Echinocandin Resistance in *Candida auris* Occurs in the Murine Gastrointestinal Tract due to *FKS1* Mutations

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19 Running title: Echinocandin Resistance in *C. auris* from *FKS1* Mutation

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Abstract

Candida auris is resistant to multiple antifungal agents. This study investigated its antifungal susceptibility and explored *FKS1* mutations across the isolates from mice enterically colonized with wild-type *C. auris* and treated with echinocandin. Resistant *C. auris* with *FKS1* mutations, including S639F, S639Y, D642Y, R1354H, or R1354Y, were isolated and found to be micafungin and caspofungin resistant *in vivo*; however, the MIC of isolates with mutation in R1354 remained below the micafungin breakpoint *in vitro*.

Keywords: *Candida auris*, yeast, antifungal resistance, gastrointestinal tract

Text

Candida auris is a multidrug-resistant yeast responsible for causing invasive fungal diseases. Despite this, most *C. auris* isolates are susceptible to echinocandins, making it the recommended initial treatment for infections caused by *C. auris* in adults (1). Clinical echinocandin resistance in *C. auris* is generally associated with amino acid mutations in the hot spot (HS) regions of *FKS1*, which encode β-1,3-D glucan synthase (2, 3). The development of echinocandin resistance has been reported in *C. auris* isolates recovered from

patients treated with echinocandins (4-6).

C. auris was reported to mainly colonize mucosal surfaces such as the skin and respiratory and urinary tracts; however, it could also be isolated from stools, indicating that it colonized the intestinal tract (7, 8). Diarrhea and gastrointestinal (GI) decompression were reported to be risk factors for *C. auris* infection (9), suggesting the possibility of *C. auris* colonization of the intestinal tract and dissemination via translocation; this was reported in a murine model (10). The GI tract is the reservoir of *Candida* spp., where they acquire antifungal drug resistance (11); therefore, studies are needed to elucidate the mechanism of *C. auris* antifungal resistance acquisition in the intestinal tract. This study investigated *C. auris* antifungal susceptibility and explored the *FKS1* mutations across isolates from mice enterically colonized with *C. auris* and subsequently treated with micafungin or caspofungin.

C. auris NCPF 8971 (Clade I) (12), *C. auris* JCM 15448 (Clade II) (13), and *Candida albicans* SC 5314 (14) were used in this study. Micafungin and caspofungin MICs were 0.12 and 0.25 μg/mL, respectively, for NCPF 8971 and 0.06 and 0.008 μg/mL each for JCM 15448, and SC 5314, respectively.

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (15) and the institutional regulations and guidelines for

57 animal experimentation after pertinent review and approval by the Institutional Animal Care
58 and Use Committee of Nagasaki University under protocol number 1906121536. *Candida*
59 colonization of the intestinal tract of a murine model was established, as described previously
60 (16). Briefly, specific-pathogen-free, 5-week-old female DBA/2J mice (CLEA Japan Inc.,
61 Tokyo, Japan) were fed a low-protein diet before colonization. The mice were inoculated
62 intragastrically with 0.2 mL of *Candida* cell suspensions adjusted to 5×10^6 cells/mL. Sterile
63 water with antibacterial agents was provided post-inoculation. The mice were treated
64 intraperitoneally with 2.71 mg/kg micafungin (Astellas, Tokyo, Japan) or 0.76 mg/kg
65 caspofungin (MSD, Tokyo, Japan) for 11 days (4–14 days after inoculation). Echinocandin
66 dose was the standard (AUC_{100}), corresponding to human equivalent exposure, and was
67 calculated using the pharmacokinetic/pharmacodynamic study (17). Fifteen days post-
68 inoculation, stool from live mice was collected, weighed, and homogenized. The latter was
69 then appropriately diluted and spread on yeast extract peptone dextrose plates with 200 mg/L
70 imipenem. After 48-h incubation, a single colony per animal was collected from the
71 developed colonies, stored at −80 °C, and tested for drug sensitivity to echinocandins. All
72 animal experiments were performed twice, with 10 mice per group in each experiment, and
73 data from two experiments were combined to get the final value.

