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Stepwise Development of a Homozygous S80P Substitution in Fks1p, Conferring Echinocandin Resistance in *Candida tropicalis*

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Three *Candida tropicalis* isolates were obtained from a patient with acute lymphoblastic leukemia. The first isolate was susceptible to all drug classes, while isolates 2 and 3, obtained after 8 and 8.5 weeks of caspofungin treatment, respectively, were resistant to the three echinocandins. Multilocus sequence genotyping suggested a clonal relation among all isolates. *FKS1* sequencing revealed a stepwise development of a heterozygous and finally a homozygous mutation, leading to S80S/P and S80P amino acid substitutions.

It is well recognized that long-term antifungal treatment entails a risk for *in vivo* selection of resistant fungi. Accordingly, an increasing number of reports demonstrate acquired echinocandin and azole resistance associated with both hetero- and homozygous mutations in the *FKS* and *ERG11* genes, which encode antifungal target proteins in *Candida* (1–6). This is of clinical importance, as resistant *Candida* isolates are associated with breakthrough candidiasis, treatment failures, and increased mortality (7). *Candida tropicalis* is identified as one of the five most common pathogenic *Candida* species, with a geographically determined proportion ranging from 3 to 66% of candidemia cases (8–10). Unfortunately, acquired fluconazole resistance is increasing, with ranges from approximately 7% in Denmark (9) to 9% in a global study (11) and 40% in Japan (12). Based on such findings, echinocandins are increasingly being utilized in the management of candidiasis caused by *C. tropicalis* (10, 13–15).

In this study, we analyzed three sequential *C. tropicalis* isolates (isolates 1, 2, and 3) obtained over a 4-month period from a patient with acute lymphoblastic leukemia who had been referred for allogeneic bone marrow transplantation. The patient was initially blood culture positive on 19 December 2010 for *C. tropicalis* (isolate 1) while receiving voriconazole prophylaxis. Caspofungin treatment was initiated (70/50 mg/day [70 mg on day 1 as a loading dose, followed by 50 mg daily thereafter]) (Fig. 1) and continued for a total of 8.5 weeks, interrupted by a 3-week fluconazole step-down treatment (Fig. 1). During the initial caspofungin treatment, nine serum samples tested positive for *Candida* mannan antigen, peaking at 479 pg/ml but stabilizing around 250 pg/ml on 20 January 2011 (Fig. 1). *C. tropicalis* was again detected in the blood on 5 March 2011 (isolate 2, after approximately 8 weeks of caspofungin treatment), and treatment was switched to amphotericin B (3 mg/kg/day) on 9 March. The patient was blood culture negative from 16 March, but the final *C. tropicalis* isolate (isolate 3, after approximately 8.5 weeks of caspofungin treatment) was obtained on 18 March from an oral swab, and treatment was changed to posaconazole (800 mg/day) on 31 March 2011. A Hickman catheter was kept in place, but sterilization was attempted with acid and fluconazole lock. Susceptibility testing was done according to EUCAST EDef 7.2 (azoles, anidulafungin, and micafungin) (16) and by Etest (amphotericin B and caspofun-

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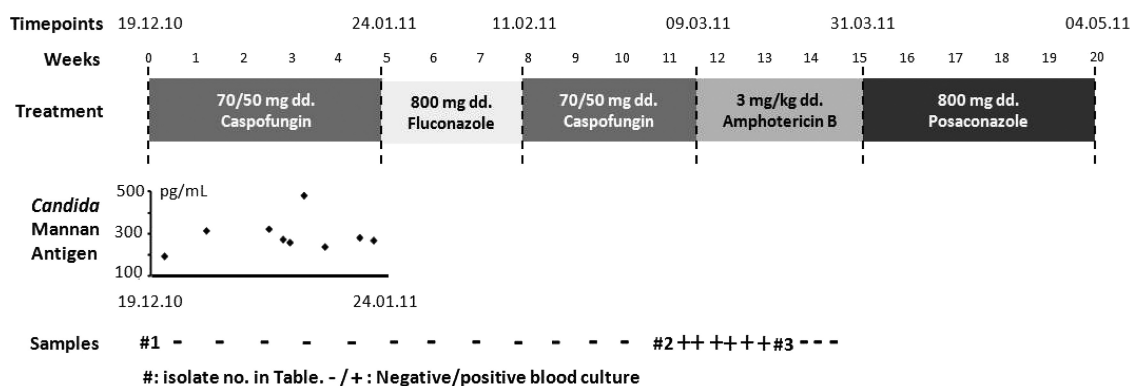

FIG 1 Systemic antifungal treatment of the leukemic patient illustrated in boxes with drugs administered as daily doses (dd.). Nine serum samples were positive for *Candida* mannan antigen during the first caspofungin treatment period, and subsequently, several positive blood cultures were obtained. Three isolates (isolates 1, 2, and 3) were chosen and sequenced for resistance mechanisms and genotyping.

