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Molecular Characterization and *In Vitro* Antifungal Susceptibility of Candida Glabrata Clinical Isolates with Reduced Echinocandin Susceptibility and High Level Multi-Azole Resistance

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Abstract

Candida glabrata is the second most commonly isolated yeast recovered from blood cultures in the United States. We characterized 85 *C. glabrata* clinical isolates recovered from various clinical specimens obtained from immunocompromised individuals. This collection was unique because it included a series of isolates recovered from the blood of a patient who only partially responded to antifungal therapy. *In vitro* activity of caspofungin, micafungin, anidulafungin, fluconazole, voriconazole and amphotericin B was evaluated. Most of the isolates were susceptible to the echinocandins, triazoles and amphotericin B. The geometric mean MIC of the antifungals for the susceptible isolates (n=79) were as follows: caspofungin, 0.061315 ± 0.076934; micafungin, 0.123521 ± 0.457202; anidulafungin, 0.044158 ± 0.895249; fluconazole, 7.013461 ± 20.56794; voriconazole, 0.324939 ± 1.051247; amphotericin B, 0.474923 ± 0.162994. Five of the six serial blood isolates showed a reduced echinocandin susceptibility (RES) to the echinocandins and the triazoles. Characterization of the hot spot 1 region of FKS1, FKS2 and FKS3 showed no amino acid alterations. However, the genes coding for the drug efflux proteins CgCDR1, CgCDR2, CgSNQ2, as well as Cgcyp51 were over-expressed in the isolates with RES and azole resistance compared to the susceptible isolates, indicating that the upregulation of the synthesis of efflux proteins and the drug target is responsible for conferring resistance to triazoles in these isolates. These results demonstrate that multi-echinocandin and multi-azole resistant *C. glabrata* clinical isolates can emerge under the selection pressure imposed by specific drug therapy over a relatively short period of time.

Keywords: Candida glabrata; Antifungal resistance; Candidemia; Azoles; Echinocandins

Introduction

Candida glabrata is the second most commonly isolated Candida species in North America, causing mucosal and disseminated candidiasis infections [1-3]. Infections due to *C. glabrata* tend to have higher mortality rates and are more difficult to manage because of their resistance to azoles [4-6].

The antifungal resistance to azoles, especially fluconazole in *C. glabrata* is not uncommon (~25%) and is generally due to the upregulation of synthesis of one or more members of two classes of efflux proteins, the ATP binding cassette (ABC) transporters and the major facilitator superfamily (recently known as MDR1) of efflux pumps [4,7-13]. Exposure of *C. glabrata* to azoles results in overexpression of the genes coding for these proteins. Additionally, the *C. glabrata* cyp51 gene codes for a 533 amino acid polypeptide. Alterations of the Cyp51p leads to azole resistance in other Candida species such as C. albicans [14-16]. Occasionally, high level resistance to azoles is reported to be due to the combined effect of drug efflux and drug target modification [17,18].

The echinocandins are the newest class of antifungals approved for the treatment of disseminated candidiasis and candidemia. These drugs inhibit the synthesis of β-1, 3-D-glucan, the major component of the fungal cell wall by non-competitively inhibiting glucan synthase [19,20]. The glucan synthase complex is composed of multiple subunits called Fks1p, Fks2p and Fks3p and encoded by FKS1, FKS2 and FKS3, respectively. Fks1p is believed to be the catalytic subunit responsible for the synthesis of β-1, 3-D-glucan. The induction of Fks2p, appears to compensate for the lack of Fks1p in terms of glucan synthase activity. Fks2p is also thought to be involved in cell wall assembly.

