Novel *FKS* Mutations Associated with Echinocandin Resistance in *Candida* Species[▽]

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We studied three clinical isolates of *Candida* spp. (one *C. tropicalis* isolate and two *C. glabrata* isolates) from patients with invasive candidiasis. The first isolate emerged during echinocandin treatment, while the others emerged after the same treatment. These strains harbored an amino acid substitution in Fksp never linked before with reduced echinocandin susceptibility in *C. tropicalis* or in *C. glabrata*. The molecular mechanism of reduced susceptibility was confirmed using a 1,3-β-D-glucan synthase inhibition assay.

Since the introduction of the echinocandin drugs in 2001, these antifungals are becoming the preferred choice for invasive candidiasis treatment (13, 14, 16, 24). *Candida tropicalis* and *Candida glabrata* are important pathogens causing invasive disease, especially in immunocompromised patients (12, 22). Echinocandin drugs target the Fksp subunits of the 1,3-β-D-glucan synthase complex and inhibit fungal cell wall biosynthesis (4). Echinocandin clinical failures are rare events. Yet, as the number of patients exposed to echinocandin therapy increases, the probability for resistance increases. In all the published cases of *C. tropicalis* and *C. glabrata* with a reduced echinocandin susceptibility (RES) phenotype, amino acid substitutions in two conserved regions of the Fksp (hot spots) were reported (2, 6, 11, 21, 25).

From March 2004 to March 2009, 285 cases of candidemia were identified at Summa Health System's hospitals. Eighty-nine cases were identified as *C. glabrata* and 17 as *C. tropicalis*. During this period, two *C. glabrata* isolates and one *C. tropicalis* isolate were considered nonsusceptible to caspofungin by the CLSI susceptibility breakpoint (MIC ≥ 2 μg/ml) (3, 23), representing an RES incidence of 2.2% (2/89) and 5.8% (1/17) for *C. glabrata* and *C. tropicalis*, respectively. In this work, we describe the molecular mechanism responsible for the RES phenotype observed in these three clinical cases.

Case 1 (*C. tropicalis* strain CT-C1). A 28-year-old female with acute myelogenous leukemia receiving broad-spectrum antibacterials showed persistent fever. An empirical caspofungin treatment was started (5th hospital day). A set of cultures were obtained. On her 22nd hospital day, while she was on caspofungin (50 mg/daily) (16th day), her esophageal biopsy specimen indicated severe invasive *Candida* esophagitis and her blood cultures were positive for *C. tropicalis*. Echinocandin and fluconazole MICs were determined (Table 1). Caspofungin was discontinued, and she was switched to fluconazole (MIC, 1 μg/ml), with resolution of symptoms. She completed 4 weeks of fluconazole, and she was discharged after 56 days.

Case 2 (*C. glabrata* strain CG-C2). A 49-year-old female diabetic and renal transplant recipient showed erosive esophagitis. She developed respiratory failure (3rd hospital day) and was treated empirically with broad-spectrum antibacterials. Her tracheal aspirate culture was positive for *Candida* spp., and her urine culture was positive for *C. glabrata*. She was accordingly started on caspofungin (50 mg/daily) (12th hospital day). After 2 weeks, her condition stabilized but did not improve. On her 55th hospital day, 24 days after completing the initial caspofungin course, she developed low-grade fever. Her blood, urine, and central line catheter tip cultures grew *C. glabrata*, and caspofungin was reinitiated. MICs were determined (Table 1), and caspofungin was switched to liposomal amphotericin B (58th hospital day). She completed a 3-week course, with noted clinical improvement and clearing of her fungemia.

Case 3 (*C. glabrata* strain CG-C3). A 53-year-old male who received a kidney transplant was admitted for gastrointestinal bleeding and pulmonary infiltrates. He developed candiduria 2 months prior to this admission, which was treated with fluconazole. Upon admission, the patient was initially treated with broad-spectrum antibacterials for 2 weeks. His pulmonary infiltrates progressed. His respiratory cultures showed *C. glabrata* and *C. albicans*. Anidulafungin (100 mg/daily) was started (21st hospital day) and continued for 3 weeks, with no clinical improvement. After a respiratory failure, his blood and urine cultures were positive for *Klebsiella pneumoniae* and *C. glabrata* (>100,000 CFU/ml), respectively. Echinocandin MICs were determined (Table 1), and the treatment was switched to liposomal amphotericin B, with significant clinical improvement.

