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Discontinuation of echinocandin and azole treatments led to disappearance of FKS alteration but maintenance of azoles resistance during clonal *Candida glabrata* persistent candidemia

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Keywords: echinocandin, antifungal resistance, caspofungin, micafungin, anidulafungin, azoles, candidaemia, amphotericin B

Abstract (240 words)

Objectives: to give indication of a fitness cost conferred by FKS mutation-associated echinocandin resistance in *Candida glabrata* during human infection.

Methods: six *C. glabrata* clinical strains sequentially isolated from blood and a hepatic abscess in a solid organ transplant recipient were analysed. The patient had received long-term azole and echinocandin therapy for invasive aspergillosis and persistent candidemia. Minimal inhibitory concentrations were determined by the EUCAST broth microdilution method. Molecular mechanism of antifungal resistance was determined by sequencing hotspots of the FKS. Strain relatedness was determined using a microsatellite-based typing method.

Results: Importantly, typing analysis showed an identical microsatellite pattern for all isolates, supporting a close relation. The first *C. glabrata* isolate showed wild type phenotype (i.e. susceptibility to echinocandins and low level of azole resistance). After voriconazole therapy, the *C. glabrata* acquired pan-azole resistance quickly. Later, echinocandin treatment led to the emergence of a FKS2 S663P alteration and echinocandin resistance. Importantly, after disruption of both azole and echinocandin therapy in favour of liposomal amphotericin B, *C. glabrata* isolates regained full susceptibility to echinocandin and lost the FKS2 S663P alteration, while nonetheless maintaining their pan-azole resistance.

Conclusion: our clinical report supports the potential existence of a fitness cost conferred by FKS mutation in *C. glabrata*, as disruption of treatment led to a rapid disappearance of the resistant clone. This suggests that a more restricted use and/or a discontinuous administration of echinocandins may limit the spread of clinical resistance to this class.

Introduction (1093 words)

Echinocandins are widely used as first line therapy for invasive candidiasis and candidemia. They are broadly active against most *Candida* species, including *C. glabrata*. This latter, which in many countries is the second most frequently implicated yeast in invasive candidiasis after *C. albicans* [1, 2], presents an intrinsically low susceptibility to azoles and often develops pan-azole resistance. For these reasons, echinocandins are of particular interest in the antifungal treatment strategy. However, with the increasing use of echinocandins [3] many *Candida* species are developing resistance [1, 4]. The best-characterised mechanism of echinocandin resistance involves hotspot regions of the FKS genes [5]. Echinocandin resistance has, for now, only been described among patients who received that class of antifungal drugs. This observation might be related to a high fitness cost for the yeast conferred by the acquisition of resistance. Currently however, a fitness cost has been demonstrated only *in vitro* or in animal models [6, 7]; it has not yet been documented in patients and is thus subject to various results [8]. To shed light on the potential existence of a fitness cost conferred by FKS alteration and echinocandin resistance we analysed six sequential isolates of *C. glabrata* with different antifungal susceptibility profiles, sampled from a transplant patient who received multiple different lines of antifungal treatment.

Materials and methods

C. glabrata isolates were obtained from a unique patient between May 2015 and August 2015. Minimum inhibitory concentrations (MICs) were determined for each isolate using the Etest method and confirmed using the EUCAST broth microdilution method. Sequences for the hotspot regions of the FKS genes and molecular typing of the *C. glabrata* isolates were performed as previously described [9]. Briefly, DNA from each isolate was subjected to PCR for eight microsatellite-containing regions using fluorescent primers. Pools of amplicons plus an internal fluorescent ladder (400HD-Rox, Applied-Biosystems) were run on a 3500xL Dx genetic analyser (Life Technologies). Chromatograms were analysed using GeneMapper software v4.1 to assign fragment size for each amplicon. An internal fluorescent ladder was used to distinguish fragment of respective sizes of 117, 129, 162, 171, 214 and 114, 129, 155, 168, 233 bp for microsatellites A and B.

