· Original article ·

Molecular mechanisms of fluconazole resistance in clinical isolates of *Candida tropicalis*

ZHOU Yong-an(周永安) DOU Juan(窦娟) ZHANG Quan-bin(张全斌)
MA Yun-xia(马云霞)

【**Abstract**】 **Objective** This study aimed to investigate the molecular mechanisms responsible for fluconazole resistance in clinical isolates of this pathogenic yeast. **Methods** A total of 41 *Candida tropicalis* strains were collected from the clinical laboratory of Taiyuan City Central Hospital. Antifungal susceptibility testing was performed by ATB FUNGU 3 method. The 14 α-demethylase (ERG11) gene in all clinical isolates of *Candida tropicalis* were amplified by PCR, and their nucleotide sequences were determined in order to detect point mutations. Likewise, efflux transporters (CDR1 and MDR1) and ERG11 genes were tested by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) for their expression in *Candida tropicalis* cells at the mRNA level. **Results** The fluconazole-resistant rate of 41 *Candida tropicalis* was 12.2%. The amino acid substitutions in ERG11p of R245K, Y221F and V362I were found in fluconazole-resistant isolates. And no amino acid substitution was detected in fluconazole-susceptible ones. The mRNA level of CDR1, MDR1 and ERG11 genes in fluconazole-resistant isolates all showed overexpression compared with fluconazole-susceptible ones. **Conclusions** Missense mutations in ERG11 gene associated with overexpression of CDR1, MDR1 and ERG11 gene seemed to be responsible for the acquired fluconazole resistance of these clinical isolates.

【**Key words**】 *Candida tropicalis*; Fluconazole; Resistance, antifungal drug

Introduction

Infections by opportunistic pathogenic fungi, especially *Candida* species, have become a major clinical problem in the past decades due to the increasing number of immunocompromised patients, e.g., AIDS patients and individuals infected with HIV, bone marrow and organ transplant recipients, or cancer patients[1-2]. *Candida albicans* has been the most important opportunistic pathogenic fungi causing candidiasis in immunocompromised patients. However, a marked shift in the distribution of *Candida* species was observed. And infections due to non-*C. albicans* species have increased dramatically on a global scale. Among non-*C. albicans* species, *Candida tropicalis* (*C. tropicalis*) represents the third most common pathogen[3], but the second in respiratory specimens.

Historically, azole antifungal agents, especially fluconazole, have been commonly used as the first-line treatment for many different fungal infections. However, as a result of the prolonged administration and the extensive use of antifungals, the clinical isolates resistant to azoles have become a serious problem in recent years[4-5]. Therefore, an understanding of the molecular mechanisms underlying the resistance to fluconazole in *C. tropicalis* is not only essential for the selection of the appropriate antifungal agents for the patients at the earliest time, but also important in the development of new antifungals.

Materials and Methods

1. Strains, identification and culture conditions

DOI:10.3877/cma.j.issn.1674-0785.2011.11.024

Department of Clinical Laboratory, Taiyuan City Central Hospital (ZHOU Yong-an, ZHANG Quan-bin, MA Yun-xia), Taiyuan 030009, China; Department of Biochemistry and Molecular Biology, Shanxi Medical University (DOU Juan)

Corresponding author: ZHOU Yong-an, Email: zya655903@163.com

A total of 41 clinical isolates of *C. tropicalis* were collected from the clinical laboratory of Taiyuan City Central Hospital from January 2010 to October 2010. The reference strain of *C. tropicalis* (ATCC750 strain) was obtained from the American Type Culture Collection. The identification of all isolates was confirmed by CHRO Magar medium and automatic bioanalysis Vitek 2 (BioMerieux).

Culture were routinely inoculated from single colonies. The isolates were grown at 30 ℃ on YEPD agar plates (20 g of peptone,10 g of yeast extract,20 g of dextrose per liter and 15 g agar per liter) or in YEPD(20 g of peptone,10 g of yeast extract,20 g of dextrose per liter),stored at 4 ℃ and subcultured weekly or stored at −80 ℃ in YEPD containing 10% glycerol.

