

# Fluconazole Susceptibility and Genotypic Heterogeneity of Oral *Candida albicans* Colonies from the Patients with Cancer Receiving Chemotherapy in China

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## Abstract

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**Aim** To identify heterogeneity of *Candida albicans* (*C. albicans*) isolated from the population with cancer in China by using identification medium, subculture molecular typing, and antifungal susceptibility test.

**Methodology** Oral cheek mucosal specimens from 52 cancer patients receiving chemotherapy were cultured on CHROMagar Candida™ plates for *Candida* identification. All the *C. albicans* colonies on the plates were subcultured and reconfirmed by API20C, then submitted to the antifungal drug susceptibility test with fluconazole and molecular typing using randomly amplified polymorphic DNA-PCR (RAPD) with primers RSD6 and RSD12.

**Results** 54% (28/52) patients were oral yeast carriage in which *C. albicans* predominated. More than 7 *C. albicans* colonies were isolated from each of 12 patients (Group A), while less than 5 colonies were isolated from each of 16 patients (Group B). RSD6 and RSD12 were successful in eliciting 17 (A1-A17) and 2 (B1-B2) genotypes, respectively from among the 205 isolates. The two primers were combined to generate 21 genotypes. The *C. albicans* isolates obtained from the same patient and episode showed a diversity for fluconazole revealed by MIC<sub>50</sub> and MIC<sub>90</sub>.

**Conclusion** The heterogeneity of the *C. albicans* colonies isolated from the same patients can be detected. *C. albicans* with varied fluconazole susceptibility and genotypic characteristics may coexist in the same oral *Candida* population.

**Keywords** *Candida albicans*, molecular typing, chemotherapy, antifungal susceptibility

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## Introduction

*Candida* species especially *Candida albicans* (*C. albicans*) are the composition of oral commensal flora, which can be isolated in the healthy oral cavities with different frequency (Cannon *et al.*, 1995; Kleinegger *et al.*, 1996; Qi *et al.*, 2005). At the same time, *C. albicans* is one of the most pervasive opportunistic pathogens, which can cause

endogenous infection from superficial to seriously deep-seated mycoses under the altered host conditions, such as the malignant cancer patients (Brawner and Cutler, 1989; McCullough *et al.*, 1996). Anticancer treatment including chemotherapy or/and radiotherapy is still associated with several side effects. Among them, oral candidiasis is one major source of illness, although strict oral hygienic care has been undertaken by using anti-

fungal reagents to prevent them (Wingard, 1994). Oral candidiasis is often accompanied by pain, loss of taste, reduction in intake, and weight loss, thereby resulting in treatment delays and necessitating dose reduction (Mead, 2002). Furthermore some studies have shown its potential importance in the development of systemic candidiasis which would be vital for these patients (Rodu *et al.*, 1984; Kontoyiannis and Lewis, 2002).

The main limitation of previous studies about oral *Candida* is the fact that only single isolates are elected from each patient for each episode. In fact there is heterogeneity among the oral *Candida* population (all the *Candida* colonies isolated from the same host) in phenotypic and genotypic aspects (Wimpenny *et al.*, 2000; Stewart and Franklin, 2008). For example, one research showed that up to 32 fold differences in fluconazole minimal inhibitory concentrations (MICs) were detected within isolates recovered at the same AIDS patient and episode (Lopez-Ribot *et al.*, 1999).

Since there is great heterogeneity among the individuals of the oral *Candida* population, it is reasonable to believe that this randomly selected single isolate cannot represent the whole view of the oral *Candida* population. Until now, the heterogeneity in the oral *C. albicans* population across China is still unknown, and many studies of genotype variation and antifungal resistance among isolates from AIDS patients have shown mixed results (Takasuka *et al.*, 1998; Metzgar *et al.*, 1998; Lopez-Ribot *et al.*, 1999).

This research may help us to understand the antifungal drug susceptibility and genotypic characteristics of oral *Candida* isolated from cancer patients in China, and would assist in getting the accurate susceptibility test in clinical practice.

