Emergence of Echinocandin Resistance due to a Point Mutation in the *fks1* Gene of *Aspergillus fumigatus* in a Patient with Chronic Pulmonary Aspergillosis

Cristina Jiménez-Ortigosa[1*], Caroline Moore[2], David W. Denning[3] and David S. Perlin[1]

[1]Public Health Research Institute-New Jersey Medical School-Rutgers Biomedical Health Sciences, Newark, NJ, USA

[2]Mycology Reference Centre, Manchester, University Hospital of South Manchester, Manchester UK

[3]University of Manchester, Manchester, UK

*Corresponding author: jimenecr@njms.rutgers.edu

Running title: Echinocandin resistance in *A. fumigatus*

Abstract: 46 words

Body Text: 1459

ABSTRACT

We have identified the first case of a *fks1* hot spot 1 point mutation causing echinocandin resistance in a clinical *Aspergillus fumigatus* isolate recovered from a chronic pulmonary aspergillosis patient with an aspergilloma who first failed azole and polyene therapy, and then subsequently failed micafungin treatment.

TEXT

Invasive aspergillosis (IA) and chronic pulmonary aspergillosis (CPA) are both life-threatening mycoses mainly caused by the fungal pathogen *Aspergillus fumigatus.* Triazole antifungal drugs represent first-line therapy for all forms of aspergillosis. Voriconazole is used as the primary azole for treatment of IA, while both itraconazole and voriconazole are used to treat CPA; posaconazole is more often used for prophylaxis (1, 2). Even though acquired resistance during therapy is rare, epidemiological studies have shown that *Aspergillus* azole resistance has increased over the past decade in patients with prolonged exposure to azoles, notably those with CPA (3). Mutations in the *cyp51A* gene leading to amino acid substitutions in the target enzyme lanosterol 14α- demethylase are the major mechanism of azole resistance (1, 4, 5), yet non-*cyp51A*-mediated resistance has been recently reported (6).

Micafungin (MCF) is a member of the echinocandin class of antifungal agents that targets the fungal cell wall by the inhibition of the β-(1,3)-glucan synthase (GS), an enzyme unique to fungi and responsible for the synthesis of the major cell wall component, β-(1,3)-D-glucan. MCF has demonstrated *in vitro* and *in vivo* activity against *A. fumigatus,* as well as synergistic activity when used in combination therapy with azoles (isavuconazole, itraconazole, voriconazole) or with amphotericin B (7, 8). The first-in-class echinocandin, caspofungin, was initially approved

by the FDA to treat patients with IA refractory to conventional therapy. Subsequently, micafungin has also been used to treat patients with IA, as a second line drug. Echinocandin resistance in *Candida* spp. has been linked to mutations in the *FKS* genes, which encode the β-(1,3)-glucan synthase enzymes (9-11). In *A. fumigatus,* an engineered point mutation in the hot spot 1 region of the *fks1* gene was also sufficient to confer resistance to echinocandin drugs (12). Yet, to date, no echinocandin resistant *A. fumigatus* isolates harboring a characteristic *fks* mutation have been recovered from patients after exposure to an echinocandin. In this study, we report the first case of a *fks1* hot spot 1 point mutation causing echinocandin resistance in a clinical *A. fumigatus* isolate recovered from a chronic pulmonary aspergillosis patient who initially failed azole and polyene therapy, and subsequently echinocandin therapy.

CASE REPORT

A 66-year-old life-long non-smoker female complained of weight loss, fatigue and severe breathlessness. The patient had severe kyphoscoliosis as a child with insertion of spinal rods in early adulthood. She had suffered recurrent chest infections for many years. She first presented in 2001 with an irritating cough and several treatments with antibiotics failed to alleviate it. After 2 years, the cough worsened. The patient then coughed up large amounts of blood (haemoptysis) requiring intensive care admission which was treated with embolization and oral tranexamic acid. She continued to cough and produced green sputum and lost weight. Her *Aspergillus* precipitin (IgG) titre was high and a CT scan demonstrated chronic cavitary pulmonary aspergillosis with a large fungal ball (aspergilloma). She started itraconazole therapy in 2005 (200mg BD, twice daily) but failed to respond despite satisfactory blood drug levels, and was switched to voriconazole in 2006 (150mg BD). Considerable improvement was seen initially and the patient gained weight. Voriconazole therapy continued for 2 years. However, the *Aspergillus*