2. Antifungal susceptibility testing

The *in vitro* susceptibilities of the 41 *C. tropicalis* to five species (amphotericin B,5-fluorocytosine,fluconazole,itraconazole,voriconazole) of antifungal agents were determined using French MEILIE ATB test tape method according to the manufacturer's instructions. *C. tropicalis* ATCC750,which was well defined laboratory fluconazole susceptible strain,was used as quality control strain.

3. Amplification,cloning and sequencing of ERG11 gene

Cells were inoculated in fresh YEPD medium with constant shaking at 30 ℃ for an overnight and were harvested while they were growing in the logarithmic phase. Genomic DNA of all clinical isolates of *C. tropicalis* was extracted using a E. Z. N. A. Yeast DNA Kit (Bio-Tek) and was used as a template for amplification of the coding region of ERG11 genes with the following primers:5′-GTTTTCTACTGGATCCCATGG-3′ and 5′-TACATCTGTGTCTACCACC-3′. PCRs were carried out in a 50 μl volume containing 10 × PCR buffer 5 μl,genomic DNA 20 ng,2 mmol/L of each dNTP 5 μl,25 mmol/L $MgCl_2$ 5 μl,10 pmol/L each primer 2.5 μl,1 U/μl Taq DNA polymerase 2.5 μl. Amplification was performed in a thermal cycler for 1 cycle of 4 min at 94 ℃ and then for 35 cycles,each of which consisted of 30 s at 94 ℃,1 min at 55 ℃,and 1 min at 72 ℃;this was followed by 1 final cycle of 10 min at 72 ℃. The PCR products were analyzed by electrophoresis on 1.0% agarose gels and were visualized by transillumination after staining of the gels with ethidium bromide. The PCR products were purified with DNA purified kit (TaKaRa) and then cloned into pMD 18-T vector (TaKaRa) according to the instructions of the manufacturer. Plasmids extracted from *E. coli* transformants were sequenced from both directions using ABI 3730 sequencer (ABI,USA). Strain ATCC 750 whose ERG11 sequence was already known (Genbank No. M23673) also underwent ERG11 amplification,PCR product cloning and sequencing of the recombinant plasmid to obtain its ERG 11 sequence as a control.

4. Semiquantitative RT-PCR analysis

Total cellular RNA was obtained using the E. Z. N. A. Yeast RNA Kit (Bio-Tek) following the instructions of the manufacturer. All RNA samples were treated with RNase-free DNase I to prevent contamination with genomic DNA. The quality of RNA was checked using the electrophoresis on 1.5% agar and a ratio of A260 nm/A280 nm by ultraviolet spectrophotometer. The cDNA for each strain was synthesized using the PrimeScript™ RT-PCR Kit (Takara) according to the manufacturer's instructions. All of the reagents,including cellular RNA,were added to a final volume of 20 μl. The samples were then incubated at 37 ℃ for 60 min,85 ℃ for 5 min and held at 4 ℃ in a thermocycler. The synthesized cDNA samples were stored at −20 ℃. The PCR primers used to amplify the *C. tropicalis* CDR1,MDR1 and ERG11 genes were designed by primer premier 5.0 design software. The housekeeping gene ACT1 was used as a control. The nucleotide sequences of the primers are given in Table 1.

PCRs were carried out in a 20 μl volume containing 10 × PCR buffer 2 μl,cDNA 10 ng,2 mmol/L of each dNTP 2 μl,25 mmol/L $MgCl_2$ 2 μl,10 pmol/L each primer 1 μl,1 U/μl Taq DNA polymerase 1 μl,RNA-free H_2O 10 μl. Amplification was performed in a thermal cycler for 1 cycle of 4 min at 94 ℃ and then for 35 cycles,each of which consisted of 30 s at 94 ℃,30 s at annealing temperature of each gene,and 30 s at 72 ℃;this was followed by

1 final cycle of 10 min at 72 ℃. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and were visualized by transillumination after staining of the gels with ethidium bromide . Using the image scanning system, the relative level expression of CDR1, MDR1 and ERG11 mRNA was got with the ACT1 gene as a control.