TABLE 1 Origins, resistance mechanisms, genotypes, and susceptibility data for the three study and two control *C. tropicalis* isolates

					MIC (μg/ml)[a]							
					EUCAST (EDef 7.1)						Etest	
Isolate	Specimen origin	Collection date (day.mo.yr)	*FKS1* resistance mechanism	Allelic profile according to PubMLST (*ICL1-MDR1-SAPT2-SAPT4-XYR-ZWF*)	ANI	MICA	POS	VOR	ITR	FLU	AMB	CAS
1	BC[b]	19.12.10	Wild type	16-20-4-10-25-5	≤0.03	≤0.008	≤0.03	≤0.03	≤0.03	1	0.5	0.25
2	BC-CVC[c]	05.03.11	S80S/P	16-20-4-10-25-5	0.25	1	≤0.03	≤0.03	≤0.03	0.5	0.5	>32
3	Cavum oris	18.03.11	S80P	16-20-4-10-25-5	0.5	>1	≤0.03	0.06	≤0.03	2	1	>32
REF-1[d]	BC[b]	08.07.10	Wild type	1-7-4-6-2-4	≤0.03	≤0.008	≤0.03	≤0.03	0.03	≤0.125	1	0.25
REF-2[d]	BC[b]	23.01.11	Wild type	1-3-1-7-2 (99.7 %)-1	≤0.03	≤0.008	≤0.03	≤0.03	0.125	0.5	0.5	0.125

[a] ANI, anidulafungin; MICA, micafungin; POS, posaconazole; VOR, voriconazole; ITR, itraconazole; FLU, fluconazole; AMB, amphotericin B; CAS, caspofungin.
[b] Unspecified blood culture.
[c] Blood culture obtained via the intravenous Hickman catheter.
[d] Susceptible reference isolate from unrelated patients, used for comparison.

gin). Etest was chosen for caspofungin susceptibility testing, as the biological potency of pure substance has been associated with an unacceptable lot-to-lot variation, with the most recently obtained powder giving rise to elevated MICs (17–19). Echinocandin susceptibility was evaluated using the EUCAST breakpoint for anidulafungin EUCAST MICs (susceptibility [S] ≤ 0.06 mg/liter) but adopting the revised CLSI breakpoint for interpretation of caspofungin Etest results (S ≤ 0.25 mg/liter) (20, 21). CLSI breakpoints were adopted for interpretation of the caspofungin Etest MICs as recommended by the manufacturer, as this has been found to be appropriate for *Candida albicans* and *C. tropicalis* (22). EUCAST breakpoints for micafungin have not yet been established, but 185 of 186 wild-type *C. tropicalis* isolates tested in our laboratory had a EUCAST MIC of ≤0.03 mg/liter, which was used to define susceptibility. Two unrelated isolates of *C. tropicalis* (REF-1 and REF-2) were included as wild-type *FKS1* reference isolates. The *FKS1* gene was amplified and sequenced using primers targeting hot spot 1 (FKS1-F, TCATTGCTGTGGCCACTTTAG; FKS1-R, TAGAATGAACGACCAATGGAGA) and hot spot 2 (FKS8-F, CT CCTGCCGTTGATTGGATTA; FKS8-R, ACCACCAACGGTCA AATCAG) and compared to the *C. tropicalis FKS1* reference sequence (GenBank accession no. EU676168). Genetic relatedness was analyzed by multilocus sequence typing (MLST) based on polymorphisms in 6 sequenced housekeeping genes (*ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*) as described previously (23) by applying the PubMLST database, covering 205 diploid sequence types (http://pubmlst.org/ctropicalis).

