Research Article

ANALYSIS OF MOLECULAR RESISTANCE MECHANISMS OF ITRACONAZOLE IN *CANDIDA ALBICANS* CLINICAL ISOLATES FROM INDIA

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ABSTRACT

Opportunistic fungal infections resistant to antifungal agents have been increasingly documented in recent years and their frequency will likely continue to increase. This phenomenon appears due largely to the extensive use of antifungal agents to treat fungal infections that typically occur in severely immunocompromised and/or critically ill patients. *Candida* species are leading fungi responsible for these invasive infections. In this study, we describe resistance mechanisms in Itraconazole-resistant isolates of *Candida albicans* isolated from AIDS patients. These mechanisms include the presence of point mutations in the *ERG11* gene and overexpression of *ERG11,* and several genes encoding efflux pumps, as measured by quantitative real-time reverse transcriptase polymerase chain reaction. Several fluconazole-resistant strains had multiple mechanisms of resistance. Four mutations previously described, Y132F, K143R, E266D, and V437I, were identified among the strains, whereas some isolates contained more than one mutation. Fourteen novel mutations were identified. Interestingly, all Itraconazole-resistant isolates showed homozygosity at mating-type loci (*MTL*) associated with Itraconazole resistance.

Keywords: Molecular resistance, Fungal infections, *Candida albicans,* Fluconazole, Itraconazole.

INTRODUCTION

The frequency of *Candida* infection invasive disease has risen approximately 14-fold over the 16 years to 1992, as judged after death in unselected autopsies[1]. Candidiasis as the most frequent fungal pathogen detected post mortem in tertiary care hospitals in Europe[3]. Thus 4% of all patients dying had Candidiasis[3]. Patients at risk based on the disease frequency include those with chronic granulomatous disease (25-40%), lung transplant recipients (17-26%), bone marrow transplant patients (4-30%), neutropenic patients with leukemia (5-25%), heart transplant recipients (2-13%), pancreas transplant recipients (1-4%), renal transplant patients in Europe and the USA (~1%) and in India (~10%), and patients with AIDS, multiple myeloma and severe combined immunodeficiency (~4%)[4-6]. Over 500,000 transplants are performed annually in the world. Acute leukemia affects about 3/100,000 of the population and on average each patient receives 3 cycles of chemotherapy, with each cycle defining a major risk period[7]. In the industrialized nations alone these treatment protocols generate about 250,000 periods of major risk per year. AIDS cases are predicted to exceed 60 million by the end of the year 2007 which would result in about 2.1 million cases of Candidiasis, although in developing countries most patients will not live long enough to get this disease[8-10].

The predominant cause of fungal infections in hospitalized patients remains *C. albicans,* pathogenic yeast that causes oral, vaginal, and systemic infections[11]. Triazole drugs such as fluconazole and itraconazole are commonly used to treat *C. albicans*. However, resistant strains often emerge during long-term or prophylactic treatment. Two major mechanisms of itraconazole resistance have been identified so far in these strains: (I) alterations in the drug target (14-α-sterol demethylase, the product of the *ERG11* gene), which results in an increased level of production of the enzyme or in its reduced binding affinity for itraconazole, and (II) a reduced level of intracellular itraconazole, which correlates with the over expression of the *CDR1* and *CDR2* genes encoding transporters of the ABC family and of the *MDR1* and *FLU1* genes coding for major facilitators[12-14]. It has already been observed that multiple mechanisms of itraconazole resistance can arise in a single *C. albicans* isolate[15-16]. We assessed resistance mechanisms of Itraconazole-resistant strains of *C. albicans*.

MATERIALS AND METHODS

The *C. albicans clinical isolates* were obtained from different hospitals of India that were obtained from immunocompromised patients.