While SC 5314 or JCM 15448 had no colony, NCPF 8971 formed colonies at 3 to 4 $\log_{10}$ CFU/mg of stool. Table 1 shows echinocandin susceptibilities of *C. auris* isolates from the stool of NCPF 8971-inoculated mice treated with micafungin or caspofungin. Antifungal susceptibility tests were performed using the Sensititre YeastOne microtiter panel (TREK Diagnostic Systems, East Grinstead, UK) (18). Although breakpoints have not been established for *C. auris*, the Centers for Disease Control and Prevention (CDC) published tentative MIC breakpoints, as follows: micafungin, caspofungin, and anidulafungin ≥ 4, 2, and 4 μg/mL, respectively (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html). Accordingly, 5%, 20%, and 5% of the micafungin-treated isolates and 10%, 35%, and 5% of caspofungin-treated isolates were considered resistant to micafungin, caspofungin, and anidulafungin, respectively. All micafungin or anidulafungin-resistant isolates were found to be caspofungin resistant.

The sequences of *FKS1* HS1, HS2, and HS3 regions of all isolates were analyzed next. *FKS1* mutations were found in 10% of micafungin-treated mice and 20% of caspofungin-treated mice. Two isolates harboring *FKS1* mutation (S639Y or R1354H) were identified from 20 strains across micafungin-treated mice; four isolates harboring S639F, D642Y, R1354Y, or R1354H were identified from 20 strains in caspofungin-treated mice

(Table 1). No isolate with mutation in *FKS1* HS3 was observed. All micafungin or anidulafungin-resistant isolates harbored mutations in *FKS1* HS1 or HS2, whereas 45% of caspofungin-resistant isolates had no mutation. The *FKS1* sequences of all non-wild-type strains were deposited to the DDBJ with the accession numbers shown in Table 1.

All statistical analyses were carried out using Prism, version 9.0.2 software (GraphPad Software, Inc., La Jolla, CA, USA). The survival curves were compared using log rank (Mantel-Cox) test. The fungal burden in the organs was analysed using Mann–Whitney U test. A P value < 0.05 was considered to be statistically significant.

To examine *in vivo* echinocandin susceptibility, survival curves of mice infected with wild-type and mutant strains evolved *in vivo* in the above experiment were evaluated. Mice were infected intravenously with *C. auris* NCPF 8971 (wild type) and four isolates, each harboring S639Y, D642Y, R1354Y, and R1354H, and subsequently treated with micafungin or caspofungin (n = 5/group). Specific-pathogen-free, 7-week-old female BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were administered 150 and 100 mg/kg cyclophosphamide 4 and 1 day before infection, respectively, and 100 mg/kg 2 and 5 days post-infection. They were infected intravenously through the lateral vein with 0.2 mL of *Candida* cell suspension adjusted to 3.5×10^5 cells/mL. Micafungin (2.71 mg/kg) and

caspofungin (0.76 mg/kg) were administered intraperitoneally for seven consecutive days commencing 2 h post-infection. The survival rates were not significantly different between wild-type- and mutant strains-infected mice in the no-treatment group. However, the survival rates of the wild-type-infected mice suggested lower mortality than those of mice infected with all types of *FKS1* mutants in micafungin or caspofungin treatment groups ($P < 0.05$) (Figure 1).

The therapeutic efficacy of echinocandin was evaluated by comparing the reduction in *C. auris* CFU burden in mice kidneys. BALB/c mice were infected intravenously with *C. auris* NCPF 8971 and four isolates harboring *FKS1* mutations. The number of *Candida* cells was adjusted to 5×10^6 CFU/mouse. Micafungin (2.71 mg/kg) and caspofungin (0.76 mg/kg) were administered intraperitoneally for three consecutive days commencing 2 h post-infection. To evaluate fungal burden in kidneys, mice were euthanized 3 days after infection (n = 5/group). In wild-type-infected mice, both micafungin and caspofungin significantly reduced the kidney fungal burden ($P < 0.01$) compared to the control group; no significant reduction was observed among the four isolates harboring *FKS1* mutations (Figure 2).

