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Original Article

Antifungal susceptibility trend and analysis of resistance mechanism for *Candida* species isolated from bloodstream at a Japanese university hospital☆

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ABSTRACT

We compared the susceptibility of six commercially available antifungal agents (fluconazole, itraconazole, voriconazole, caspofungin, micafungin, and amphotericin B) against 133 *Candida* bloodstream isolates between 2008 and 2013 at Aichi Medical University Hospital. *C. albicans* was the most common isolate, followed by *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*. MIC_{90}s of voriconazole against *C. albicans*, *C. parapsilosis*, and *C. tropicalis* were the lowest and that of micafungin against *C. glabrata* was the lowest among the agents tested. Of the 133 isolates, two strains were identified as drug-resistant. One was a fluconazole-resistant *C. glabrata* strain, in which the ATP-binding cassette (ABC) transporter gene expression was upregulated. The other was a micafungin-resistant *C. glabrata* strain, that had 13 amino acid substitutions in *FKS1* and *FKS2*, including a novel substitution V1342I in *FKS1* hotspot 2. We also evaluated the susceptibility of T-2307, a novel class of antifungal agents used in clinical trials, against the fluconazole- and micafungin-resistant *C. glabrata* strain; the MICs of T-2307 were 0.0039 and 0.0078 μg/mL, respectively. In conclusion, the incidence of bloodstream infection caused by drug-resistant *Candida* spp. was rare from 2008 to 2013 at our hospital. Of 133 isolates, only two strains of *C. glabrata* were resistant to azoles or echinocandins, that upregulated the ABC transporter genes or had novel *FKS* mutations, respectively.

1. Introduction

Fungal infections occur in immunocompromized or hospitalized patients with serious underlying diseases and comorbidities. *Candida* spp. are considered the major causative pathogens of septicemia and account for high mortality rates [1,2]. Some population-based studies conducted in the USA, Europe, and Asia have reported an increased incidence of candidemia [3–6]. Some reports have demonstrated that the antifungal activity of azoles against *C. albicans*, *C. glabrata*, and *C. guilliermondii* isolates from

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MIC, minimum inhibitory concentration; MS, mass spectrometry; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

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blood was different from that observed against other types of specimens [7,8]. Therefore, antifungal susceptibility has to be tested for each isolate. Population-based laboratory surveillance conducted in Atlanta and Baltimore, USA, from 2008 to 2011 revealed an increase in the prevalence of non-*albicans Candida* spp., which are resistant or less susceptible to azoles [4]. Furthermore, increasing echinocandin resistance in *C. glabrata* has been an emerging concern because it is innately less susceptible to azoles [9]. In *C. glabrata*, amino acid substitutions within the subunits of 1,3-β-glucan synthase, namely *FKS1* and *FKS2*, which are the targets of echinocandins, such as micafungin and caspofungin, have been associated with echinocandin resistance [10]. Echinocandin exposure within the preceding month is the greatest risk factor for developing breakthrough infections caused by echinocandin-resistant *C. glabrata* strains with *FKS* mutations [11]. The acquired resistance of *C. glabrata* to azoles, such as fluconazole, itraconazole, and voriconazole, has been associated with several mechanisms, the most common being the induction of efflux pumps encoded by the ABC transporter genes (*CDR1*, *CDR2*, and *SNQ2*) or MDR belonging to the major facilitator superfamily that leads to decreased drug concentration [12,13]. Azole-resistant strains upregulating *CDR1* have been shown to enhance virulence [14]. Therefore, monitoring resistance trends based on the resistance mechanism at each hospital is needed to predict clinical response and estimate clinical aspects of using novel antifungal agents against drug-resistant strains.

However, there are no epidemiological reports on antifungal resistance surveillance along with resistance mechanism in *Candida* spp. strains isolated from patient blood samples in Japan. In this study, we collected 133 *Candida* bloodstream isolates from Aichi Medical University and examined their resistance to commercially available antifungal agents and the resistance mechanisms. In addition, we evaluated the antifungal activity of T-2307, which is a novel class of antifungal agent used in clinical trials (Fig. 1) [15,16], against drug-resistant *Candida* isolates.

2. Materials and methods

2.1. Organisms

Overall, 133 bloodstream isolates of *Candida* spp. (one isolate per patient, except two isolates per patient were collected from five patients) collected at Aichi Medical University Hospital between June 2008 and September 2013 were used. Table 1 presents the distribution of *Candida* spp. by year. All isolates were identified to the species level using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry fingerprinting with a MALDI Biotyper (Bruker Daltonics KK, Yokohama, Japan) and CHROMagar™ Candida (Mast Diagnostica GmbH, Reinfeld, Germany), according to the manufacturer's specifications. The isolates were stored as frozen stock in 20% glycerol at −80 °C. Prior to use, each isolate was subcultured at least twice on Sabouraud dextrose agar plates to ensure purity and optimal growth.

2.2. Antifungal susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by broth microdilution method [17], with a final inoculum concentration of 0.5×10^3–2.5×10^3 cells/ml in RPMI 1640 medium

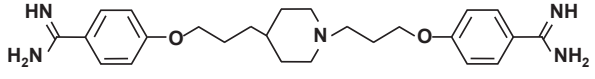

Fig. 1. Chemical structure of T-2307.

Table 1
Distribution of *Candida* Species by year.

