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Acquisition of Flucytosine, Azole, and Caspofungin Resistance in *Candida glabrata* Bloodstream Isolates Serially Obtained from a Hematopoietic Stem Cell Transplant Recipient▽†

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We describe the acquisition of flucytosine, azole, and caspofungin resistance in sequential *Candida glabrata* bloodstream isolates collected from a bone marrow transplant patient with clinical failure. Point mutations in *C. glabrata FUR1* (*CgFUR1*) and *CgFKS2* and overexpression of *CgCDR1* and *CgCDR2* were observed in resistant isolates.

Candida glabrata has emerged as an important fungal pathogen, particularly in patients with hematologic malignancies (8). This *Candida* species is known to have intrinsic low-level fluconazole (FLC) resistance but can easily acquire high-level resistance and cross-resistance to other triazoles (16). Echinocandins appeared as a therapeutic advance in the management of *C. glabrata* infections (12), and clinical failures associated with resistance remain rare events (4, 5, 6, 9, 10, 13, 20). Here we report a fatal case of a hematopoietic stem cell transplant (HSCT) recipient with recurrent *C. glabrata* candidemia with isolates developing resistance to flucytosine (5FC), azoles, and caspofungin (CSF) following exposure to these drugs.

A 9-year-old girl suffering from Fanconi anemia underwent an unrelated HSCT on 25 October 2001 (Fig. 1). At this time, she had digestive colonization with *C. glabrata* and received FLC prophylaxis from 22 October to 31 October and from 12 November to 24 November. From 1 November to 12 November and from 24 November to 10 December, she received broad-spectrum antibiotics in combination with liposomal amphotericin B (LAMB) as empirical therapy. The first blood culture grew positive for *C. glabrata* on 8 December (isolate 1) (ID32C strips; bioMérieux, France). The patient was treated with intravenous FLC in combination with intravenous 5FC and LAMB, and the central venous catheter was removed. After an 8-day treatment, use of FLC and 5FC was stopped and intravenous voriconazole (VRC) was introduced on 21 December. Because there was no sustained engraftment, a second HSCT was performed on 26 December without any granulocyte recovery. Despite antifungal combination therapy, *C. glabrata* was again recovered from blood cultures on 3 January (isolate 2), 7 January (isolate 3), 8 January, 9 January, 10 January, and 11 January. LAMB was replaced by CSF on January 12. On 14 March (isolate 4) and 16 March (isolate 5), two blood cultures grew positive for *C. glabrata*. Use of all therapeutics was stopped on 14 March, and the patient died on 17 March.

C. glabrata MICs are summarized in Table 1. Molecular typing of the *C. glabrata* isolates was performed as previously described (2). Briefly, DNA from each isolate was subjected to PCR for 8 microsatellite-containing regions by using fluorescent primers. With an internal fluorescent ladder, fragment size analysis revealed similar genotypes for all 5 isolates, with respective sizes of 114, 115, 117, 171, 169, 224, 175, and 226 bp for microsatellites 2bis to 9. The genetic basis of 5FC resistance was determined by sequencing the *C. glabrata FCY1* (*CgFCY1*) gene (GenBank accession no. XM_445483), encoding cytosine deaminase, and the *CgFUR1* gene (GenBank accession no. XM_447193), encoding uracil phosphoribosyltransferase, in the clinical isolates and the CBS138 strain (with primer pairs 5Fcy1/3Fcy1 and 5Fur1/3Fur1; see the supplemental material). All isolates displayed a *CgFCY1* sequence similar to that of the CBS138 strain. In contrast, a missense point mutation in *CgFUR1* conferring a G190D substitution in Fur1p was detected only in isolates 2 and 3. To investigate azole resistance, the *CgERG11* gene (GenBank accession no. XP445876), encoding the cytochrome P450 C_{14} α-demethylase, was sequenced in the clinical isolates and in the CBS138 strain (with primer pair FC14/RC14; see the supplemental material). Levels of expression of *CgERG11* in the CBS138 strain and in clinical isolates were compared by semiquantitative reverse transcription (RT)-PCR (primer pair RTFC14/RTRC14; see the supplemental material). The sequences of *CgERG11*, as well as its

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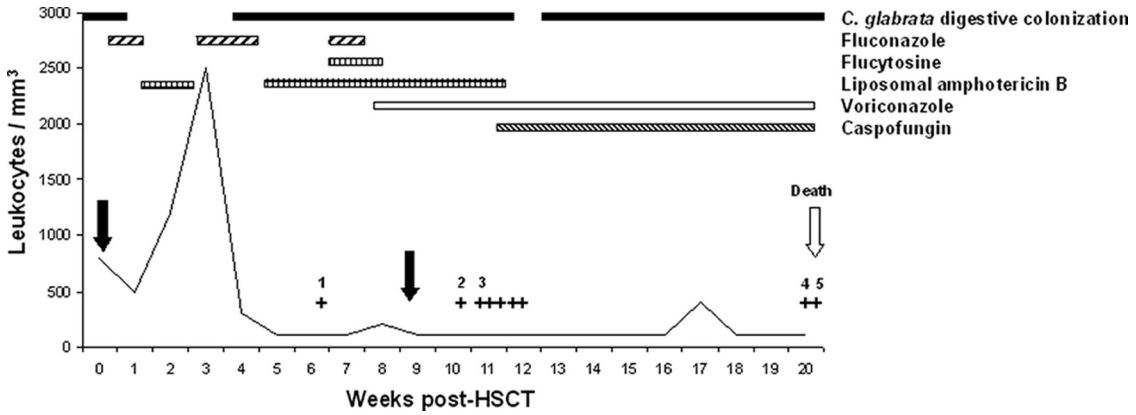

FIG. 1. Schematic representation of *Candida glabrata* bloodstream infection in an allogeneic HSCT recipient. Day 0 was the day of the first HSCT. The two HSCTs are represented with black arrows. Each plus symbol represents a *C. glabrata*-positive blood culture. Each isolate studied is indicated by a number. Dosages of antifungal drugs were as follows: FLC at 200 mg/day for the first two periods and 600 mg/day for the last period, 5FC at 150 mg/kg of body weight/day, LAMB at 3 mg/kg/day, CSF at 50 mg/day, and VRC at 300 mg/day.

expression levels, in clinical isolates were identical to that of the CBS138 strain.