This is the first report of *C. auris* acquiring echinocandin resistance in the murine GI tract. Here, resistant strains were isolated from NCPF 8971-inoculated mice; none were

125 isolated from those inoculated with JCM 15448 and SC 5314. Since only few *C. auris* clade
126 II strains are echinocandins resistant (19), different *C. auris* clades may have varying
127 capacities to acquire echinocandin resistance.

128 In *C. auris*, three mutations (S639Y, S639P, and S639F) in *FKS1* HS1 have been
129 reported to be correlated with echinocandin resistance (2, 3); recently, mutations in *FKS1*
130 HS1 (ΔF635, F635Y, F635L, and D642Y) and *FKS1* HS2 (R1354S and R1354H) have also
131 been reportedly implicated (4, 5, 20). In our study, isolates harbored mutations in *FKS1*, such
132 as S639F, S639Y, D642Y, R1354H, and R1354Y. These mutations except R1354Y have all
133 previously been described multiple times. All mutations showed echinocandin resistance
134 both *in vitro* and *in vivo*. Our findings show that the strains with mutation in R1354 were the
135 most isolated, and there could be more strains with this mutation clinically. Interestingly, *in*
136 *vitro* MICs of all the isolates with mutation in R1354 exceeded caspofungin breakpoints but
137 not micafungin and anidulafungin, as proposed by CDC; the therapeutic effect of micafungin
138 *in vivo* was poor. This indicated that although the MICs of echinocandins are below the
139 breakpoints, if close to 1 μg/mL, strains may become echinocandin resistant.

140 The present study demonstrated that *C. auris* acquires echinocandin resistance
141 relatively easily in the murine GI tract. Limitations of the present study include the use of

only two *C. auris* strains and the inability to clarify the echinocandin concentrations in the GI tract. Previous studies reported that the GI caspofungin concentrations were significantly lower than plasma levels, and drug levels within the GI tract were not sufficiently maintained for long time in a murine model (11). Similarly, in our model, GI drug concentrations were unlikely to be high enough to inhibit *C. auris* growth; this may have created a niche that allowed *C. auris* to acquire echinocandin resistance. To the best of our knowledge, there are no reports examining echinocandin concentrations in human GI tract; however, insufficient echinocandin concentrations may induce *C. auris* to acquire echinocandin resistance in humans. Further studies are warranted to examine the relationship between drug concentrations in the GI tract and *C. auris* resistance acquisition, as well as GI echinocandin concentrations in humans. This study may have implications in clinical practice and future validation; therefore, using various strains with different clades might help identify novel *FKS1* mutations.

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233 Table 1. Echinocandin susceptibilities of *C. auris* wild-type and mutant strains isolated from

234 mouse stool

Agents administered	Stain no.	DDBJ Accession no.	Amino acid mutation *FKS1* HS1	Amino acid mutation *FKS1* HS2	MIC (μg/mL) MFG	MIC (μg/mL) CAS	MIC (μg/mL) AFG
	NCPF 8971		WT	WT	0.12	0.25	0.12
MFG	1	LC736030	S639Y	WT	8	> 8	> 8
	2	LC736033	WT	R1354H	1	> 8	1
	3		WT	WT	1	> 8	1

4	WT	WT	0.5	2	1
5	WT	WT	0.25	1	0.25
6	WT	WT	0.25	0.5	0.25
7	WT	WT	0.12	1	0.25
8	WT	WT	0.12	1	0.25
9	WT	WT	0.12	1	0.25
10	WT	WT	0.12	1	0.25
11	WT	WT	0.12	1	0.12
12	WT	WT	0.12	1	0.12
13	WT	WT	0.12	1	0.12
14	WT	WT	0.12	0.5	0.25
15	WT	WT	0.12	0.5	0.25
16	WT	WT	0.12	0.5	0.12
17	WT	WT	0.12	0.5	0.12
18	WT	WT	0.12	0.5	0.12
19	WT	WT	0.12	0.25	0.25