Culture of *C. albicans*

The pathogenic strains of *Candida* were cultured on Sabouraud dextrose agar plates. An amount of 47.0 g Sabouraud dextrose agar (E. Merck, Cat No.V868415) was dissolved in 1.0 L of sterile distilled water according to the manufacturer's instructions, in a conical flask. The medium was autoclaved at 10 psi for 15 min. It was cooled to 45°C and approximately 15.0 ml of the same dispensed into the sterilized Petri plates from Tarson (Cat No. 460090-90). The plates were allowed to cool at room temperature for 3 h. Sabouraud dextrose agar plates were inoculated with *C. albicans*, incubated for 96 h in BOD incubator (Thermo Scientific) at 37°C.

Preparation of Spore Suspension

Spores/conidia from the fungal colonies of 96 h cultures in the plates were picked up either by an applicator and transferred to 1.0 ml of sterile physiological saline in a tube or harvested by adding 1.0 ml of sterile physiological saline directly to the plates and recovering back the spore suspension into screw cap tube. The tubes were vortexes gently for 3-4 min at room temperature to prepare a homogeneous suspension of the conidia. The conidia were counted using hemocytometer and the number was adjusted to $1x10^6$ spores/ml.

Susceptibility testing

Antifungal susceptibility testing was performed by using the National Committee for Laboratory Standards (NCCLS) reference broth microdilution[18.] Reference powders of itraconazole and fluconazole, and ketoconazole, were kindly provided by the manufacturers: Sigma Group (USA). Amphotericin B was obtained from Sigma.

Sequencing of the ERG11 gene

The open reading frame was polymerase chain reaction (PCR) amplified using Taq DNA platinum polymerase high fidelity (Invitrogen) and primers described in James B A et al., and Gustavo *et al*., 2004. PCR conditions were as follows: 94°C for 2 minutes and 35 times 94°C for 1 minute, 55°C for 1 minute, and 68°C for 2 minutes, followed by an extension step at 68°C for 10 minutes. After the reaction, the approximately 1.7-kb PCR product was purified with a Qiagen PCR cleanup kit and inserted into TOPO TA cloning kit (Invitrogen) following manufacturer's instructions. Sequencing reactions were prepared using BigDyeTM Terminator Cycle Sequencing (Applied Biosystems) and primers were used according to James B A *et al.*, and Gustavo *et al*., 2004. The nucleotide sequences were determined in both strands by primer elongation with an ABI 3100 automated DNA sequence (Applied Biosystems). Sequence data were compared with a published *ERG11*sequence using BLAST[16].

PCR analysis of the (mating type locus) MTL locus

The PCR protocol used to analyze the MTL locus was the following: 94°C for 5 minutes, 35 times 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and an extension step at 72°C for 10 minutes. The presence of *MTL***a** was ascertained by amplifying *MTL***a**1 and the associated gene *PAP***a**[20.] The presence of *MTL*α was ascertained by amplifying *MAT*α1 and *MAT*α2 and the associated gene *PAPα*. These three different genes, *MTL*α1, *MTL*α2, and *MTL***a**1, are the *C. albicans* homologues of *Saccharomyces cerevisiae* mating-type genes *MAT*α1, *MAT*α2, and *MAT***a**1, respectively[21].

DNA and RNA isolation, and real-time RT-PCR

$1x10^6$ conidial spore was transferred in a 1.5 ml tube with 700 µl TRIzol (Sigma), followed by addition of 200 µl chloroform. Sample is mixed by inverting the tube for 15 sec, followed by 3 minute incubation at room temperature, **c**entrifugation at 12,000 g for 15 min, and transfer of aqueous phase into a fresh microcentrifuge tube. Thereafter, 500 µl isopropanol was added and centrifuged at 12,000 g for 10 min in cold room. The pellet was washed with 500 µl in 70 % ethanol, centrifuged at max. 7,500 g for 5 min in the cold room dried in air for 10 min and dissolved in 50 µl DEPC-H_2O. To verify RNA integrity, 20 micrograms of RNA were fractionated in a 2.2-M formaldehyde, 1.2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. The presence of intact 28S and 18S ribosomal RNA bands (semi quantitatively in a 2:1 ratio) was used as a criterion to determine whether the RNA was degraded. RNAse-free DNAse treatment was done. The absence of DNA contamination after the RNAse-free DNAse treatment was verified by PCR amplification of the *ACT1* gene. cDNA was synthesized by using the Super-Script reverse transcriptase (Gibco, BRL).