Isolate 1 and the two reference isolates were susceptible to all tested antifungals, whereas isolates 2 and 3 were categorized as echinocandin resistant (Table 1). Isolate 1, REF-1, and REF-2 were wild type in both hot spot 1 and 2 in the *FKS1* sequence, while isolate 2 harbored a heterozygous T238C mutation and isolate 3 a homozygous T238C mutation in hot spot 1 of *FKS1*, leading to S80S/P and S80P amino acid substitutions, respectively. The MLST data suggested that isolates 1, 2, and 3 were clonally related, since the diploid sequences in the 6 housekeeping genes were 100% identical (Table 1).

In vivo selection for echinocandin resistance has been demonstrated for several *Candida* species, including *C. albicans* (1, 24–27), *C. glabrata* (24, 28–31), *C. krusei* (5, 24, 32), and *C. parapsilosis* (33). However, to our knowledge, this is the first study to demonstrate the stepwise *in vivo* progression of a wild-type *C. tropicalis* strain to a homozygous *fks1* mutant exhibiting echinocandin resistance. Even the heterozygous mutant isolate was classified as echinocandin resistant, with a significant ≥3 to 7 twofold-dilution step increase in echinocandin MICs, illustrating the significance of the S80 codon in *FKS1* in *C. tropicalis.* Nevertheless, the MICs indicated that the homozygous mutant (isolate 3) may be slightly more resistant to echinocandins (at least 1 dilution step, as suggested by the increase in anidulafungin and micafungin MICs). Other homozygous mutations in *C. tropicalis fks1* have been associated with elevated echinocandin MICs and amino acid substitutions, including L79W (4), F76S (6), and F76L (34). Moreover, heterozygous S80S/P mutants that display echinocandin resistance have been found (34, 35), but interestingly, the homozygous S80P mutation has not been described previously. This is in contrast to the findings for *C. albicans*, where a homozygous alteration at the corresponding codon (S645) has been detected in several resistant isolates (17, 36–38). Several factors may contribute to this difference. First, fitness cost when the second allele is mutated may vary, as supported by previous observations associating homozygous *fks1* mutations in *C. albicans* with both fitness and virulence costs (39). Second, the resistance conferred by the heterozygous mutation may be sufficient to allow escape in S80S/P *C. tropicalis* during caspofungin treatment, whereas the homozygous variant may be required for high-level echinocandin resistance in *C. albicans* (37, 40).

Our and related studies contribute to the overall understanding of resistance development *in vivo* as a consequence of antifungal treatment, including understanding the duration of treatment and which compounds allow selection of resistant mutants. Finally, this study may assist in determining treatment guidelines for the management of *C. tropicalis* infections, as the development of echinocandin resistance should be acknowledged as a rising concern in the treatment of patients with long-term echinocandin exposure.

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H.K.J. has nothing to declare.

REFERENCES

1. **Baixench M-T, Aoun N, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S, Ramires S, Piketty C, Dannaoui E.** 2007. Acquired resistance to echinocandins in Candida albicans: case report and review. J. Antimicrob. Chemother. **59**:1076–1083.