Characterization of clinical isolates. Susceptibility testing was performed according to the recommendations of CLSI document M27-A3 (3). *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 750, *C. albicans* ATCC 90028, *C. albicans* 205, and *C. albicans* 177 (3, 7) were used to compare the MICs for our clinical isolates. The strains CT-C1, CG-C2, and CG-C3 showed 16- to >500-fold-higher MICs than the reference strains (Table 1). 1,3-β-D-Glucan synthase complexes were isolated, and inhibition kinetics values (50% inhibitory concentrations [IC$_{50}$s]) were obtained to confirm the RES phenotype

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TABLE 1. *In vitro* susceptibility testing and 1,3-β-D-glucan synthase complex inhibition profiles for anidulafungin, caspofungin, and micafungin drugs[d]

Strain	Organism	Wild-type *FKS* or *FKS* with nucleotide substitution	Fksp hot spot 1[a]	MIC (µg/ml)[b]			IC$_{50}$ (ng/ml)[c]		
				ANF	CSF	MCF	ANF	CSF	MCF
ATCC 750	*C. tropicalis*	Wild type	FLTLSLRDP	0.06	0.25	0.06	4.66	2.33	7.75
CT-C1	*C. tropicalis*	*FKS1*-T227C	**S**LTLSLRDP	1.00	4.00	1.00	122.7	61.28	405.9
ATCC 90030	*C. glabrata*	Wild type	FLILSLRDP	<0.03	0.03	0.03	1.91	1.92	0.71
CG-C2	*C. glabrata*	*FKS1*-T1885C	FLIL**P**LRDP	4.00	8.00	2.00	13,998	12,778	15,144
CG-C3	*C. glabrata*	*FKS2*-T1987C	FLIL**P**LRDP	8.00	>16.0	>16.0	13,351	16,882	11,422
ATCC 90028	*C. albicans*	Wild type	FLTLSLRDP	0.03	0.12	0.03	1.83	0.50	18.80
177	*C. albicans*	*FKS1*-T1922C	**S**LTLSLRDP	0.83	4.00	1.00	2,622	1,091	1,435
205	*C. albicans*	*FKS1*-T1933C	FLTL**P**LRDP	1.33	8.00	4.00	989	245.4	1,088

[a] Bold letters represent amino acid changes. Shown are the sequences of Fks1p hot spot 1 for all strains except 03-Cg, in which the mutation was present in Fks2p.
[b] MIC values represent the geometric means of results from three repetitions.
[c] IC$_{50}$ values represent the arithmetical means of results from three repetitions.
[d] ANF, anidulafungin; CSF, caspofungin; MCF, micafungin.

(5, 6, 18). The 1,3-β-D-glucan synthase complexes isolated from strains CT-C1, CG-C2, and CG-C3 yielded higher IC$_{50}$s (from 26- to >20,000-fold) for all echinocandins than wild-type 1,3-β-D-glucan synthase complexes (Fig. 1; Table 1). Consistent with this biochemical data, DNA sequence analysis of the *FKS1* gene (GenBank accession no. EU676168) of the CT-C1 strain showed a homozygous T227C mutation, resulting in a deduced F76S amino acid substitution. This is the only sequence available, and it is partial. Its first amino acid is equivalent to Ala566 of the *C. albicans* Fks1p (GenBank accession no. XM_716336). Thus, the F76S amino acid substitution in strain 01-Ct is equivalent to a F641S substitution in *C. albicans* (Table 1) (6).

The *C. glabrata FKS1* and *FKS2* genes (GenBank accession no. XM_446406 and XM_448401, respectively) from strains CG-C2 and CG-C3 were sequenced. These isolates showed S629P and S663P amino acid substitutions in Fks1p and Fks2p, respectively (Table 1). These amino acid substitutions have not been previously reported for *C. tropicalis* or *C. glabrata*. However, equivalent substitutions were described for *C. albicans* (7, 11, 18) and are located in the highly conserved hot spot 1 region of the Fksp associated with RES in *Candida* spp. (21).