All the RT-PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystem, USA). The Taq- ManR PCR Reagent kit was used for PCR reactions. The thermal cycling conditions comprised an initial step at 50°C for 2 min, followed by 10 minutes at 95°C, and 40 cycles at 95°C for 15 seconds and 60°C for 1 min. Primer and probe sequences are described in James B A *et al*., 2003.

RESULTS

The minimal inhibitory concentration (MIC) values obtained for 20 *C. albicans* isolates with the three different azoles (fluconazole, itraconazole, and ketoconazole) are summarized in Table 1. The 20 *C. albicans* isolates included 9 that were resistant to itraconazole e (MICs ≥64 µg/mL), 6 that were susceptible-dose dependent (S-DD for MICs of 16 and 32 µg/mL), and 5 that were susceptible to fluconazole (MICs < 8µg/mL). Regarding itraconazole, 1 isolate was considered resistant to fluconazole (MIC ≥1 µg/mL), 10 were considered as S-DD (MICs of 0.25 and 0.5µg/mL), and 9 were susceptible to fluconazole (MIC > 0.25< 0.5) (Table 1). All of them were susceptible to amphotericin B (MICs < 1 µg/mL). Table 1 shows that 12 out 15 isolates S-DD or resistant to itraconazole were also considered as S-DD (9 for itra and keto) or resistant (1 for itra and 2 for keto) to the other azoles[23-25]. Otherwise, only 1 out of 5 isolates susceptible to fluconazole exhibited an MIC value compatible with S-DD.

ERG11, CDR1, CDR2, MDR1, and FLU1 genes implicated in azole resistance

Overexpression of the genes *ERG11, CDR1, CDR2, MDR1,* and *FLU1* has been associated to itraconazole resistance and was investigated as a mechanism of resistance in our clinical isolates by using real-time RTPCR.[21] The cDNA levels of the different genes were normalized using the *ACT1* gene (encoding actin); comparable results were observed when the *PMA1* gene was used as a normalizer gene[27]. Because a matched set of isolates was not available for this collection of itraconazole - resistant

isolates, the mRNA expression levels of isolates 137 and 168 were used as controls[22]. Table 2 illustrates the number of times each respective gene was expressed greater than the average expression of the same gene in the control isolates (where the mRNA expression levels were given a value of 1.0). When this criterion is used, the isolates that are resistant to itraconazole expressed *ERG11* at much higher levels (11.2 to 14.4 times) than the average in isolates 114, 116, 117, 168, and 172. The *CDR1* gene was more highly expressed in isolates 116, 168, and 172, whereas the *CDR2* gene was more highly expressed in the isolates 214, 116, 117, 133, 168 and 172. *MDR1* was more highly expressed in the isolate 133, whereas *FLU1* was more highly expressed in isolates 18, 136, and172. Considering the isolates SSD to itraconazole, the *ERG11* is more expressed in the isolate 185, whereas the *CDR1* gene is more expressed in the isolates 21 and 85[28-30]. The *CDR2* is more expressed in the isolates 121, 169, 185, and 186. *MDR1* is more expressed in the isolates 119 and 169, whereas the *FLU1* is more expressed in the isolate 119. Interestingly, some isolates that show susceptibility to itraconazole, such as 151, 155, and 161, show higher expression of *MDR1* and *CDR2, CDR2,* and *ERG11,* respectively[31]. Taken together, our results show that some isolates concomitantly over expressed different genes involved in drug resistance in *C. albicans*[32].