2. **Cleary JD, Garcia-Effron G, Chapman SW, Perlin DS.** 2008. Reduced Candida glabrata susceptibility secondary to an FKS1 mutation developed during candidemia treatment. Antimicrob. Agents Chemother. **52**:2263–2265.
3. **Costa-de-Oliveira S, Marcos Miranda I, Silva RM, Pinto E Silva A, Rocha R, Amorim A, Gonçalves Rodrigues A, Pina-Vaz C.** 2011. FKS2 mutations associated with decreased echinocandin susceptibility of Candida glabrata following anidulafungin therapy. Antimicrob. Agents Chemother. **55**:1312–1314.
4. **Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E.** 2008. Mutations in the fks1 gene in Candida albicans, C. tropicalis, and C. krusei correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob. Agents Chemother. **52**: 3092–3098.
5. **Durán-Valle MT, Gago S, Gómez-López A, Cuenca-Estrella M, Jiménez Díez-Canseco L, Gómez-Garcés JL, Zaragoza O.** 2012. Recurrent episodes of candidemia due to Candida glabrata with a mutation in the hot spot 1 of the FKS2 gene developed after prolonged therapy with caspofungin. Antimicrob. Agents Chemother. **56**:3417–3419.
6. **Garcia-Effron G, Chua DJ, Tomada JR, DiPersio J, Perlin DS, Ghannoum M, Bonilla H.** 2010. Novel FKS mutations associated with echinocandin resistance in Candida species. Antimicrob. Agents Chemother. **54**: 2225–2227.
7. **Sun H-Y, Singh N.** 2010. Characterisation of breakthrough invasive mycoses in echinocandin recipients: an evidence-based review. Int. J. Antimicrob. Agents **35**:211–218.
8. **Arendrup MC, Fuursted K, Gahrn-Hansen B, Schønheyder HC, Knudsen JD, Jensen IM, Bruun B, Christensen JJ, Johansen HK.** 2008. Semi-national surveillance of fungaemia in Denmark 2004–2006: increasing incidence of fungaemia and numbers of isolates with reduced azole susceptibility. Clin. Microbiol. Infect. **14**:487–494.
9. **Arendrup MC, Bruun B, Christensen JJ, Fuursted K, Johansen HK, Kjaeldgaard P, Knudsen JD, Kristensen L, Møller J, Nielsen L, Rosenvinge FS, Røder B, Schønheyder HC, Thomsen MK, Truberg K.** 2011. National surveillance of fungemia in Denmark (2004 to 2009). J. Clin. Microbiol. **49**:325–334.
10. **Chai LYA, Denning DW, Warn P.** 2010. Candida tropicalis in human disease. Crit. Rev. Microbiol. **36**:282–298.
11. **Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA.** 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of Candida species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J. Clin. Microbiol. **48**: 1366–1377.
12. **Chong Y, Shimoda S, Yakushiji H, Ito Y, Miyamoto T, Shimono N, Kamimura T, Akashi K.** 2012. Fatal candidemia caused by azole-resistant Candida tropicalis in patients with hematological malignancies. J. Infect. Chemother. In press.
13. **Kothavade RJ, Kura MM, Valand AG, Panthaki MH.** 2010. Candida tropicalis: its prevalence, pathogenicity and increasing resistance to fluconazole. J. Med. Microbiol. **59**:873–880.
14. **Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Diekema DJ.** 2009. Comparison of results of fluconazole and voriconazole disk diffusion testing for Candida spp. with results from a central reference laboratory in the ARTEMIS DISK Global Antifungal Surveillance Program. Diagn. Microbiol. Infect. Dis. **65**:27–34.
15. **Yang Y-L, Lin C-C, Chang T-P, Lauderdale T-L, Chen H-T, Lee C-F, Hsieh C-W, Chen P-C, Lo H-J.** 2012. Comparison of human and soil Candida tropicalis isolates with reduced susceptibility to fluconazole. PLoS One **7**:e34609. doi:10.1371/journal.pone.0034609.
16. **Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W.** 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin. Microbiol. Infect. **18**:E246–247.
17. **Arendrup MC, Garcia-Effron G, Buzina W, Mortensen KL, Reiter N, Lundin C, Jensen HE, Lass-Flörl C, Perlin DS, Bruun B.** 2009. Breakthrough Aspergillus fumigatus and Candida albicans double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. Antimicrob. Agents Chemother. **53**:1185–1193.
18. **Arendrup MC, Rodriguez-Tudela J-L, Park S, Garcia-Effron G, Delmas G, Cuenca-Estrella M, Gomez-Lopez A, Perlin DS.** 2011. Echinocandin susceptibility testing of Candida spp. Using EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time, and drug lots. Antimicrob. Agents Chemother. **55**:1580–1587.
19. **Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, Du C, Clancy CJ.** 2012. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. Antimicrob. Agents Chemother. **56**:4862–4869.
20. **Arendrup MC, Rodriguez-Tudela J-L, Lass-Flörl C, Cuenca-Estrella M, Donnelly JP, Hope W.** 2011. EUCAST technical note on anidulafungin. Clin. Microbiol. Infect. **17**:E18–20.