	20		WT	WT	0.03	1	0.25
CAS	1	LC736029	S639F	WT	> 8	> 8	1
	2	LC736031	D642Y	WT	4	> 8	> 8
	3	LC736034	WT	R1354H	2	> 8	2
	4	LC736032	WT	R1354Y	1	4	1
	5		WT	WT	0.5	> 8	0.5
	6		WT	WT	0.25	> 8	0.5
	7		WT	WT	0.25	1	0.25
	8		WT	WT	0.12	1	0.25
	9		WT	WT	0.12	1	0.25
	10		WT	WT	0.12	1	0.25
	11		WT	WT	0.12	1	0.25
	12		WT	WT	0.12	1	0.12
	13		WT	WT	0.12	1	0.12
	14		WT	WT	0.12	1	0.12
	15		WT	WT	0.12	0.5	0.12

16	WT	WT	0.12	0.5	0.12
17	WT	WT	0.12	0.5	0.12
18	WT	WT	0.12	0.25	0.12
19	WT	WT	0.06	0.25	0.12
20	WT	WT	0.03	2	0.25

Tentative MIC breakpoints: micafungin ≥ 4 μg/mL, caspofungin ≥ 2 μg/mL, and anidulafungin ≥ 4 μg/mL

MFG, micafungin; CAS, caspofungin; AFG, anidulafungin; WT, wild type

Figure legends

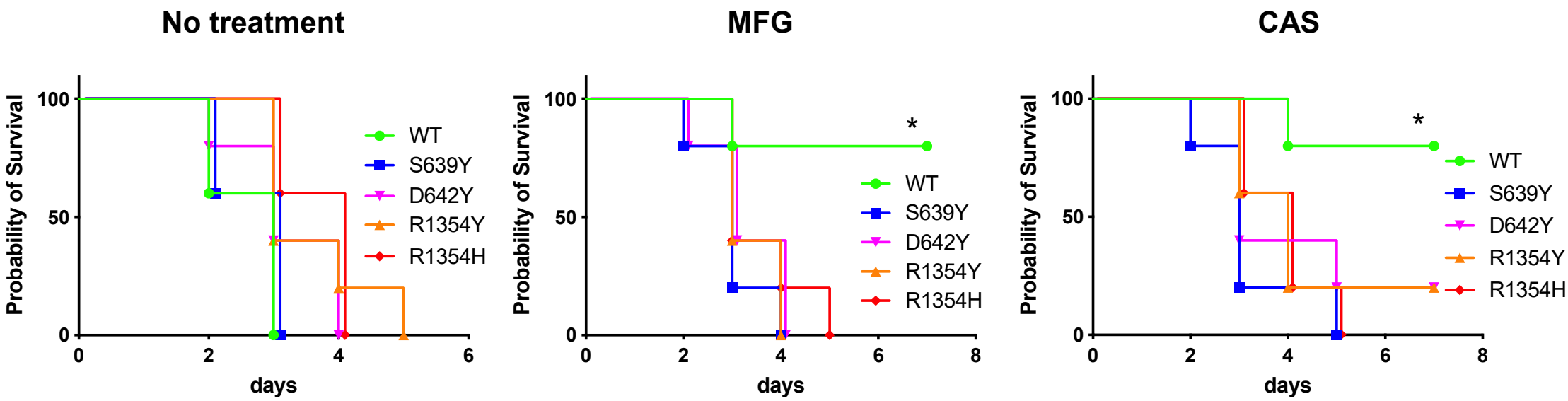
Figure 1. Survival analyses of mice infected with *C. auris* wild-type strain and *FKS1* mutants

Immunocompromised mice were inoculated with *C. auris* NCPF 8971 (wild-type) and isolates harboring *FKS1* mutations. Micafungin (2.71 mg/kg) and caspofungin (0.76 mg/kg) were administered intraperitoneally for seven consecutive days. Survival curves of *FKS1* mutant infection were compared with those of *FKS1* wild-type infection. Data were analyzed by log-rank (Mantel-Cox) test. Asterisks indicate statistically significant differences (* $P < 0.05$).