Investigators have reported the emergence of various Candida species with either reduced echinocandin susceptibility (RES) or echinocandin resistance (ECR) [21-25]. Several studies have shown that the primary mechanism for resistance to the echinocandins appears to be the alteration of glucan synthase, in particular Fks1/Fks2 [22,26-29]. Most of the amino acid mutations on the Fks1p related to RES appear to be confined to two regions of the protein and have been designated 'hot spot' 1 (HS1) and 'hot spot' 2 (HS2), located on the amino terminal and the carboxyl terminal halves of the protein, respectively [30]. In C. glabrata, RES is usually caused by the acquisition of point mutations in HS regions of the fks gene [24,25]. In a recent study of 119 *C. glabrata* isolates, FKS alterations were detected in 31% of strains with elevated MICs to echinocandins, 28 of the 37 strains had HS substitutions [24]. Other known mechanisms such as

efflux and reduced permeation of the drug into the cell appear to play no significant role in conferring RES.

The primary objective of this study was to investigate the *in vitro* susceptibilities of the *C. glabrata* clinical isolates collected in a tertiary care institution over a three-year period and characterize the *C. glabrata* clinical isolates with RES and azole resistance.

Case Report

A 47-year-old female was transferred to our institution with an intra-abdominal abscess which resulted from a perforated duodenal ulcer. Prior to transfer, caspofungin 50 mg daily was initiated for persistent *C. glabrata* candidemia. *C. glabrata* also grew from a CT-guided aspiration of the abscess. Candidemia resolved after three-wks of therapy and adequately draining the intra-abdominal abscess. Three months later the patient was re-admitted for fever and found to have a recurrence of the *C. glabrata* candidemia. A repeat CT scan of her abdomen revealed reaccumulation of the abscess. This time, it was surgically drained and all catheters were changed. The candidemia resolved after three-weeks of micafungin 100 mg daily IV and was continued for 4 wks after blood cultures were negative. Two weeks later, the patient was re-admitted to the hospital with Clostridium difficile colitis. The blood cultures again grew *C. glabrata* and an echocardiogram revealed vegetation on the coronary cusp of the aortic valve. The patient refused to have cardiac surgery for presumptive candidal endocarditis and antifungal therapy with micafungin 100 mg/day and liposomal amphotericin B 5 mg/kg/day was initiated. The candidemia cleared after 10 days of combination therapy and the patient was discharged home on micafungin 100 mg/day. After discharge the patient was lost of follow up.

Materials and Methods

Fungal Isolates

Eighty-five *C. glabrata* isolates were obtained from a variety of clinical specimens including blood, peritoneal fluid, abdominal abscess, pleural fluid and bile. The isolates were collected from patients in Henry Ford Hospital, Detroit, Michigan, USA over a 3-yr period from January 2006 to December 2008. Included in this collection of *C. glabrata* isolates were six serial isolates from a patient admitted with intra-abdominal abscess and recurrent *C. glabrata* candidemia.

In vitro susceptibility

The antifungal drugs were obtained as from the manufacturers. *In vitro* susceptibilities were determined in RPMI1640 using the M27-A2 broth microdilution method (CLSI) [31].

DNA isolation and purification

DNA was isolated using MasterPureTM Yeast Purification Kit (Epicentre Biotechnologies, Madison, WI). The DNA was treated with RNase A to remove the contaminating RNA and purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA).

Isolation and purification of total RNA

RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The quality of the RNA was examined by RNA agarose gel electrophoresis using the yeast 28S and 18S ribosomal RNA as molecular size markers.

Amplification and nucleotide sequence determination of Hot Spot 1 region of FKS1, FKS2 and FKS3

Oligodeoxynucleotide primers designated FKS1.fwd and FKS1.rev (Table 1) designed based on the published FKS1 gene sequence of *C. glabrata* (accession XM_446406) [32]. A 600-bp DNA fragment containing the HS1 region of FKS1 was amplified by PCR as previously described [27]. Similarly, a 577-bp and 663-bp DNA fragments containing the HS1 regions of FKS2 and FKS3 were amplified using the primer pairs FKS2.fwd, FKS2.rev and FKS3.fwd, FKS3.rev, respectively. The PCR products were purified and the sequences of the amplicons were determined by BigDye chain termination reaction followed by sequence analysis. The sequences were compared with that of the C. glabrata ATCC90030 using Clustal W Multiple DNA and Protein Sequence Alignment Program (**www.align.genome.jp/clustalw**).

