the transfectants and SD-URA3-Minus-Gla-Raf medium (Genmed, Minneapolis, MN, USA) was used to induce heterogeneous expression of the Erg11 protein.

2.2. Antifungal susceptibility tests

Fluconazole and voriconazole were purchased from Pfizer (Shanghai, China) and itraconazole, amphotericin B and 5-fluorocytosine were purchased from Sigma–Aldrich (St. Louis, MO, USA). The minimum inhibitory concentrations (MICs) of these antifungal drugs were determined using the broth microdilution method established by the CLSI M27-A2 standard guideline (2002) [16]. *C. albicans (*ATCC 90028), *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used as internal controls. *C. albicans* was scored as "susceptible," "susceptible-dose-dependent/intermediate," or "resistant" to fluconazole, itraconazole, or voriconazole using the following MIC threshold values: fluconazole-susceptible = ≤8 μg/mL, susceptible-dose-dependent (SDD) = 16–32 μg/mL and resistant = ≥64 μg/mL; itraconazole-susceptible = ≤0.125 μg/mL, SDD = 0.25–0.5 μg/mL, resistant = ≥1 μg/mL; and voriconazole-susceptible = ≤1 μg/mL, intermediate = 2 μg/mL and resistant = ≥4 μg/mL.

2.3. Real-time PCR

RNA was extracted from cultures in the mid-log exponential growth phase, grown in YPD medium at 35 °C and shaking at 200 rpm using the yeast RNAiso reagent kit (TaKaRa, Tokyo, Japan) according to the manufacturer's recommendations. The RNA concentration was determined using a Nanodrop 8000 (Thermo-Scientific, Waltham, MA, USA). The isolated RNA was quantitatively reverse-transcribed to cDNA using the PrimeScript RT Reagent kit (TaKaRa, Tokyo, Japan). Expression levels of azole resistance genes *CDR1*, *CDR2*, *MDR1*, *ERG11* and of housekeeping genes *PMA1* and *ACT1* were determined by quantitative RT-PCR. The analysis was performed on an Mx3000P instrument (Stratagene, USA) with the SYBR Premix Ex Taq kit (TaKaRa) under the following conditions: denaturation at 94 °C for 3 min, followed by 40 cycles consisting of 10 s at 94 °C and 20 s at 55 °C. The primers used for RT-PCR analysis are shown in Table 1. Each sample was processed in triplicate. Relative gene expression was measured quantitatively after normalization to an 18S RNA control that was amplified at the same time as each target gene. The RNA transcript levels for each isolate were compared with the average expression level of a collection of 18 azole-susceptible isolates. RNA transcripts were considered significantly overexpressed when the △Ct value [Ct(gene of interest)−Ct(18S RNA)] exceeded 3.0 standard deviations.

2.4. Rhodamine 6G efflux

The activity of efflux pumps was evaluated using flow cytometry as previously described [17] with the following modifications. *C. albicans* cells were grown overnight at 30 °C to 5×10^7 cells/mL in YPD. Cells were centrifuged at 3000 × g for 5 min and washed three times with PBS. The cells were then incubated in PBS for 4 h at 30 °C under continuous shaking (200 rpm/min) to deplete the energy reserves of the cells. Rhodamine 6G (Sigma–Aldrich, St Louis, MO, USA) at a final concentration of 10 μM was added and the cells were incubated for 2 h at 30 °C. The fluorescence uptake was measured immediately using a Beckman Coulter