Table 1 Nucleotide sequences of primers used to amplify the study genes in 41 *C. tropicalis* isolates using reverse transcription-polymerase chain reaction

Gene	Genbank accession number	Primer sequences(5′-3′,F:Forward;R:Reverse)	Product length (base pairs)	Annealing temperature(℃)
ERG11	M23673	F:TGCTGAAGAAGCTTATACCC	541	57
		R:CAAGGAATCAATCAAATCTCTC		
MDR1	AF104419	F:TAAAGCAGGCTGGAGATGGA	513	58
		R:ACAACCTCCAACTATAGCTA		
CDR1	XM_002545811.1	F:TGAAGCCAGACCCGTAGTTG	352	60
		R:CCACTTTGCCCATCCTAACA		
ACT1	AJ389059	F:TTTACGCTGGTTTCTCCTTGCC	319	60
		R:GCAGCTTCCAAACCTAAATCGG		

ERG11:14 α-demethylase gene; CDR1:*Candida* drug resistance 1 gene;MDR1:multidrug resistance 1 gene;ACT1:housekeeping gene

Table 2 The susceptibilities of 41 clinical isolates of *C. tropicalis* to five antifungal agents[isolate,(%)]

Antifungal agents	S	SDD	R
5-fluorocytosine	40(97.6)	0(0)	1(2.4)
amphotericin B	41(100)	0(0)	0(0)
fluconazole	33(80.5)	3(7.3)	5(12.2)
voriconazole	35(85.3)	3(7.3)	3(7.3)
itraconazole	25(61.0)	5(12.2)	11(26.8)

S:susceptible;SDD:susceptible dose dependent;R:resistant

Table 3 ERG11 gene mutation in *C. tropicalis* strains

Isolate	Susceptibility	Nucleotide mutations	Amino acid substitutions
1	susceptible	G1362A	–
4	susceptible	T225C	–
7	susceptible	T225C	–
8	susceptible	T225C	–
10	susceptible	T225C,C639T	–
15	resistant	T225C,T336G,G483A,G734A[a],T744C,C1047T	R245K
17	resistant	T225C,A327G,C471T,G483A,G734A[a],T744C,C951T,C1047T	R245K
18	resistant	T285C,T312C,T573C,A612G,G633A,A662T[a],G1084A[a],T1134CY221F,V362I	
20	susceptible	T225C	–
24	susceptible	T225C	–
25	susceptible	T225C	–
38	susceptible	T225C	–
40	susceptible	T225C	–

[a]:missense nucleotide mutations; –:no amino acid substitution

Results

1. Strain identification

On CHRO Magar plates, colonies of 41 isolates being studied were light blue. And the date from the automatic bioanalysis Vitek 2 system confirmed that the 41 isolates were *C. tropicalis*.

2. Antifungal susceptibility testing

The susceptibilities of the 41 clinical isolates of *C. tropicalis* to five common antifungal agents are given in Table 2. The yeasts had high sensitivity to amphotericin B and 5-fluorocytosine, whereas these isolates show lower susceptibility to azole drugs (fluconazole, itraconazole and voriconazole) than amphotericin B and 5-fluorocytosine. Among the 41 isolates of *C. tropicalis*, there are 5 fluconazole-resistant ones, 3 fluconazole-susceptible dose dependent (SDD) isolates and 33 fluconazole-susceptible *C. tropicalis*, respectively. Additionally, the phenomenon of cross-resistance to fluconazole, itraconazole and voriconazole was tested in two isolates.

3. Mutations in ERG11 gene

The PCR product of all the *C. tropicalis* isolates resulted in a clear band on ethidium bromide stained, UV-transilluminated agarose gel (Figure 1). From the sequencing results of ERG11 gene in 41 *C. tropicalis* isolates, 33 mutations were found (Table 3), of which 29 were silent mutations and 4 missense mutations. 3 missense mutations were detected in 3 fluconazole-resistant strains (Figure 2), and no missense mutation was found in fluconazole-susceptible strains. None of these 3 missense mutations have been reported previously.