Gene	Accession	Primer sequence and designation	Application
CgFKS1	XM_446406	GTTGCAGTCGCTACATTGCTA (FKS1.fwd) TAGCGTTCCAGACTTGGGAA (FKS1.rev)	PCR, sequencing
CgFKS2	NC_006030	GTTCAATCAAAGGCAGGCCA (FKS2.fwd) GTAAATGTTCTCTGTACATGGA (FKS2.rev)	PCR, sequencing
CgFKS3	NC_006030	TGGAGCCCAGCACTTAACAA (FKS3.fwd) GTCCATCTCGGATGTTGCTA (FKS3.rev)	PCR, sequencing
CgCDR1	AF109723	TAGCACATCAACTACACGAACGT (CDR1.fwd) AGAGTGAACATTAAGGATGCCATG (CDR1.rev) TGCTGCTGCTTCTGCCACCTGGTT (CDR1.pr)	RT-PCR, probe
CgCDR2	AF251023	GTGCTTTATGAAGGCTACCAGATT (CDR2.fwd) TCTTAGGACAGAAGTAACCCATCT (CDR2.rev) TACCTTTGCGTGCTGGGCGTCACC (CDR2.pr)	RT-PCR, probe
CgSNQ2	AF251022	ACCATGTGTTCTGAATCAATCAAT (SNQ2.fwd) TCGACATCATTACAATACVAGAAA (SNQ2.rev) AACTAATCGCCGCAGGTTGTGACA (SNQ2.pr)	RT-PCR, probe
CgCYP51	L40389	ATTGGTGTCTTGATGGGTGGTC (CYP51.fwd) TCTTCTTGGACATCTGGTCTTTCA (CYP51.rev) ACTTCCGCTGCTACCTCCGCTTGG (CYP51.pr)	RT-PCR, probe

CgURA3	L13661	GAAAACCAATCTTTGTGCTTCTCT (URA3.fwd) CATGAGTCTTAAGCAAGCAAATGT (URA3.rev) ACGTCACCACCACCAGCGAATTG T (URA3.pr)	RT-PCR, probe
Note: The 5'- and the 3'-terminii of CgCDR1, CgCDR2, CgSNQ2 and CgCYP51 probes were labeled with 6-carboxyfluorecein (6FAM) and 6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA), respectively whereas the 5'- and the 3'-ends of URA3 probe is tagged with Texas Red (Molecular Probes and BHQ2, Black Hole Quencher 2			

Table 1: List of oligodeoxynucleotide primers and probes used in this study

Determination of gene expression

The transcriptional upregulation of the genes coding for the efflux pump proteins of C. glabrata, CgCDR1, CgCDR2 and CgSNQ2 as well as Cgcyp51 gene coding for P450 lanosterol demethylase were examined by determining the levels of mRNA produced in the drug resistant isolates compared to that in the drug susceptible baseline clinical isolate by real time RT-PCR. Gene specific RT-PCR primers and Taqman probes (Table 1) were designed using Beacon Designer 6.0 and tested for specificity by the online National Center for Biotechnology Information (NCBI) program. The comparative threshold cycle method was used to calculate relative gene expression. A twofold or greater increase in expression compared to that obtained in the drug susceptible parent baseline isolate was considered indicative of overexpression.

Strain delineation

Genetic relatedness of the *C. glabrata* serial isolates was examined by electrophoretic karyotyping by contour-clamped homogeneous electric field (CHEF) using a CHEF-DRIII (BioRad Life Science Research, CA) as previously published [33].

Results

In vitro susceptibility assays

The MICs of 85 clinical isolates of *C. glabrata* were evaluated. As shown in Table 2, most of the isolates were susceptible to all the antifungals, with the exception of a series of six isolates recovered from the bloodstream of the patient described previously. The MICs of the susceptible isolates ranged between 0.03 - 0.12 μg/ml for anidulafungin, 0.03 - 0.25 μg/ml for micafungin, 0.03 - 0.25 μg/ml for caspofungin, 0.12 – 1.0 μg/ml for amphotericin B, and 0.12 - 4 μg/ml for voriconazole. The widest MIC range was for fluconazole (0.5 - 64 μg/ml) (Table 2).