Table 1: Patient demographics, MIC values of clinical isolates, and *ERG11* sequence analysis for isolate collection

S. No.	Reference isolate number	Patient's age and gender	ITRA	FLC	KTC
1	168	F-45	64	0.25	0.25
2	18	M-NA	64	0.25	0.5
3	133	F-NA	64	0.25	0.25
4	116	M-25	64	0.25	0.5
5	121	M-33	32	0.5	1.0
6	115	M-42	32	0.25	0.5
7	169	M-49	32	0.25	0.25
8	185	M-36	32	0.5	0.125
9	119	M-32	16	0.06	0.125
10	186	M-44	16	0.125	0.06
11	114	M-32	>64	0.25	0.5
12	123	M-42	>64	0.125	0.5
13	712	F-38	>64	0.125	0.125
14	136	M-38	>64	0.03	2
15	117	M-27	64	1.0	0.5
16	161	M-36	8	0.03	0.06
17	151	M-37	4	0.06	0.06
18	155	F-29	4	0.25	0.125
19	237	M-62	0.25	0.03	0.03
20	268	M-25	0.125	0.03	0.03

NA, not available; M, male; F, female; ND, not detected; NE, not evaluated; FLC, fluconazole; ITRA, itraconazole; KTC, ketoconazole.

Table 2: *ERG11* and efflux transporter gene mRNA expression levels in the clinical isolates as assessed by real-time RT-PCR.

Isolate number	MIC fluconazole (µg/mL)	*FLU1*	*MDR1*	*CDR1*	*CDR2*	*ERG11*
168	64	2.3	1.3	5.8	386.3	12.1
18	64	5.3	6.7	2.9	4.3	4.3
133	64	1.9	12.0	2.6	19.5	5.1
116	64	2.0	6.0	4.3	24.8	21.3
115	32	0.9	0.7	3.1	8.0	3.5
121	32	2.9	2.0	4.7	1,241	7.6
169	32	4.4	70.0	4.0	15.5	2.9
185	32	3.0	2.7	7.7	186.5	15.2
119	16	5.0	57.3	1.8	2.3	8.0
186	16	2.3	3.3	2.8	21.0	3.9
161	8	4.1	2.0	0.9	3.5	10.3
151	4	3.6	27.3	3.5	82.5	2.1
155	4	2.3	5.3	4.0	9.5	4.4
237 and 268	0.125 and 0.25	1.0	1.0	1.0	1.0	1.0
114	>64	1.7	0.7	3.7	24.0	14.4
123	>64	1.0	1.3	1.2	0.8	0.77
172	>64	5.3	4.7	4.2	43.3	26.0
136	>64	5.0	2.7	2.8	4.5	5.8
117	64	2.1	0.7	3.0	21.3	11.2

cDNA levels were calculated relative to those of the average cDNA levels of the isolates 137 and 168. The numbers represent the averages of 3 replicates, with the ranges shown in parentheses.

DISCUSSION

The clinical isolates used in this study were screened for the currently characterized molecular mechanisms of azole resistance. The first hot spot comprising G464S, G465S, and R476K associates with the N-terminal part of the cysteine pocket[29-31]. A second hot spot is mapped to the C-terminus of the G helix and H helix, and a third hot spot comprising F72L, F105L, S495F, and T229A associates with the domain interface. The fourth hot spot comprising D116E, F126L, K128T, G129A, Y132H, K143R, F145L, K147R, A149V, and D153E is located in the region between the B' and C helices that have been postulated to be involved in inhibitor or substrate-induced structural changes. The mutations identified in *C. albicans* itraconazole -resistant isolates indicate that azole resistance in fungi develops in protein regions involved in orchestrating the passage of CYP51p through different conformational stages rather than in residues directly contacting the triazole[25-29]. Some of our isolates contained more than one mutation, and 14 novel mutations were identified among them.