Results

The 46-year-old patient was admitted to the ICU after he received a combined kidney and liver transplantation for hepatocellular carcinoma and multifactorial end stage renal disease. On postoperative day (POD) seven, he developed probable invasive aspergillosis. On POD eight, he developed *C. glabrata* candidemia. Voriconazole was initiated on POD 11 (Figure 1). The *C. glabrata* strain initially showed intermediate susceptibility toward azoles (Table 1) but quickly acquired pan-azole resistance. Caspofungin (70 mg per day instead of 50 mg, motivated by extracorporeal membrane oxygenation) was added to voriconazole. The candidemia became persistent (last positive blood culture 28 days after the first one) due to a *C. glabrata* liver abscess, which was resolved surgically, in turn allowing for the negativation of blood cultures. After clinical improvement and one month of treatment discontinuation, micafungin (100 mg/day) was empirically initiated on POD 90. Only seven days later, a *C. glabrata* candidemia breakthrough occurred. Antifungal therapy was changed to liposomal amphotericin B, which failed to completely eradicate the yeast (sporadically positive blood cultures) after 50 days of treatment. The patient died from *Pseudomonas aeruginosa* ventilator-associated pneumonia and refractory septic shock on POD 148.

Antifungal susceptibility profiles and molecular analysis. The results are compiled in Table 1. The first isolate had a wild-type phenotype (intermediate susceptibility to azoles and full susceptibility to echinocandins). All further isolates were completely resistant to all azole derivatives. The isolate retrieved during the candidemia breakthrough, while the patient was receiving micafungin, was resistant to echinocandins and harboured the well-identified S663P alteration. Isolates collected up to 11 days after micafungin cessation (during the liposomal amphotericin B treatment) maintained the same profile. However, when MICs were determined on *C. glabrata* isolates retrieved after 11 days, they indicated regained susceptibility to echinocandins, although azole resistance was still present.

Microsatellite analysis. Fragment size analysis demonstrated that all of the six isolates tested had a similar multi-microsatellite locus pattern, supporting the clonal origin of the isolates.

Discussion

Acquired resistance involving FKS hotspot mutations is a subject of concern, but to date it has only been described in patients who were already receiving echinocandins. The acquisition of FKS mutations is related to cell wall modifications, notably an increase in chitin content [10]. These alterations have a clear impact, as the mutated yeast grows more slowly, has lower virulence in animal models and falters when simultaneously challenged by the wild type genotype [6, 7, 11].

In our study, all of the *C. glabrata* isolates were undistinguishable by genotyping analysis. Extended treatment with caspofungin did not lead to the apparition of *Candida* with higher echinocandin MICs. Intriguingly, a candidemia breakthrough with the same *C. glabrata* strain, except for the FKS2 S663P alteration, occurred only seven days after micafungin initiation. So, despite the prolonged period of antifungal therapy, the *C. glabrata* strain was not replaced by another. Moreover, emergence appeared quickly after treatment initiation while no resistance occurred during the 28-day treatment period with high-dose caspofungin. This might be due at least in part to an insufficient dosage of the drug in this particular patient [12].

Facing a multi-drug resistant *C. glabrata*, we switched treatment to liposomal amphotericin B. During that treatment, blood cultures were sporadically positive, possibly due to remaining occult deep lesions. A blood culture sampled 23 days after echinocandin cessation retrieved the same *C. glabrata* except that it was once again susceptible to echinocandin and lacked the S663P alteration. Interestingly however, its azole resistance profile was not modified.

Our report reflects the daily clinical practice of mycologists and physicians and thus has some limitations. We did not perform extensive and exhaustive analyses of the innumerable colonies that grew in our array of blood culture vials and thus may have missed mixed and persistent resistant isolates which might reflect a pooled reservoir, as previously described [13]. Nonetheless, the most important observation is that at that time we were no longer able to detect a resistant isolate. Thus, our report brings an important observation to light that required further investigation based on larger clinical datasets. Indeed, the disappearance of the echinocandin resistant *Candida* harbouring an FKS alteration following the discontinuation of echinocandin treatment for several days is a strong argument for the existence of a fitness cost conferred by the FKS mutation in the setting of human infection. Stopping the selection pressure may lead to the elimination of the resistant mutated clone. Thus, discontinuous administration of echinocandin or alternating treatments might limit the incidence of resistance.