MFG, micafungin; CAS, caspofungin

Figure 2. Treatment efficacy of micafungin and caspofungin against *C. auris* wild-type strain and *FKS1* mutants

BALB/c mice were infected with *C. auris* NCPF 8971 (wild-type) and isolates harboring *FKS1* mutations. Micafungin (2.71 mg/kg) and caspofungin (0.76 mg/kg) were administered intraperitoneally for three consecutive days. The number of cells recovered from the bilateral kidneys is indicated for individual mice in the plots, and error bars represent SDs. Asterisks and NS indicate statistically significant differences (** $P < 0.01$) and no significance ($P > 0.05$), respectively. NT indicates no treatment. MFG, micafungin; CAS, caspofungin


No treatment
MFG
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Probability of Survival
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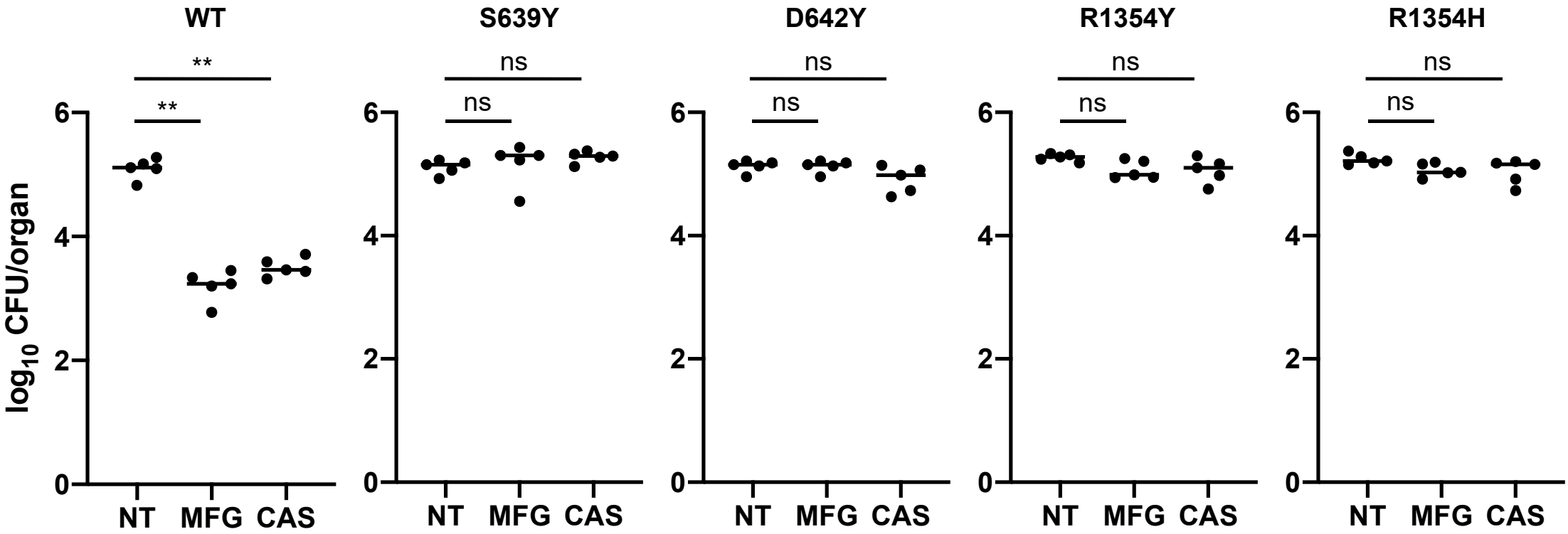
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