21. **Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, Lockhart SR, Motyl M, Perlin DS.** 2011. Clinical breakpoints for the echinocandins and Candida revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist. Updat. **14**:164–176.
22. **Arendrup MC, Pfaller MA.** 2012. Caspofungin Etest susceptibility testing of Candida species: risk of misclassification of susceptible isolates of C. glabrata and C. krusei when adopting the revised CLSI caspofungin breakpoints. Antimicrob. Agents Chemother. **56**:3965–3968.
23. **Tavanti A, Davidson AD, Johnson EM, Maiden MCJ, Shaw DJ, Gow NAR, Odds FC.** 2005. Multilocus sequence typing for differentiation of strains of Candida tropicalis. J. Clin. Microbiol. **43**:5593–5600.
24. **Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, Bretagne S, Dromer F, Lortholary O, French Mycoses Study Group.** 2012. Candida spp. with acquired echinocandin resistance, France, 2004–2010. Emerg. Infect. Dis. **18**:86–90.
25. **Hernandez S, Lo L, Najvar LK, Mccarthy DI, Bocanegra R, Graybill JR.** 2004. Caspofungin resistance in Candida albicans: correlating clinical outcome with laboratory susceptibility testing of three isogenic isolates serially obtained from a patient with progressive Candida esophagitis. Antimicrob. Agents Chemother. **48**:1382–1383.
26. **Laverdière M, Lalonde RG, Baril J-G, Sheppard DC, Park S, Perlin DS.** 2006. Progressive loss of echinocandin activity following prolonged use for treatment of Candida albicans oesophagitis. J. Antimicrob. Chemother. **57**:705–708.
27. **Park S, Kelly R, Kahn JN, Robles J, Hsu M, Register E, Li W, Vyas V, Fan H, Abruzzo G, Flattery A, Gill C, Chrebet G, Parent SA, Kurtz M, Teppler H, Douglas CM, Perlin DS.** 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. Antimicrob. Agents Chemother. **49**:3264–3273.
28. **Daneman N, Chan AK, Poutanen SM, Rennie R, Sand C, Porter S.** 2006. The emergence of caspofungin resistance during treatment of recurrent Candida glabrata candidaemia. Clin. Microbiol. Infect. **12**: P1204.
29. **Dodgson KJ, Dodgson AR, Pujol C, Messer SA, Soll DR, Pfaller MA.** 2005. Caspofungin resistant C. glabrata. Clin. Microbiol. Infect. **11**:P1158.
30. **Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen JD.** 2006. Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. Clin. Infect. Dis. **42**:938–944.
31. **Singh-Babak SD, Babak T, Diezmann S, Hill JA, Xie JL, Chen Y-L, Poutanen SM, Rennie RP, Heitman J, Cowen LE.** 2012. Global analysis of the evolution and mechanism of echinocandin resistance in Candida glabrata. PLoS Pathog. **8**:e1002718. doi:10.1371/journal.ppat.1002718.
32. **Hakki M, Staab JF, Marr KA.** 2006. Emergence of a Candida krusei isolate with reduced susceptibility to caspofungin during therapy. Antimicrob. Agents Chemother. **50**:2522–2524.
33. **Moudgal V, Little T, Boikov D, Vazquez JA.** 2005. Multiechinocandin- and multiazole-resistant Candida parapsilosis isolates serially obtained during therapy for prosthetic valve endocarditis. Antimicrob. Agents Chemother. **49**:767–769.
34. **Garcia-Effron G, Kontoyiannis DP, Lewis RE, Perlin DS.** 2008. Caspofungin-resistant Candida tropicalis strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. Antimicrob. Agents Chemother. **52**:4181–4183.
35. **Axner-Elings M, Botero-Kleiven S, Jensen RH, Arendrup MC.** 2011. Echinocandin susceptibility testing of Candida isolates collected during a 1-year period in Sweden. J. Clin. Microbiol. **49**:2516–2521.
36. **Balashov SV, Park S, Perlin DS.** 2006. Assessing resistance to the echi-

nocandin antifungal drug caspofungin in Candida albicans by profiling mutations in FKS1. Antimicrob. Agents Chemother. **50**:2058–2063.

37. **Garcia-Effron G, Park S, Perlin DS.** 2009. Correlating echinocandin MIC and kinetic inhibition of fks1 mutant glucan synthases for Candida albicans: implications for interpretive breakpoints. Antimicrob. Agents Chemother. **53**:112–122.
38. **Perlin DS.** 2007. Resistance to echinocandin-class antifungal drugs. Drug Resist. Updat. **10**:121–130.
39. **Ben-Ami R, Garcia-Effron G, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis DP.** 2011. Fitness and virulence costs of Candida albicans FKS1 hot spot mutations associated with echinocandin resistance. J. Infect. Dis. **204**:626–635.
40. **Niimi K, Monk BC, a Hirai Hatakenaka K, Umeyama T, Lamping E, Maki K, Tanabe K, Kamimura T, Ikeda F, Uehara Y, Kano R, a Hasegawa Cannon RD, Niimi M.** 2010. Clinically significant micafungin resistance in Candida albicans involves modification of a glucan synthase catalytic subunit GSC1 (FKS1) allele followed by loss of heterozygosity. J. Antimicrob. Chemother. **65**:842–852.