Figure legend

Figure 1: Time line for a solid organ transplant recipient who developed *Candida glabrata* candidemia due to related isolates presenting different antifungal susceptibility patterns. Day 1 (D1) corresponds to the day the patient received the graft. The encircled 'MIC's indicate determinations of minimal inhibitory concentrations by Etest and EUCAST (*) or by Etest only.

Acknowledgements

We thank Astellas for providing micafungin to perform EUCAST methods.

Funding

This work was supported by internal funding.

Transparency declarations.

A.F. has received funds for speaking from Merck; for consultancy from Pfizer; and for travel from Astellas, Gilead, Merck and Pfizer. CH received travel grants from MSD, Astellas, Pfizer and Gilead and fees for oral speaker by MSD and Astellas. All other authors have none to declare.

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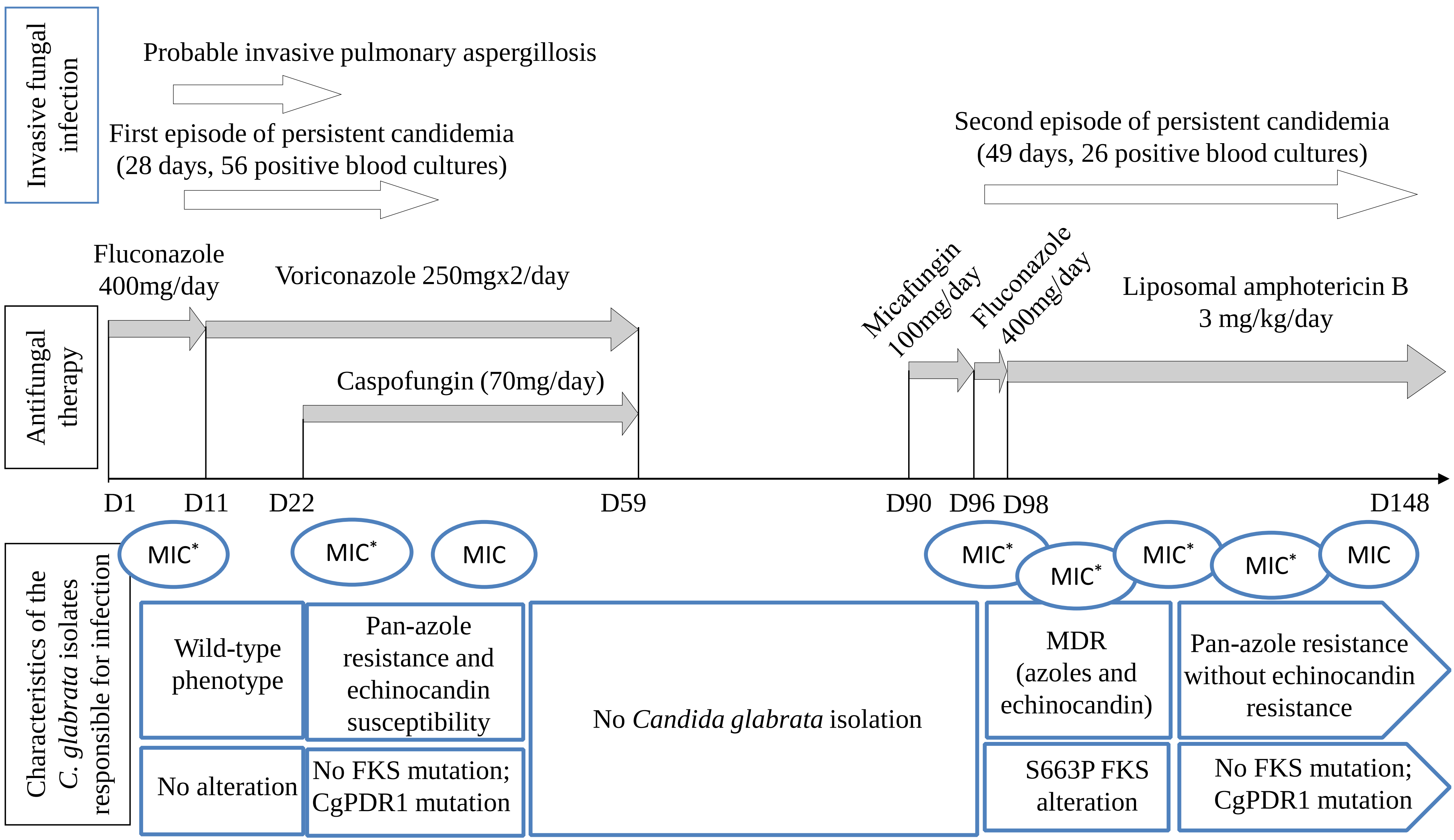
Figure 1