precipitin titre remained high and the cough continued. Further tests showed that trough plasma levels of voriconazole were above 0.5 mg/L; however, *Aspergillus fumigatus* isolates recovered were resistant to itraconazole, voriconazole and posaconazole. She received IV amphotericin B (120mg daily) for 3 weeks without any impairment of renal function. She had further intermittent courses of therapy with amphotericin B at the same daily dose, without improvement over the following year. In 2010, the patient started micafungin IV 150mg 6x weekly with oral terbinafine (250mg BD). She improved substantially and continued to take micafungin, with terbinafine to minimize the risk of resistance. She remained on this combination, until she developed more haemoptysis and therapy was discontinued in 2013. She remained off therapy for over 2 years. Haemoptysis recurred and she was trialed on isavuconazole in 2015/6 (200mg three times daily for 2 days and then 200mg a day). A time-line of the antifungal therapy of the patient is shown in **Fig. 1A**.

Once micafungin therapy was initiated, twelve consecutive *Aspergillus* spp. isolates (11 *A. fumigatus* and 1 *A. flavus*) were recovered from sputum cultures (**Fig. 1B**) and were submitted for antifungal susceptibility testing in accordance to the guidelines described in CLSI document M38-A2 (13). The drugs used were isavuconazole (ISA) (Astellas Pharma USA, Inc., Northbrook, IL), itraconazole (ITR) (Sigma-Aldrich, St. Louis, MO), voriconazole (VRC) (Pfizer Inc., New York, NY), posaconazole (POS) (Merck Sharp & Dohme Corp., Rahway, NJ), amphotericin B (AMB) (Sigma-Aldrich, St. Louis, MO), caspofungin (CSF) (Merck Sharp & Dohme Corp., Rahway, NJ), micafungin (MCF) (Astellas Pharma USA, Inc., Northbrook, IL) and terbinafine (TERB) (Novartis Pharmaceuticals, East Hanover, NJ). Multilocus sequencing typing (MLST) of the 11 *A. fumigatus* strains collected from the patient was conducted (14).

Promoter and open reading frame of the *cyp51A* (Afu6g12400) and the *fks1* (Afu4g06890) genes were sequenced to identify mutations that account for resistance (**Table 1**).

Twelve isolates were collected from the patient after MCF treatment, 11 of them were classified as *A. fumigatus* and 1 isolate was classified as *A. flavus* after internal transcribed spacer (ITS)-PCR identification. All *A. fumigatus* isolates but one (24053B) grew well on PDA plates, showing the typical dark green colonies (**Fig. 1B**); by contrast, 24053B presented a diminish growth rate and a reduction in sporulation, hence its white colony color (**Fig. 1C**). To assess the genetic diversity and the potential relatedness of the *A. fumigatus* isolates recovered from the patient, MLST was carried out since a previous study in patients with aspergilloma has reported the extreme genetic diversity in isolates recovered from fungal balls (15). MLST revealed only two different sequence types ST9 and ST12 (**Table 2**). According to the CLSI epidemiological cut-off values (ECVs) (16), all isolates except one (45755) were classified as wild-type (WT) for all the triazole drugs tested. Sequencing of the *cyp51A* gene revealed five mutations unlinked to azole resistance in all *A. fumigatus* isolates (17) (**Table 2**). Since no ECVs have been established for terbinafine or micafungin, three WT strains (ATCC13073, Af293 and R21 [18]) were included in the study for comparison reasons. Clinical isolates showed an MIC range of 0.5-1 µg/ml for terbinafine compared to 0.5-2 µg/ml for control strains (**Table 2**); hence, we considered the clinical isolates to be WT for this antifungal drug. Regarding echinocandins, isolate 24053B showed a 16.6-33.3- and 66.6-fold increase in minimum effective concentrations (MECs) for CSF and MCF, respectively, compared to control strains. The other 11 isolates recovered from the patient showed a WT phenotype to all echinocandins assayed (**Table 2**). The reduced *in vitro* susceptibilities to echinocandin drugs were confirmed in GS enzyme assays. Product-entrapped β-(1,3)-glucan synthase complexes were extracted from the prototype WT