## Material and methods

### Patients selection

Fifty-two cancer patients from two affiliated teaching hospitals and a cancer hospital of Shandong University Medical School were selected for this study and sampled for oral *Candida* carriage, including 6 patients with hematological malignancies, and 46 with solid tumors. The patients'

ages ranged from 21 to 76 years old, averaging 49.8 years. There were 31 male patients and 21 female patients. The inclusion criteria for the study were previous diagnoses of advanced cancer for more than 1 year and treatment by chemotherapy. The exclusion criteria for this study were significant physical defects, denture wearers, uncontrolled diabetes mellitus, systemic antifungal medication or steroids within the recent 2 weeks. The patients with head and neck cancer receiving radiation therapy were excluded. This study was approved by the Ethics Committee of Shandong University (No. 2008-03) and Northeastern University MA US IRB (No. 09-01-02).

### Sampling preparation

All patients received regular oral hygiene care including 0.2% chlorhexidine daily. Patients were examined by two physicians, according to the guidelines of oral candidiasis diagnostic standards, for evidence of oral candidiasis (Kleinegger *et al.*, 1996). They were sampled with specimens obtained 8 hours following regular oral hygiene care. The specimens were collected by passing a sterile swab across the inner cheek mucosal surface five times. The procedure was repeated three times in total within a half-hour interval. Swabs were transferred to a sterile test tube containing 500  $\mu$ L sterile phosphate buffered saline (PBS). The tubes containing the swabs were vortexed vigorously for 30 seconds to suspend the samples. After 30 seconds, the swabs were removed, the samples were sedimented by centrifugation and concentrated by resuspending in 50  $\mu$ L PBS. This suspension was spread plated on CHROMagar *Candida*<sup>TM</sup> differential medium, and incubated for 24–48 hours (Worthington and Clarkson, 2002). Colonies were identified as *Candida* by CHROMagar based on color change and colony morphology of this propriety selective and differential medium (Tamura *et al.*, 2001). *Candida* strains were confirmed by API20C (BioMerieux France) based on carbon source utilization and other differential biochemical assays according to the manufacturer's directions (Xu *et al.*, 2002). *C. albicans* species were additionally confirmed by observing germ tube formation induced by serum *via* microscopy (Mackenzie, 1962). If multiple *Candida* colonies were present,

all of the isolates were subculture and chosen for further analysis.

### Antifungal susceptibility test

The 10 mg·mL<sup>-1</sup> stock solution of amphotericin B (AMB) (Fisher Scientific, USA) and chlorhexidine (CHX) (Sigma-Aldrich, USA) were prepared in dimethyl sulfoxide. The MIC of clinical isolates was determined by broth microdilution based on the CLSI M27-A2 guidelines (McArthur, 2006). Briefly, cells from an overnight culture grown in yeast peptone dextrose medium were harvested by centrifugation, then being washed twice in sterile PBS and resuspended to 0.5×10<sup>3</sup>–2.5×10<sup>3</sup> cell·mL<sup>-1</sup> in RPMI 1640 (GBICO, USA) medium buffered to pH 7.0 with 0.165 mol·L<sup>-1</sup> MOPS (Sigma-Aldrich, USA). Cells were dispensed into a 96-well microtiter plate at 100 µL per well, and antifungals were added by a series of 2-fold dilutions, 100 µL per well in RPMI medium. Plates were incubated at 37°C for 48 hours. MICs for fluconazole were determined visually and measured by microplate spectrophotometer (Molecular Device, USA) at 600 nm, prior to the identification of the lowest concentration that would result in 50% (MIC<sub>50</sub>) and 90% (MIC<sub>90</sub>) reduction.

### Genotypic determinations

Extraction of *C. albicans* DNA, 1–2 monoclonies from the YPD agar plates was suspended in 200 µL of TE buffer in a microcentrifuge tube. DNA extraction was carried out using Promega Wizard DNA Extacyon Kit (Promega, USA). Nucleic acid pellet was obtained, washed with ice-cold 70% ethanol, dried, and resuspended in sterile TE buffer at a concentration of 5 µg·mL<sup>-1</sup>.