Antifungal drugs	MIC (μg/ml) range	MIC_{50}	MIC_{90}	GM ± SD
Caspofungin	0.03-0.25	0.03	0.12	0.061 ± 0.076
Micafungin	0.03-0.25	0.12	0.25	0.123 ± 0.457
Anidulafungin	0.03-0.12	0.03	0.06	0.044 ± 0.895
Amphotericin B	0.12-1	0.5	0.5	0.474 ± 0.162
Voriconazole	0.12-4	0.25	0.5	0.324 ± 1.051
Fluconazole	0.5-64	8	64	7.013 ± 20.567
Note: The clinical isolates of C. glabrata used in this study included six serial isolates showing high level in vitro resistance to echinocandins and triazoles obtained from a patient who failed therapy with the echinocandins and triazoles. MIC50 = Minimum inhibitory concentration required to inhibit 50% of the organisms; MIC90 = Minimum inhibitory concentration required to inhibit 90% of the organisms.				

Table 2: In vitro susceptibilities of clinical isolates of *Candida glabrata* (n=79) to various antifungal drugs.

Of the 85 isolates, six serial isolates were obtained from a single patient who had relapsed despite adequate echinocandin therapy. *In vitro* susceptibility studies (Table 3) revealed that the initial isolate showed high-level resistance to caspofungin (MIC >16 μg/ml) and RES to micafungin and anidulafungin (MIC 2 μg/ml), whereas it remained susceptible to amphotericin B, posaconazole, voriconazole and fluconazole. A second isolate recovered approximately 2 wks after the initial isolate revealed an altered antifungal susceptibility profile showing a reduced susceptibility to micafungin and anidulafungin, and resistance to two azoles (fluconazole MIC >64 μg/ml and voriconazole MIC 4 μg/ml) and increased posaconazole MIC at 2 μg/ml. Four subsequent isolates obtained 12 wks later (isolate 37054), 14 weeks later (isolate 37178) and 15 weeks later (isolates 37179 and 37180) showed identical susceptibility profiles. On average, an 8-fold (fluconazole and voriconazole) to 32-fold (posaconazole) increase in MICs was demonstrated by the resistant *C. glabrata* strains, when compared to the MICs of the baseline isolate.

Isolate	Date of isolation	MIC (μg/ml) of						
		CFG	MFG	AFG	FLU	VOR	POS	AMB
36271	03.18.2006	>16	2	2	8	0.5	0.06	0.5
36421	04.04.2006	>16	8	4	>64	4	2	0.5
37054	06.21.2006	>16	8	4	>64	4	2	0.5
37178	06.30.2006	>16	8	4	>64	4	2	0.5
37179	07.09.2006	>16	8	4	>64	4	2	0.5
37180	07.09.2006	>16	8	4	>64	4	2	0.5

ATCC90030	NA	0.06	0.12	0.06	8	0.5	0.5	0.5
CFG = caspofungin; MFG = micafungin; AFG = anidulafungin; FLU = fluconazole, VOR = voriconazole; POS = posaconazole; AMB = amphotericin B.								

Table 3: In vitro antifungal susceptibilities of serial bloodstream isolates of Candida glabrata showing high level resistance to echinocandins and triazoles

To assure that these serial isolates were genetically related to the initial isolate electrophoretic karyotyping of all six isolates was performed. As shown in Figure 1, all six isolates had an identical karyotype indicating that these isolates are closely related and derived from the initial isolate recovered from the patient.