strain ATCC13073 and the 24053B clinical isolate as previously described (10, 11), and the echinocandin inhibition parameter IC_{50} (half maximal inhibitory concentration) was determined. The *fks1* enzyme extracted from the 24053B clinical strain showed a multi-log increase in IC_{50} values than that from the WT (>10^6-fold change for both CSF and MCF) (**Fig. 2**) indicating that it was nearly insensitive to drug. DNA sequence analysis of the published *A. fumigatus* Af293 *fks1* gene, revealed a point mutation at nucleotide position 2072 (T to C) in 24053B isolate. This nucleotide change conferred a Phe-to-Ser amino acid substitution in codon position 675, the first codon of the highly conserved hot spot 1 region of *fks1* (nt2071-3003→ aa675-684) (**Table 3).** The equivalent mutation in this hot spot 1 region is prominent and well known to confer echinocandin resistance in *Candida albicans* (F641S) and other *Candida* spp., and has been linked with echinocandin clinical failure (9-11). It is noteworthy that the 24053B isolate was not recovered from any other samples collected from the patient and even though micafungin therapy failed. As it has been described previously, multiple strains are present in aspergillomas and the strain that is grown from sputum often does not reflect the full spectrum of strains present. It is likely that the patient still harbors MCF resistant strains. The addition of terbinafine did not prevent the emergence of resistance to MCF, although it is possible it delayed its emergence.

In conclusion, a resistance-associated point mutation in the well-conserved hot spot 1 region of *fks1* conferring a F675S amino acid substitution was found in *A. fumigatus* isolate 24053B recovered from a patient on micafungin therapy for CPA. The mutant strain yielded a β-(1,3)-glucan synthase enzyme with highly reduced (>5-6 log orders) sensitivity to echinocandin drugs resulting in elevated MECs and echinocandin clinical failure. To date, this is the first reported

case of echinocandin resistance due to a characteristic point mutation in the *fks1* gene in an *A. fumigatus* clinical isolate.

AKNOWLEGMENTS

This work was supported by a grant from Astellas Pharma, Inc. to D.S.P.

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200 **Figure legends**

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202 **Fig. 1. A.** Time-line for patient antifungal therapy. Collection of the *Aspergillus* spp. isolates for
203 the current microbiological study is shown (triangles). **B.** Isolates recovered from the patient
204 grown for 2 days at 37º C on a PDA plate. **C.** *A. fumigatus* clinical isolates 24053A and 24053B
205 grown for 4 days at 37º C on PDA plates. Isolate 24053B grows at a lower rate and sporulates
206 very poorly compared to the rest of isolates collected from the patient.

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208 **Fig. 2.** Echinocandin kinetic inhibition profiles for wild-type ATCC13073 and clinical isolate
209 24053B. Product-entrapped β-(1,3)-glucan synthase complexes were assessed by incorporation
210 of [^{3}H]-UDP-glucose into radiolabeled product and evaluated using a sigmoidal-response
211 (variable-slope) curve. Echinocandin inhibition kinetics yielding 50% inhibitory concentrations
212 (IC_{50}s) are expressed in nanograms per milliliter.