Genotypic was determined using randomly amplified polymorphic DNA-PCR (RAPD) analysis. Two primers RSD6 (5'-GCG ATC CCC A-3') and RSD12 (5'-GCA TAT CAA TAA GCG CAG GAA AAG-3') for RAPD were those described by Fell and Akopyanz (Akopyanz *et al.*, 1992; Fell, 1993). Amplification reactions were performed in 50 µL of distilled water containing 1 µmol·L<sup>-1</sup> primer, 200 ng of genomic DNA, and PCR kit including Taq (5 U·µL<sup>-1</sup>) 0.125 µL, 10× Ex Taq buffer 5 µL, MgCl<sub>2</sub> (25 mmol·L<sup>-1</sup>) 2 µL, 200 µmol·L<sup>-1</sup> of dNTP

mixture, 1.5 U of Taq polymerase (PCR kit of TAKARA, China). The PCR conditions were listed follows: the first five cycles incubated 30 seconds of denaturation at 94°C, 2 minutes of annealing at 27°C (for RSD6) and 52°C (for RSD12), and 2 minutes of primer extension at 72°C, then a final extension at 72 °C for 15 minutes in a thermo-reactor (Gradient Thermal Cycle, Promega Corp, USA).

The amplification products were characterized by electrophoresis on 1.2% agarose gels in 1× TBE buffer at 50 V for 120 minutes, stained in a solution of 0.5 µg·mL<sup>-1</sup> of ethidium bromide, and then visualized by UV transillumination (Bio Rad, USA) (Waltimo *et al.*, 2001).

## Results

### Oral *Candida* carriage and antifungal susceptibility of the *Candida* isolates from cancer patients

Oral *Candida* strains were isolated from 28 of 52 patients and the frequency of oral *Candida* carriage was 53.8%. Among them, *Candida glabrata* (*C. glabrata*) alone was detected in 3 patients.

The fungal burden of the patients was determined using the numbers of yeast colonies on the identification medium of each sample (Rüchel *et al.*, 2000). All the patients were divided into two groups according to the fungal burden. The average number of yeast cfu of Group A (fungal burden more than 7) was 13.4±5.2 (*n*=12), significantly higher than that of Group B (fungal burden less than 5), *i.e.*, 2.8±1.4 (*n*=16) (*P*<0.001) (table 1).

### Heterogeneity of antifungal susceptibility and genotype among the isolates of oral *C. albicans* population

The two primers, RSD6 and RSD12, were successful in eliciting 17 (A1-A17) and 2 (B1-B2) genotypes, respectively from among the 205 isolates. RSD6 (A1-A17) and RSD12 (B1-B2) were combined to generate 21 genotypes. Multiple *C. albicans* strains isolated from most of the patients (8/12) of Group A belong to the same genotype. While for 3 patients, 2 genotypes coexist at the

same *C. albicans* population. However the oral *C. albicans* population of patient A8 showed highly genotypic heterogeneity (Figure 1). Much fewer stains were isolated from Group B patients, and genotypic heterogeneity could be detected in the *C. albicans* population of 4 patients (B1, B6, B9 and B12).

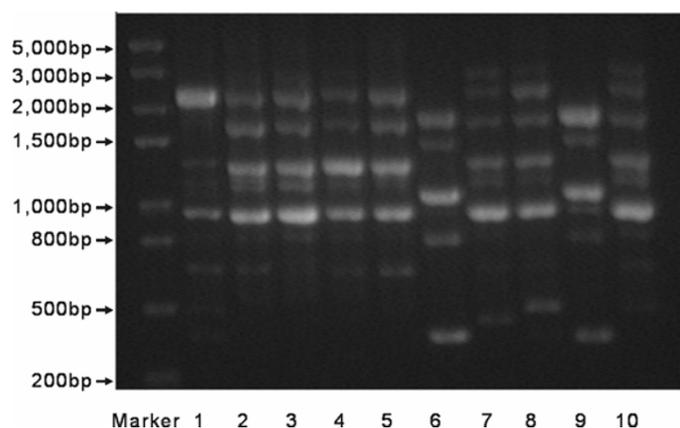
The variation in fluconazole susceptibility and genotype of the *C. albicans* isolates from the patients of Group A were analyzed. Firstly, for the isolates from patient A8, great heterogeneity could be observed in both genotype and fluconazole