Expression of the *C. glabrata* genes for ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p was studied by Northern blot analysis of the clinical isolates and the CBS138 strain by using a previously described method (3) (amplification of labeled DNA probes with primer pairs CG1S/CG1R and CG2S/CG2R; see the supplemental material). Basal *CgCDR1* (GenBank accession no. AAF05069) and *CgCDR2* (GenBank accession no. AF046120) expression levels in the CBS138 strain and in isolates 1, 2, and 3 were too low to be detected, but higher levels of *CgCDR1* and, to a lesser extent, *CgCDR2* expression were detected in isolates 4 and 5 (Fig. 2). Finally, a region of *CgFKS1* and *CgFKS2*, encoding ß-glucan synthase and previously shown to be a hot spot of mutation associated with echinocandin resistance (13), in the CBS138 strain and clinical isolates was sequenced (primer pairs Cg-FKS1F/Cg-FKS1R and Cg-FKS2F/Cg-FKS2R; see the supplemental material). The *CgFKS1* fragment sequences were similar for all isolates, but a nonsynonymous nucleotide mutation (T1988C) in *CgFKS2*, leading to an S663P amino acid substitution in Fks2p, was detected only in isolates 4 and 5.

We described the molecular mechanisms likely responsible for the development of multiple resistances in *C. glabrata* isolates during therapy. If mechanisms of secondary azole resistance in *C. glabrata* are mostly associated with increased expression of the *CgCDR1* and *CgCDR2* genes (18, 19), as in our isolates, mechanisms of 5FC resistance are based upon mutations resulting in enzyme deficiency (1). It was recently demonstrated with *C. lusitaniae* that a *fur1* null mutant was resistant to both 5FC and 5-fluorouracil (11). Isolates 2 and 3 were also fully cross-resistant to 5-fluorouracil (data not shown), supporting the idea that a defective Fur1p bearing the G190D substitution was very likely responsible for the fluoropyrimidine resistance phenotypes. The apparent loss of 5FC resistance in isolates resistant to azoles and caspofungin is probably the consequence of the presence of different subpopulations derived from a common progenitor. Here, resistance to echinocandins was associated with the Fks2p mutation S663P, as previously described (7). Considering the fact that neither azoles nor AMB is the best alternative for treating *C. glabrata* infection, the occurrence of echinocandin resistance represents a real therapeutic challenge. Furthermore, these results show that *C. glabrata* can acquire resistance to multiple antifungal drugs through successive independent genetic events. The recovery of different isolates exhibiting clonality for microsatellite markers but genetic diversity for antifungal resistance markers demonstrates the high propensity of *C. glabrata* to readily mutate *in vivo* in a single patient. Expression of resistance in *C. glabrata* is facilitated by the haploid nature of the genome. Such a number of events over a rather small time scale (5 months) suggests that this organism could divide actively in a severe neutropenia context despite antifungal treatment. This report also demonstrates the importance of suscep-

TABLE 1. MICs of consecutive *C. glabrata* isolates

Drug	MIC (μg/ml) for isolate (day/mo/yr isolated)[a]				
	1 (08.12.2001)	2 (03.01.2002)	3 (07.01.2002)	4 (14.03.2002)	5 (16.03.2002)
Amphotericin B	1	2	0.75	0.75	0.75
Flucytosine	0.004	>32	>32	0.004	0.004
Fluconazole	8	2	1	>256	>256
Voriconazole	0.25	0.125	0.047	12	16
Caspofungin	0.19	0.125	0.125	>32	>32

[a] Susceptibility testing was performed using the Etest method (AB-Biodisk, Sweden) according to the manufacturer's instructions. *C. parapsilosis* ATCC 22019 served as the control strain. MIC interpretative criteria for the susceptibility to FLC, VRC, and 5FC were as previously published by the CLSI (14, 17). The susceptibility breakpoint of ≤2 mg/liter was applied for CSF (15).

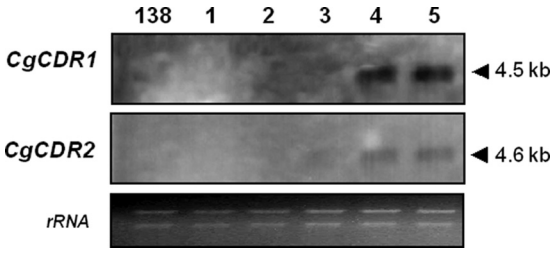

FIG. 2. Expression analysis of *C. glabrata CgCDR1* and *CgCDR2* genes in clinical isolates and in wild-type strain CBS138 (138). Northern blot analysis of total RNA from the clinical isolates 1, 2, 3, 4, and 5 and from strain CBS138 hybridized with *CgCDR1* and *CgCDR2* RNA probes. The ethidium bromide-stained 28S/18S rRNAs are shown as a control for loading.

tibility testing in patients with recurrent isolation of *Candida* spp. while receiving or having previously been exposed to antifungal treatments.

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