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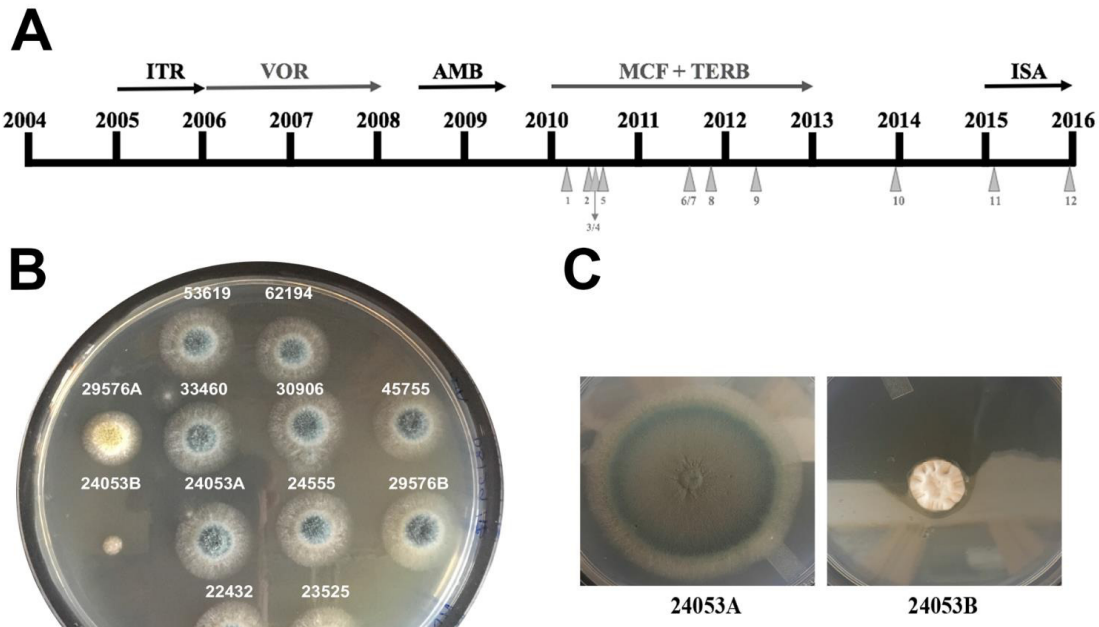
Fig. 1. A. Time-line for patient antifungal therapy. Collection of the *Aspergillus* spp. isolates for the current microbiological study is shown (triangles). **B.** Isolates recovered from the patient grown for 2 days at 37º C on a PDA plate. **C.** *A. fumigatus* clinical isolates 24053A and 24053B grown for 4 days at 37º C on PDA plates. Isolate 24053B grows at a lower rate and sporulates very poorly compared to the rest of isolates collected from the patient.



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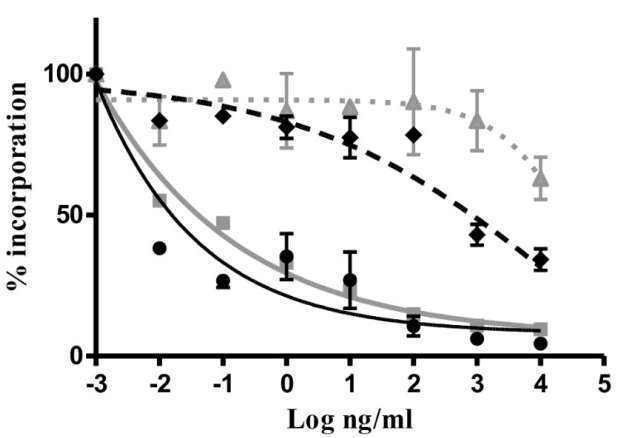

Strain	IC_{50} (ng/ml)
ATCC13073 CSF	<0.01
24053B CSF	>10000
ATCC13073 MCF	<0.01
24053B MCF	>10000

Table 1. Oligonucleotides used for *fks1 and cyp51A* PCR and sequencing.

#	Oligo Name	Sequence 5'→3'	Purpose
1	AfFKS1 -751F	CCTGAGTTGGTGGTCAAT	
2	AfFKS1 R6378	GACTGGCGAAACACGTTG	Af*fks1* PCR amplification
3	AfFKS1F -211	CTGCGACTCGAGATTCAG	
4	AfFKS1F +697	GCATGCGCAACATGTATG	
5	AfFKS1F +1562	CGCACAATCGCTTTACAC	
6	AfFKS1 1947F	CGTCAGTATGTGGCTAGC	
7	AfFKS1F +2405	GATTTCTCAAGTTTGGAATGC	
8	AfFKS1 3043F	CGATCAAGCTCCTGTACC	Af*fks1* sequencing
9	AfFKS1F +3401	GTCTGACAACCAGAATCAC	
10	AfFKS1F +4269	GTCCAGGAACTGACAGAG	
11	AfFKS1F +5117	CGAAGTCATGTTCTTCCTTG	
12	AfFKS1R +5931	CTTCGAGGCGCTGGATAC	
13	AfCyp51A F -991	CGTCGATCTGTGTGACAC	
14	AfCyp51A R 1993	CTAGAAGGAGCAGGACTG	Af*cyp51A* PCR amplification
15	AfCyp51A F -819	CATGCTGGGAGGAATCTC	
16	AfCyp51A F -147	GCTGGTCTCTCATTCGTC	
17	AfCyp51A F 502	AGAGTCTCATGTGCCACT	Af*cyp51A* sequencing
18	AfCyp51A F 1151	CACTCCTCTATTCACTCTATC	

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Table 2. MIC/MEC distributions of the antifungal drugs tested in the study for the *Aspergillus spp.* clinical isolates. Alterations in the Cyp51A and Fks1 together with the sequence type (ST) are also shown. Three wild type (WT) strains have also been included for comparison purposes.