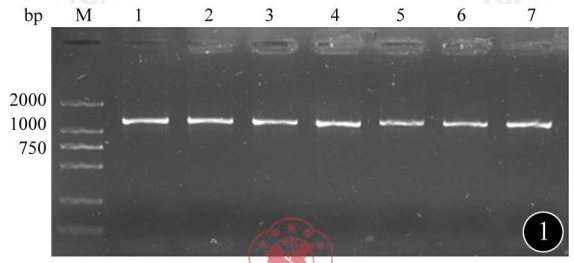
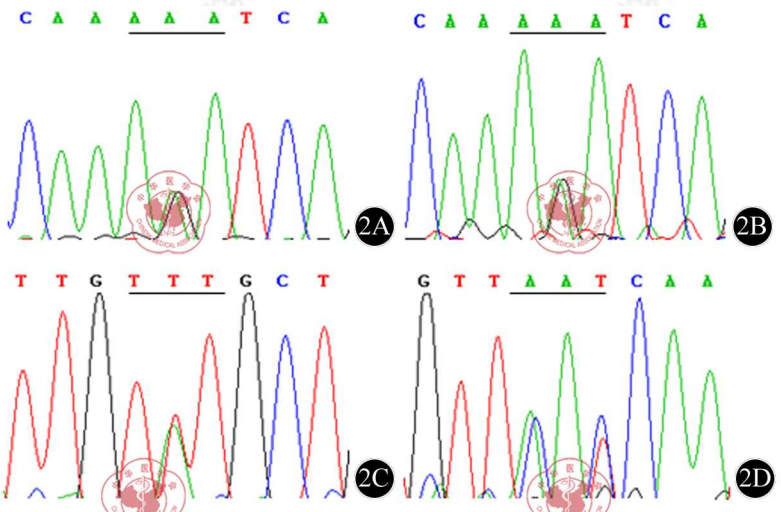


Figure 1 The target fragment of ERG11 gene amplified from *Candida tropicalis* strains.M: DNA Marker; 1: *Candida tropicalis* ATCC750; 2-7: a part of clinical isolates of *Candida tropicalis*



2A: strain 15, AGA→AAA, R245K; 2B: strain 17, AGA→AAA, R245K; 2C: strain 18, TAT→TTT, Y221F; 2D: strain 18, GTT→ATT(inverse sequencing), V362I

Figure 2 Missense nucleotide mutations in ERG11 gene of *Candida tropicalis* strains

4. Gene expression

The CDR1 gene in the 5 fluconazole-resistant isolates was upregulated 1.7 fold compared with the fluconazole-susceptible ones (Figure 3). Likewise, a 0.7 fold increase in MDR1 transcriptional levels was observed related to the susceptible control strains. Furthermore, the ERG11 mRNA level was approximately 1.4 fold higher in the azole-resistant isolates than in susceptible ones. To sum up, significant difference of the CDR1, MDR1 and ERG11 mRNA level was found between fluconazole-resistant isolates and the susceptible ones.

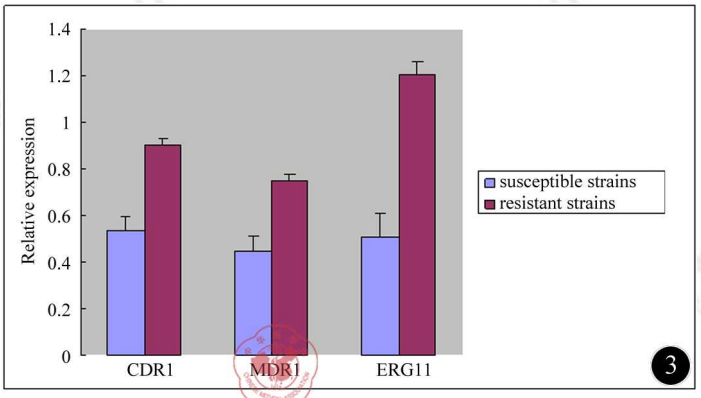


Figure 3 RNA expression level of CDR1,MDR1 and ERG11 gene in *Candida tropicalis* strains

Discussion

Little information is available concerning the molecular mechanism involved in fluconazole resistance in the clinical isolates of *C. tropicalis*. Nevertheless, due to the extensive use of antifungal agents in the prophylaxis or treatment of candidiasis, fluconazole-resistant clinical isolates are increasingly reported for this species. The increasing number of fluconazole-resistant isolates recovered in many institutions during the past decade has motivated studies with the aim of better understanding their mechanisms of resistance at the molecular level.