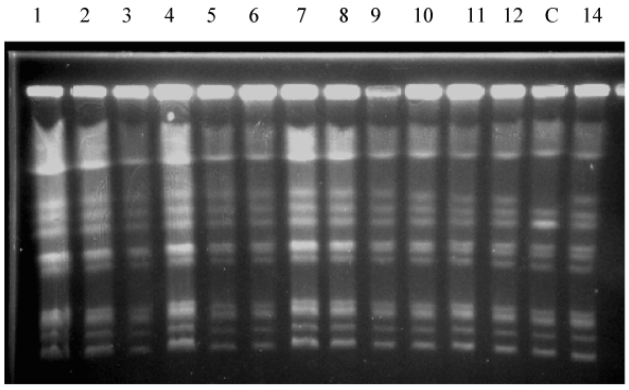

Figure 1: Karyotype analysis of serial clinical isolates of *C. glabrata* resistant to multiple members of the echinocandin and azole classes of antifungal drugs. Lanes 1 through 14: karyotypes of thirteen *C. glabrata* serial isolates obtained from the patient during a 4 month period of time. Lane C: karyotype of drug susceptible *C. glabrata* ATCC90030.

FKS Analysis

We characterized the HS 1 region of Fks1p, Fks2p and Fks3p by nucleotide sequence analysis of the regions of FKS1, FKS2 and FKS3 coding for HS 1 in all six serial isolates. The amino acid sequences were compared with that of the echinocandin susceptible *C. glabrata* ATCC 90030. We did not find any amino acid variations in HS 1 among the clinical isolates or in *C. glabrata* ATCC 90030, suggesting that the RES in these specific isolates is due to an alternative mechanism(s) independent of the genetic mutation described in the HS 1 region.

Triazole resistance analysis

Although, the initial isolate revealed RES, it remained susceptible to fluconazole, voriconazole, and posaconazole. However, the second isolate obtained after two wks of daily micafungin demonstrated an 8-32-fold increase in azole MICs to fluconazole, voriconazole and posaconazole without exposure to any azole during therapy.

We investigated the possible over expression of CDR1, CDR2 and SNQ2 as well as cyp51 in the three key isolates obtained at different time points. Isolate 36271, isolate 36421, recovered 19 days later, and isolate 37179, recovered 95 days after the 2nd isolate. Measurements of the mRNA levels of these genes by real time RT-PCR and the comparison with those of a previously studied isolate with fluconazole resistance (ATCC 200918) were evaluated. As shown in Figure 2, all three efflux proteins were over expressed at least 2-fold in isolate 36421, compared to that found in the initial isolate. Isolate 37179, collected almost four months after the recovery of the initial isolate, showed over expression of CDR1, CDR2 and cyp51 genes, but not SNQ2, compared to the initial isolate. A comparison of the level of expression of SNQ2 in isolate 36271 and 37179 suggests that the SNQ2 expression is dependent on the continuous exposure of the fungal cells to the drug, but not for the other efflux proteins, as well as the drug target Cyp51p. These results suggest that the increased resistance of the *C. glabrata* serial isolates recovered from the patient with recurrent candidemia despite adequate antifungal therapy is most likely due to the cumulative effect of the over expression of key efflux pump proteins in these isolates.

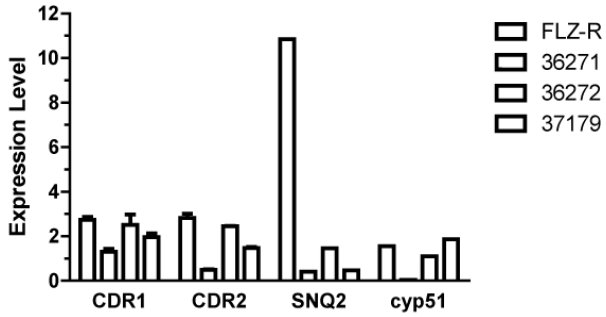

Figure 2: Relative expression of CDR1, CDR2, SNQ2 and cyp51 in *C. glabrata* serial isolates 36271 (initial baseline isolate), 36272 (isolate obtained 3 days later) and 37179 (isolate obtained 100 days later). The level of expression of various genes is shown relative to the level of expression obtained in the drug susceptible *C. glabrata* ATCC90030. The fluconazole resistant *C. glabrata* ATCC200918 was used as a positive control. The vertical bar on each histogram represents standard deviation.