Our isolates showed MIC values comparable with those of clinical *C. albicans* strains harboring equivalent *FKS* mutations (strains 177 and 205) (Table 1). Also, the 1,3-β-D-glucan synthase complexes isolated from our strains showed decreased echinocandin sensitivity (a higher IC$_{50}$), demonstrating that *FKS* hot spot mutations are sufficient and necessary to produce an RES phenotype. However, the IC$_{50}$ shifts did not show a strict correlation with MIC values, especially for the 01-Ct strain. This phenomenon was observed before for *C. albicans* and *C. tropicalis*, suggesting that factors other than 1,3-β-D-glucan synthase complex inhibition may influence echinocandin MIC values (1, 6, 7, 17).

In summary, we report three patients who developed invasive *Candida* species infection despite echinocandin treatment, showing that RES is an emerging problem as echinocandin use continues to increase (2, 6, 8–11, 15, 18–20, 25). Furthermore, we demonstrate the clear linkage between Fksp substitutions, 1,3-β-D-glucan synthase complex resistance to echinocandin drugs, elevated MIC, and echinocandin clinical failure.

	MCF - 750	MCF-CT-C1
IC50	7.545	401.4

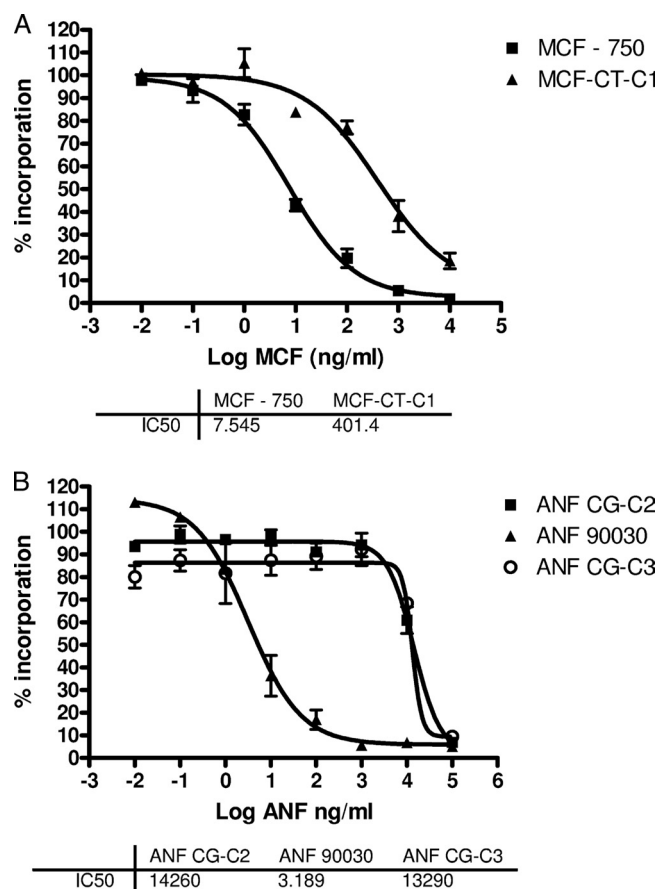|	ANF CG-C2	ANF 90030	ANF CG-C3
IC50	14260	3.189	13290

FIG. 1. Echinocandin inhibition profiles for product-entrapped 1,3-β-D-glucan synthase complexes obtained using a sigmoidal-response (variable-slope) curve. Echinocandin inhibition kinetics yielding 50% inhibitory concentrations (IC$_{50}$s) were obtained and are expressed in nanograms per milliliter. (A) Inhibition curves and 50% inhibitory concentrations (IC50) for micafungin (MCF) and for 1,3-β-D-glucan synthase complexes obtained from reference *C. tropicalis* ATCC 750 (750) and *C. tropicalis* CT-C1 strains. (B) Anidulafungin (ANF) titration curves for 1,3-β-D-glucan synthase complexes isolated from reference *C. glabrata* ATCC 90030, *C. glabrata* CG-C2, and *C. glabrata* CG-C3 strains.

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