Real-time RT-PCR assays were used to obtain more accurate data on gene expression in *C. albicans.* We assessed the quantitative expression of two ABC transporters, *CDR1* and *CDR2,* and two MFS transporters, *MDR1* and *FLU1,* and also *ERG11.* Some clinical isolates showed increased mRNA expression of the *ERG11* gene, which encodes the target 14-α- demethylase have previously shown, in a collection of itraconazole-resistant and -susceptible isolates, considerable variation in the levels of *ERG11* mRNA expression, but this expression did not seem to be related to the azole resistance of the isolates. However, *ERG11* overexpression has been found in many other itraconazole- resistant *C. albicans*isolates compared with matched susceptible isolates .Overexpression of *ERG11* from *C. albicans*has been shown to confer a fivefold enhanced resistance to itraconazole in *S.cerevisiae*. Therefore, constitutive *ERG11* overexpression may contribute to itraconazole resistance in our clinical *C. albicans* isolates.

REFERENCES

1. Hajjeh R A, Sofair AN, Harrison L H, Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J Clin Microbiol, 42, 2004, 1519-1527.
2. Barberino MG, Silva N, Reboucas C, Barreiro K, Alcantara A P, Netto E M, Albuquerque L, Brites C, Evaluation of Blood Stream Infections By *Candida* In Three Tertiary Hospitals In Salvador, Brazil: A Case-Control Study. Braz J Infect Dis, 1, 2006, 36-40.
3. Lorenz M C, Fink G R, The glyoxylate cycle is required for fungal virulence, Nature, 412, 2001, 83–86.
4. Geiger A M, Foxman B, Gillespie B W, The epidemiology of vulvovaginal candidiasis among university students, Am J Public Health, 85, 1995, 1146–8.
5. Trick W E, Fridkin S K, Edwards J R, National Nosocomial Infections Surveillance System Hospitals. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999, Clin Infect Dis, 35, 2002, 627-630.
6. Casaulta C, Fluckiger S, Crameri R, Blaser K, Schoeni MH, Time course of antibody response to recombinant Candida fumigatus antigens in cystic fibrosis with and without ABPA, Pediatr Allergy Immunol, 16,2005, 217-25.
7. Jantunen E, Piilonen A, Volin L , Diagnostic aspects of invasive candida infections in allogeneic BMT recipients. Bone Marrow Transplant 25, 2000, 867–871.
8. Fink L N, Immune Responses to Candida Bronchopulmonary candidiasis fumigatus and Pseudomonas aeruginosa Antigens in Cystic Fibrosis and Allergic, Chest, 106, 2007, 513-519.
9. Meyer JD, Fungal infection in bone marrow transplants patients, Semin,Oncol, 17, 1990, 10.
10. Banerjee B, Kurup V P , Molecular biology of *Candida* allergens, Front Biosci, 8, 2003, s128-s139.
11. Centeno-Lima S, Silveira H, Casimiro C, Aguiar P, do Rosario V E, Kinetics of cytokine expression in mice with invasive aspergillosis lethal infection and protection, FEMS Immunol Med Microbiol, 32, 2002,167-173.
12. Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M, PCR detection of DNA specific for candida species in serum of patients with invasive aspergillosis, J Clin Microbiol, 34,1996, 2464–2468.
13. Zander D S Allergic bronchopulmonary aspergillosis: an overview, Arch Pathol Lab Med, 129,2005,924-8.
14. White TC, Holleman S, Dy F, Mirels LF, Stevens DA, Resistance mechanisms in clinical isolates of *Candida albicans,* Antimicrob Agents Chemother 46, 2002, 1704–1713.
15. National Committee for Clinical Laboratory, Standards Reference method for both dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27A. National Committee for Clinical laboratory Standards, Wayne, Pa. (2002).
16. Lai MH, Kirsch DR Nucleotide sequence of cytochrome P450L1A1 (lanosterol 14 alpha-demethylase) from *Candida albicans*, Nucl Acids Res 17: 1989, 804.
17. Mellado E G, Garcia-Effron L, Alcazar-Fuoli W J, Melchers P E, Verweij M, Cuenca-Estrella, and Rodriguez-Tudela J L, A new *Candida fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob, Agents Chemother, 51, 2007, 1897–1904.
18. Alcazar-Fuoli L E, Mellado G, Garcia-Effron M J, Buitrago J F, Lopez J O, Grimalt M, Cuenca-Estrella, and Rodriguez-Tudela J L, *Candida fumigatus* C-5 sterol desaturases (Erg3A and Erg3B): role in sterol biosynthesis and antifungal drugs susceptibility, Antimicrob, Agents Chemother,50,2006,453–460,
19. Diaz-Guerra T M, Mellado E, Cuenca-Estrella M, and Rodriguez- Tudela J L, A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Candida fumigatus*. Antimicrob, Agents