susceptibility (Table 2). Secondly, all the isolates from patients A12 and A3 were genotype A6B1 and A9B2 respectively, having the same level of MIC<sub>90</sub> but different MIC<sub>50</sub> for fluconazole (Table 3). Thirdly, two genotypes coexist in the same patient *C. albicans* population, but all the isolates show the same susceptibility to fluconazole, whatever MIC<sub>50</sub> or MIC<sub>90</sub> (patients A1, A4 and A7). Lastly, multiple isolates from the same patient were of the same genotype, meanwhile showing the same level of fluconazole susceptibility (patients A5, A6, A10 and A11).

**Table 1** Overview of fungal burden, clinical evidence of oral candidiasis and genotypic heterogeneity among the oral *C. albicans* isolates

Group (Patient)	Fungal burden	Clinical diagnosis of oral candidiasis	<i>C.albicans</i> genotype RAPD analysis
A1	6	Yes	A6B2, B7B2
A2	16 ( <i>C. glabrata</i> )	Yes	
A3	7	Yes	A9B1
A4	13	Yes	A9B2, A13B2
A5	17	Yes	A13B1
A6	21	Yes	A10B2
A7	18	yes	A13B9, A14B9
A8	10	Yes	A4B2, A9B2, A11B2, A12B2, A13B2
A9	19		A15B2
A10	7		A1B1
A11	17		A10B2
A12	10		A6B1
B1	3		A1B2, A2B2
B2	2		A3B2
B3	3 ( <i>C. glabrata</i> )	Yes	
B4	4 ( <i>C. glabrata</i> )	Yes	
B5	2		A1B2
B6	3		A1B2, A1B4
B7	1		A5B2
B8	3		A6B2
B9	5		A3B2, A8B2, A17B2
B10	1		A9B2
B11	2		A9B2
B12	5		A8B2, A9B2
B13	4		A6B2
B14	1		A6B2
B15	4		A4B2
B16	1		A16B2

The average number of yeast cfu of Group A was 13.4±5.2 (n=12), significantly higher than that of Group B 2.8±1.4 (n=16). Most of the Group A patients and all of the oral *C. glabrata* had the clinical evidence of oral candidiasis, indicating that high fungal burden and *C. glabrata* carriage may be interrelated with oral *Candida* infectious disease.



**Figure 1** RAPD fingerprints of *C. albicans* isolated from patient A8 obtained using primer RSD6

Five genotypes were elicited among the 10 strains isolated from patient A8. Please note that No.2, 3, 4, 5, 7 and 8 belong to the same genotype.

**Table 2** Heterogeneity in both genotype and fluconazole susceptibility for *C. albicans* isolated from patient A8

Strains	MIC <sub>50</sub> / $\mu\text{g}\cdot\text{mL}^{-1}$	MIC <sub>90</sub> / $\mu\text{g}\cdot\text{mL}^{-1}$	Genotype
1	0.5	2	A13B2
2	0.125	0.5	A9B2
3	0.5	>128	A9B2
4	0.125	>128	A9B2
5	0.125	>128	A9B2
6	0.5	>128	A11B2
7	0.125	1	A9B2
8	4	16	A9B2
9	0.125	4	A4B2
10	0.5	4	A12B2

The heterogeneity could be found in both genotype and fluconazole susceptibility for the isolates from patient A8. But most of strains (6/10) belonged to the same genotype (A9B2). Up to 32 fold differences in fluconazole, MIC<sub>50</sub> were detected within isolates recovered at the same patient and episode, which is consistent with the results of Lopez-Ribot.