The mechanisms of resistance to azole antifungal agents have been studied principally in *Candida albicans*[6] and *Candida glabrata*[7-9]. And four main mechanisms are usually described[10]: (1) decrease in the affinity of azoles target ERG11p (lanosterol 14α-demethylase) due to point mutations in the ERG11 gene; (2) overexpression of the ERG11 gene, which encodes the azole target lanosterol 14α-demethylase; (3) increase in the efflux of the azole drug, due to the overexpression of efflux pumps such as the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) membrane transporters, encoded by CDR and MDR gene, separately; (4) otherwise, many such drug resistant manifestations are associated with the formation of *Candida* biofilms which restrict the penetration of drugs. These four mechanisms can be found separately, but a combination of different resistance mechanisms contributing to a step-by-step acquisition of azole resistance is responsible in clinical isolates of Candida species.

In this study, we searched for mutations in ERG11 gene not only in fluconazole-resistant strains but also in fluconazole-susceptible ones using the methods of PCR amplification and sequencing. We found 3 amino acid substitutions, interestingly, all of the three substitutions are novel and all of them were found in fluconazole-resistant isolates. Both of the isolate 15 and 17 showed the the amino acid substitution R245K. And in the fluconazole-resistant strain 18, there were 2 amino acid substitutions, Y221F and V362I, were found. Our results suggest that the amino acid substitutions R245K, Y221F and V362I may have a relationship with the fluconazole resistance in clinical of *C. tropicalis* isolates. However, further study of these mutations and fluconazole resistance in *C. tropicalis* is needed to provide a better understanding of the resistance mechanisms involved. Previous studies[11-12] demonstrated the relationship between Y132F substitution and resistance to fluconazole in clinical of *C. tropicalis* isolate, but we did not

find this amino acid substitution in our study.

The expression of ERG11 at the mRNA level in *C. albicans* varies considerably in the presence and absence of fluconazole. Some researchers have concluded there is no relationship between the expression levels of ERG 11 gene and fluconazole-resistance[13]. Nevertheless, overexpression of ERG11 gene has been observed in several fluconazole-resistant isolates[14-16]. In the present study, 5 fluconazole-resistant clinical isolates of *C. tropicalis* showed significant upregulation of ERG11 compared to the fluconazole-susceptible ones. In agreement with our data, Vandeputte et al[11] demonstrated that the ERG11 mRNA level was approximately twofold to fivefold higher in the azole-resistant isolate of *C. tropicalis* than in the susceptible isolates.

In fungi, multidrug resistance is mediated by two types of efflux pumps: the ATP-binding cassette (ABC) transporters using ATP as the energy source to drive transport; and the major facilitators (MFS), which are energized by the proton gradient across the cell membrane[17]. In *C. tropicalis*, CDR1 and MDR1 homologs were found to show overexpression after sequential passages of a drug-susceptible strain in the presence of increasing concentrations of fluconazole, concomitantly with the development of resistance to azoles and terbinafine[18]. Yet, the genes and the encoded efflux pumps have not been characterized in detail. To our knowledge, this study demonstrates for the first time that fluconazole resistance in clinical *C. tropicalis* isolates have the relationship with the upregulation of CDR1 gene and MDR1 gene. Similar results are existing in *C. albicans*[19-21] and *C. glabrata*[22], as shown by studies carried out by Silver et al[20].

Taken together, this study demonstrates the molecular mechanisms responsible for fluconazole-resistance in clinical isolates of *C. tropicalis*, showing that the decreased susceptibility to fluconazole of this isolates seemed to be due to missense mutations and an overexpression of ERG11, associated with the increase of efflux. Moreover, we found three novel ERG11 gene mutations which may be correlated with fluconazole-resistance. Even though, further experiments of additional isolates are needed to explore other still-unknown resistance mechanisms and which is the most frequent mechanism involved in fluconazole resistance in clinical isolates of *C. tropicalis*.

(本文中文文题:热带念珠菌临床分离株对氟康唑耐药分子机制的研究)

References

[1] Dixon DM, McNeil MM, Cohen ML, et al. Fungal infections: a growing threat. Public Health Rep, 1996, 111: 226-235.