Chemother,47,2003,1120–1124.

20. James B A, Caroline S S, Ainslie B P, Charles B, Claire, Cowen L E, Linda M. K .: Mode of Selection and Experimental Evolution of Antifungal Drug Resistance in *Saccharomyces cerevisiae*, Genetics, 163, 2003, 1287–1298.
21. Gustavo H, Goldman, Márcia E da S F, Everaldo dos R M, Marcela S, David P, Steven P, Patricio C G M, Maria Helena S G and Arnaldo L C, Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil, Diagnostic Microbiology and Infectious Disease,50,2004,25–32.
22. Mellado E L, Alcazar-Fuoli G, Garcia-Effron A, Alastruey-Izquierdo M, Cuenca-Estrella, and Rodriguez-Tudela J L, A new *Candida fumigatus* resistance echanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob. Agents Chemother. 51, 2007, 1897–1904.
23. Pryce T M, Palladino S I D Kay, and Coombs G W, Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Med. Mycol. 41,2003,369–381.
24. Wang L K, Miyaji M and Nishimura K, Mitochondrial cytochrome *b* gene analysis of *Candida fumigatus* and related species, J Clin Microbiol, 38,2000,1352–1358.
25. Podust L M, Poulos TL, Waterman M R, Crystal structure of cytochrome P450 14α-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. Proc Natl Acad Sci USA *98*, 2001, 3068–3073.
26. Fisher MC, Koenig GL, White TJ, Taylor J W, Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non–California population of *Coccidioides immitis*. Mycologia 94, 2002, 73–84.
27. Maslov DA, Westenberger SJ, Xu X, Campbell DA, Sturm NR, Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador, Journal of Eukaryotic Microbiology 54, 2007, 57–65 .
28. Rydholm C, Dyer PS, Lutzoni F, DNA sequence characterization and molecular evolution of MAT1 and MAT2 mating-type loci of the self-compatible ascomycete mold *Neosartorya fischeri*. Eukaryotic Cell 6, 2007,868–874.
29. Semighini CP, Marins M, Goldman MHS, Goldman GH, Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Candida nidulans* by Real-Time Reverse Transcription- PCR assay, Appl Environ Microbiol *68*, 2002, 1351–1357.
30. Semighini CP, Marins M, Goldman MHS, Goldman GH, Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Candida nidulans* by Real-Time Reverse Transcription- PCR assay, Appl Environ Microbiol *68*, 2002,1351–1357.
31. Maslov D A, Westenberger S J, Xu X, Campbell D A, Sturm N R, Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador, Journal of Eukaryotic Microbiology 54, 2007, 57–65.
32. Rydholm C, Dyer P S and Lutzoni F, DNA sequence characterization and molecular evolution of MAT1 and MAT2 mating-type loci of the self-compatible ascomycete mold Neosartorya fischeri, Eukaryotic Cell 6, 2007,868–874.
33. Semighini CP, Mozart Marins, Maria Helena S G and Gustavo H G, Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Candida nidulans* by Real-Time Reverse Transcription- PCR assay, Appl Environ Microbiol *68*, 2002,1351–1357.

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