Invasive fungal infection
Probable invasive pulmonary aspergillosis
First episode of persistent candidemia (28 days, 56 positive blood cultures)
Second episode of persistent candidemia (49 days, 26 positive blood cultures)
Antifungal therapy
Fluconazole 400mg/day
Voriconazole 250mgx2/day
Caspofungin (70mg/day)
Micafungin 100mg/day
Fluconazole 400mg/day
Liposomal amphotericin B 3 mg/kg/day
D1
D11
D22
D59
D90
D96
D98
D148
Characteristics of the C. glabrata isolates responsible for infection
MIC*
MIC*
MIC
MIC*
MIC*
MIC*
MIC*
MIC
Wild-type phenotype
Pan-azole resistance and echinocandin susceptibility
No Candida glabrata isolation
MDR (azoles and echinocandin)
Pan-azole resistance without echinocandin resistance
No alteration
No FKS mutation; CgPDR1 mutation
S663P FKS alteration
No FKS mutation; CgPDR1 mutation

Isolate number		**1**	**2**	**3**	**4**	**5**	**6**
Post-operative day number		D8	D25	D97	D104	D108	D119
Type of sample		Blood culture	Liver abcess	Blood culture	Blood culture	Blood culture	Blood culture
Antifungal drug administred at time of sampling (number of days from start of treatement)		fluconazole (8)	voriconazole (15) and caspofungin (4)	micafungin[a] (7)	liposomal amphotericin B (7)	liposomal amphotericin B (11)	liposomal amphotericin B (22)
Determination of Minimal Inhibitory Concentrations (mg/L) by EUCAST method	amphotericin B	1	1	1	1	1	1
	fluconazole	8	>64	>64	>64	>64	>64
	itraconazole	1	>16	>16	>16	>16	>16
	voriconazole	0.125	4	4	4	4	8
	posaconazole	0.5	>8	>8	>8	>8	>8
	caspofungin	0.25	0.5	**>16**	**>16**	**>16**	0.5
	micafungin	<0.03	<0.03	**2**	**2**	**1**	<0.03
Molecular analysis of genes related to antifungal resistance	FKS 1 (HS1, HS2 and HS3)	WT	WT	WT	WT	WT	WT
	FKS 2 HS1	WT	WT	**S663P**	**S663P**	**S663P**	WT
	FKS 2 HS2	WT	WT	WT	WT	WT	WT
	FKS 3	WT	WT	WT	WT	WT	WT

Table 1: evolution of minimal inhibitory concentrations and genetic alteration of six related sequentially isolated *Candida glabrata* responsible for invasive infection in a solid organ transplant recipient receiving multiple antifungal therapy

HS: hot spot ; WT: wild type

[a]: last dose of micafungin was administrated the day before blood culture was sampled