#	Lab no.	Date received	ID	MIC/MEC (mg/L)								Cyp51A*	Fks1*	ST
				ISA	ITR	POS	VOR	CSF	MCF	AMB	TERB			
1	22432	2/9/2010	*Aspergillus fumigatus*	0.25	0.25	0.12	0.25	0.12	0.03	2	1	Y46F, V172M, T248N, E255D, K427E	ND[$]	9
2	23525	5/4/2010	*Aspergillus fumigatus*	0.25	0.25	0.12	0.12	0.12	0.03	2	0.5	Y46F, V172M, T248N, E255D, K427E	ND	9
3	24053A	6/15/2010	*Aspergillus fumigatus*	0.25	0.25	0.25	0.25	0.12	0.03	2	1	Y46F, V172M, T248N, E255D, K427E	S53G	12
4	24053B	6/15/2010	*Aspergillus fumigatus*	0.5	0.25	0.25	1	**2**	**2**	2	1	Y46F, V172M, T248N, E255D, K427E	S53G and F675S	9
5	24555	7/28/2010	*Aspergillus fumigatus*	0.25	0.25	0.25	0.25	0.12	0.03	2	0.5	Y46F, V172M, T248N, E255D, K427E	ND	9
6	29576A	7/27/2011	*Aspergillus flavus*	0.25	0.5	0.25	0.25	0.12	0.03	2	0.06	WT[#]	ND	ND
7	29576B	7/27/2011	*Aspergillus fumigatus*	0.25	0.25	0.25	0.25	0.12	0.03	2	0.5	Y46F, V172M, T248N, E255D, K427E	ND	9
8	30906	10/25/2011	*Aspergillus fumigatus*	0.25	0.5	0.25	0.12	0.12	0.03	2	1	Y46F, V172M, T248N, E255D, K427E	ND	12
9	33460	4/10/2012	*Aspergillus fumigatus*	0.25	0.5	0.25	0.12	0.12	0.03	2	1	Y46F, V172M, T248N, E255D, K427E	ND	9
10	45755	12/30/2013	*Aspergillus fumigatus*	2	1	1	0.5	0.12	0.03	2	1	Y46F, V172M, T248N, E255D, K427E	ND	12
11	53619	1/28/2015	*Aspergillus fumigatus*	0.25	0.5	0.25	0.25	0.12	0.03	2	0.5	Y46F, V172M, T248N, E255D, K427E	ND	9
12	62194	12/24/2015	*Aspergillus fumigatus*	0.25	0.5	0.25	0.12	0.12	0.03	2	0.5	Y46F, V172M, T248N, E255D, K427E	ND	9
	ATCC13073		*Aspergillus fumigatus*	0.25	0.25	0.25	0.25	0.12	0.03	2	1	ND	S53G	ND
	R21		*Aspergillus fumigatus*	0.25	0.25	0.25	0.25	0.12	0.03	2	2	ND	WT	ND
	Af293		*Aspergillus fumigatus*	0.12	0.12	0.12	0.12	0.06	0.03	1	0.5	ND	WT	ND

*Ref strain used Af293 [$]ND = not determined [#]Ref strain used *A. flavus* NRRL3357

Table 3. *fks1* hot spot 1sequencing of the *A. fumigatus* 24053B isolate.

Strain	*fks1* HS1	
	nt	aa
Af293 (Ref)	TTCCTGACCCTGTCTTTCAAGGATCCGATCCG	**F**LTLSFKDPI
ATCC13073	TTCCTGACCCTGTCTTTCAAGGATCCGATCCG	**F**LTLSFKDPI
24053B	TCCCTGACCCTGTCTTTCAAGGATCCGATCCG	**S**LTLSFKDPI