[2] Nissapatorn V, Lee C, Fatt QK, et al. AIDS-related opportunistic infections in Hospital Kuala Lumpur. Jpn J Infect Dis, 2003, 56: 187-192.

[3] Kothari A, Sagar V. Epidemiology of candida bloodstream infections in a tertiary care institute in India. Indian J Med Microbiol, 2009, 27: 171-172.

[4] Myoken Y, Kyo T, Fujihara M, et al. Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. Haematologica, 2004, 89: 378-380.

[5] Yang YL, Ho YA, Cheng HH, et al. Susceptibilities of Candida species to amphotericin B and fluconazole: the emergence of fluconazole resistance in *Candida tropicalis*. Infect Control Hosp Epidemiol, 2004, 25: 60-64.

[6] Casalinuovo IA, Di Francesco P, Garaci E. Fluconazole resistance in Candida albicans: a review of mechanisms. Eur Rev Med Pharmacol Sci, 2004, 8: 69-77.

[7] Brun S, Bergès T, Poupard P, et al. Mechanisms of azole resistance in petite mutants of Candida glabrata. Antimicrob Agents Chemother, 2004, 48: 1788-1796.

[8] Sanguinetti M, Posteraro B, Fiori B, et al. Mechanisms of azole resistance in clinical isolates of Candida glabrata collected during a hospital survey of antifungal resistance. Antimicrob Agents Chemother, 2005, 49: 668-679.

[9] Vermitsky JP, Edlind TD. Azole resistance in Candida glabrata: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrob Agents Chemother, 2004, 48: 3773-3781.

[10] Sanglard D, Odds FC. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis, 2002, 2: 73-85.

[11] Vandeputte P, Larcher G, Bergès T, et al. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob Agents Chemother, 2005, 49: 4608-4615.

[12] LI JS. The study on drug resistance gene of Candida. J Prev Med Chin PIA,2006,24:12-15.
[13] White TC,Holleman S,Dy F,et al. Resistance mechanisms in clinical isolates of Candida albicans. Antimicrob Agents Chemother,2002,46:1704-1713.
[14] Perea S,López-Ribot JL,Kirkpatrick WR,et al. Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother,2001,45:2676-2684.
[15] Goldman GH,da Silva Ferreira ME,dos Reis Marques E,et al. Evaluation of fluconazole resistance mechanisms in candida albicans clinical isolates from HIV-infected patients in Brazil. Diagn Microbiol Infect Dis,2004,50:25-32.
[16] Ribeiro MA,Paula CR. Up-regulation of ERG11 gene among fluconazole-resistant Candida albicans generated in vitro:is there any clinical implication? Diagn Microbiol Infect Dis,2007,57:71-75.
[17] Del Sorbo G,Schoonbeek H,De Waard MA. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol,2000,30:1-15.
[18] Barchiesi F,Calabrese D,Sanglard D,et al. Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. Antimicrob Agents Chemother,2000,44:1578-1584.
[19] Schuetzer-Muehlbauer M,Willinger B,Krapf G,et al. The Candida albicans Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin. Mol Microbiol,2003,48:225-235.
[20] Silver PM,Oliver BG,White TC. Characterization of caspofungin susceptibilities by broth and agar in Candida albicans clinical isolates with characterized mechanisms of azole resistance. Med Mycol,2008,46:231-239.
[21] Holmes AR,Lin YH,Niimi K,et al. ABC transporter Cdr1p contributes more than Cdr2p does to fluconazole efflux in fluconazoleresistant Candida albicans clinical isolates. Antimicrob Agents Chemother,2008,52:3851-3862.
[22] Torelli R,Posteraro B,Ferrari S,et al. The ATP-binding cassette transporter-encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of Candida glabrata. Mol Microbiol,2008,68:186-201.

(Received:2011-06-13)
(Editor: ZHANG Lan)

ZHOU Yong-an,DOU Juan,ZHANG Quan-bin,et al. Molecular mechanisms of fluconazole resistance in clinical isolates of *Candida tropicalis*[J/CD]. 中华临床医师杂志:电子版,2011,5(21):6329-6335.