## Discussion

Oral candidiasis is a common side-effect of anticancer chemotherapy for malignant cancer patients (Clarkson *et al.*, 2000; Worthington and Clarkson, 2002). Our results however showed that the frequency of oral *Candida* carriage was 58% which was similar with the afore-reported frequency of healthy host. There were 19% (10/52) patients who had the clinical diagnosis of oral candidiasis. This frequency of oral yeast carriage and oral candidiasis varied according to different

**Table 3** Heterogeneity in fluconazole susceptibility for *C. albicans* isolated from patient A12

Strains	MIC <sub>50</sub> / $\mu\text{g}\cdot\text{mL}^{-1}$	MIC <sub>90</sub> / $\mu\text{g}\cdot\text{mL}^{-1}$	Genotype
1	0.5	>128	A6B1
2	1	>128	A6B1
3	0.5	>128	A6B1
4	0.5	>128	A6B1
5	>128	>128	A6B1
6	0.125	>128	A6B1
7	>128	>128	A6B1
8	>128	>128	A6B1
9	>128	>128	A6B1
10	0.125	>128	A6B1

All the *C. albicans* isolates from the patient A12 were of genotype A6B1. And they have the same level of MIC<sub>90</sub> (>128  $\mu\text{g}\cdot\text{mL}^{-1}$ ), but different MIC<sub>50</sub> for fluconazole.

studies. The difference in sampling preparation and position, treatment conditions of the patients, and geographic area may contribute to the diversity of frequency (Rodu *et al.*, 1984; da Costa *et al.*, 2009).

More and more researches indicated that heterogeneity of phenotype and genotype among the population of microorganisms (Wimpenny *et al.*, 2000; Kontoyiannis and Lewis, 2002). It is believed that heterogeneity may play a role in determining the fitness of cell populations, and by producing variant subpopulation of different phenotypes, the cell population can be more suitable for persisting during harsh condition (Bishop *et al.*, 2007). This is important for the microorganism population which inhabit at a frequently changing environ-

ment such as oral cavity (McArthur, 2006). Thus the heterogeneity of phenotype and genotype in the oral yeast population should be reasonable. In this study, all the *C. albicans* colonies from the same patients were selected for heterogeneity study. We found that *C. albicans* isolates with different fluconazole MICs and genotypes could coexist in the same oral *Candida* population, which reinforces this hypothesis.

RAPD is an ideal method for analyzing the *C. albicans* genotype, greatly dependent on the choice of primers and the genomic locus selected. In this research we employed the primer RSD12 and RSD6 for they have been successfully subgrouping the yeast strains. Distinctively different from the other research using primer RSD12, RSD12 in our research only elicited 2 genotypes from among the 205 isolates. The *C. albicans* strains isolated from different geographic areas may contribute to this phenomenon (Tsang *et al.*, 1995).

From the results of genotypic determinations of Group A patients using RAPD analysis, the multiple colonies from the same patient generally belong to the similar genotype. Although there still had an exception such as strain A8, multiple genotypes with different MIC for fluconazole coexisted in the same population, and most of the strains (6/10) still belonged to the same genotype. Genotypic heterogeneity also could be detected in the population of some patients of Group B, although much fewer isolates from Group B patients. The results indicate that multiple isolates may be reproduced by one or two colonies. The genotypic heterogeneity may be ascribed to the interference of transient strains (Metzgar *et al.*, 1998). But the isolates with the same genotype show different antifungal susceptibility, which reminds us that this hypothesis needs to be further studied.

Resistance of *C. albicans* isolates to azoles is the most prevalent type of antifungal resistance, also showing great heterogeneity (Lopez-Ribot *et al.*, 1999; Kontoyiannis and Lewis, 2002). Fluconazole resistance in *C.albicans* can be attributed to overexpression of the drug target (lanosterol demethylase), mutations in the drug target, and most commonly for high-level resistance, overexpression of energy-dependent drug efflux pumps. Our research indicated that although some *C. albicans* population showed great heterogeneity in

fluconazole susceptibility, analyzing both MIC<sub>50</sub> and MIC<sub>90</sub> can be helpful for grasping the whole view of the population. For example, all the isolates from patient A12 showed very high MIC<sub>90</sub> despite a great diversity in MIC<sub>50</sub>. This fact remind us that comprehensive consideration of MIC<sub>50</sub> and MIC<sub>90</sub> for azole antifungal drugs susceptibility or some measurement such as agar dilution screening technique (Patterson *et al.*, 1996a; Patterson *et al.*, 1996b) may be useful to provide a more comprehensive assessment and avoid the aberration in clinical practice.

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