A comparison of the amino acid sequences of Cyp51p of C. albicans and *C. glabrata* showed a 64% amino acid identity, including the highly preserved amino acid sequences at the heme-binding and the membrane anchoring regions. Thus, it is likely that the high level of resistance to the azoles in these resistant *C. glabrata* isolates may be due to the combined action of upregulation of efflux pump protein synthesis, together with amino acid changes at the critical regions of P450 lanosterol demethylase gene.

Discussion

Overall, the majority of the *C. glabrata* isolates we evaluated showed similar susceptibility patterns for the echinocandins, triazoles and amphotericin B.

The MICs of the serial isolates recovered from the one patient over a period of four months is rather intriguing. Prior to the admission to our institution the patient had received caspofungin for a candidemia due to C. glabrata. Thus, it was not surprising that the initial isolate recovered in our institution showed RES to caspofungin, micafungin and anidulafungin. The 2nd isolate obtained two wks after the initiation of micafungin also showed RES to micafungin and anidulafungin. It is important to note that the secondary reduced susceptibility to caspofungin, and later, the in vivo development of RES to micafungin and anidulafungin is an extremely uncommon occurrence and has not been previously described.

Since the echinocandins are non-competitive inhibitors of glucan synthase the drug must bind to the enzyme molecule in a non-competitive fashion to exert its inhibitory action. Any genetic alteration of the primary structure of the protein will affect the ability of the drug and its analogues to bind and/or inhibit the enzyme function. However, we did not find any amino acid change at the HS 1 region which has previously been associated with drug target modification dependent reduction in echinocandin susceptibility in different Candida species [24,25,34]. The increase in MICs detected after the 2nd isolate and all of the isolates thereafter, could be the cumulative effect of multiple amino acid changes outside the HS1 region. Alternatively, it is possible that drug efflux may have a role to play in the development of RES in *C. glabrata*. The efflux proteins in general are less specific in many cases and capable of pumping out structurally unrelated molecules accumulated within the cell. So it is possible that over expression of some as yet unknown efflux pump(s) may be responsible for the RES in these isolates [22,24,35].

The acquired resistance to multiple azoles in association with the RES has not been previously described. Since the modes of action of these two classes of antifungals are very different, the emergence of resistance to azoles in association with the RES is unlikely to be linked by a single mechanism [36,37]. Over expression of the efflux proteins commonly associated with azole resistance appears to be at least partly responsible for the increased MICs to these drugs in the isolates that we obtained from this specific patient. Interestingly, our patient was not exposed to any azole during her infection. So the moderate upregulation of the syntheses of CDR1, CDR2 and SNQ2 may be constitutively achieved since the isolates obtained after two wks and all of the isolates recovered thereafter showed the same degree of increased level of expression. Moreover, the increased level of expression of cyp51 may be partly responsible for increased resistance.

Alternatively, the multi-azole resistance we found in our serial isolates may be due to drug target modifications. Such a spontaneous mutation could happen in the isolate that has RES. Amino acid changes belonging to any of the five highly conserved regions may affect the ability of the drug to inhibit the activity of the enzyme. Amino acid alteration(s) in the MAR and HBR are known to confer general as well as selective resistance to triazoles in fungi, including Candida species. It is possible that the high level of resistance to triazoles we observed may be the result of the combined effect of antifungal drug target mutation and efflux.

In conclusion, our results raise questions regarding the development, incidence, acquisition, and mechanisms of resistance of *C. glabrata* [37]. Although the recent expansion of our antifungal armamentarium has added newer treatment modalities, a great many issues remain unclear. Moreover, our observations did not identify the previously described echinocandin resistance mechanisms in *C. glabrata*. The investigation into the interaction of echinocandins and azoles in the different Candida species is still necessary to further address these concerns.

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