Species	No. of *Candida* spp. by year						total No. (%)
	2008	2009	2010	2011	2012	2013	
C. albicans	10	2	9	17	13	10	61 (45.9)
C. parapsilosis	4	3	7	5	8	5	32 (24.1)
C. glabrata	–	2	4	4	5	2	17 (12.8)
C. tropicalis	–	1	1	3	4	3	12 (9.0)
C. guilliermondii	–	1	3	2	–	–	6 (4.5)
C. metapsilosis	–	2	–	–	–	1	3 (2.3)
C. krusei	–	–	1	–	–	–	1 (0.8)
C. lusitaniae	–	–	–	–	1	–	1 (0.8)
total	14	11	25	31	31	21	133 (100.0)

(Sigma, Steinheim, Germany) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (Merck, Darmstadt, Germany). The antifungal agents used in this study and the concentration ranges tested in two-fold steps were as follows: amphotericin B (Bristol-Myers KK, Tokyo, Japan), 0.0625–32 μg/mL; caspofungin (MSD KK, Tokyo, Japan), 0.000125–64 μg/mL; micafungin (Astellas Pharma Inc., Tokyo, Japan), 0.000125–64 μg/mL; fluconazole (Toyama Chemical Co., Ltd.), 0.000125–64 μg/mL; itraconazole (Janssen Pharmaceutical KK, Tokyo, Japan), 0.0005–4 μg/mL; voriconazole (Pfizer Japan Inc., Tokyo, Japan), 0.000125–64 μg/mL; and T-2307 (Toyama Chemical Co., Ltd.), 0.000000125–64 μg/mL. Each strain was incubated at 35 °C for 24–48 h in 96-well plates under aerobic conditions, and the MIC endpoint was determined according to document M27-A3 [17]. MIC was visually assessed as the lowest concentration of agents showing no growth for amphotericin B and prominent growth reduction of ≥50% for other antifungal agents compared with the agent-free growth control. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used as controls. The resistance breakpoints were defined according to the interpretive criteria of Clinical and Laboratory Standards Institute (CLSI) [18]. MIC values ≥64, ≥0.5, and ≥0.25 μg/mL for fluconazole, caspofungin, and micafungin, respectively, were considered as resistant for *C. glabrata*.

2.3. DNA extraction, polymerase chain reaction (PCR) amplification, sequencing, and sequence analysis

DNA was extracted from *Candida* spp. isolates using PrepMan™ Ultra (AppliedBiosystems, Foster City, CA, USA), according to the manufacturer's instructions. PCR was performed in triplicate with the extracted DNA, 0.2 μM each of the appropriate forward and reverse primers (Table 2) [19], and Premix Taq DNA Polymerase (Takara Bio Inc., Shiga, Japan), as recommended by the manufacturer. DNA sequencing was performed by Takara Bio Inc. Sequences were analyzed and translated using GENETYX 12.0 (Genetyx Co., Ltd., Tokyo, Japan). The resultant protein sequences were compared with those of *C. glabrata* CBS138 (GenBank accession number XM_446406 and XM_448401 for *FKS1* and *FKS2*, respectively).

2.4. Quantitative real-time reverse transcription (RT)-PCR

Expression levels of some azole-resistant genes encoding azole efflux pumps and an enzyme of ergosterol biosynthesis were measured by quantitative real-time RT-PCR. Total RNAs were extracted from logarithmic-phase cultures of *Candida* spp. isolates in RPMI 1640 medium (pH 7.0) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Quantitative expression analysis of the target genes *CDR1*, *CDR2*, *SNQ2*, and *ERG11* was then performed. The extracted RNA (100 ng) was reverse transcribed to cDNA in a final volume of 20 μL using the 5 × PrimeScript™ RT Master Mix (Takara Bio Inc.), and 3 μl of the resultant cDNA was used as the template for

Table 2
Oligonucleotides primers used in this study.

Target gene	Primer	Purpose(s)	Sequence (5′-3′)
fks1	FKS1F[a]	PCR, sequencing	CACTTAATTAAAAAATGTCTTACAATAATAACGGACAACAAATG
	FKS1R[a]	PCR, sequencing	CACGCGGCCGCTTATTTGATTGTAGACCAGGTCTTT
	CgFKS1SQ1	sequencing	CCAACCAGAAGACCAACAGAA
	CgFKS1SQ2	sequencing	GCTGTCTTTACACGCCGATT
	CgFKS1SQ3	sequencing	CGACGGTCGTTACGTTAAGC
	CgFKS1SQ4	sequencing	GGTTCTTATGTTTGATTTTTGCAG
	CgFKS1SQ5	sequencing	CAACACTGTCTTTTCCGTTGG
	CgFKS1SQ6	sequencing	GAGAGGATGACCAATTCTCCA
	CgFKS1SQ7	sequencing	CCTCCACTAAACGAAGGTGAA
	CgFKS1SQ8	sequencing	TATGGTCACCCGGATTTCAT
	CgFKS1SQ9	sequencing	TTCTGGATTGCCTTTGTTCC
	CgFKS1SQ10	sequencing	TTGGTTGGTGATGAATCTGAA
	CgFKS1SQ11	sequencing	CAAACACTGCCTTCTGGACA
fks2	FKS2F[a]	PCR, sequencing	CACTTAATTAAAAAATGTCTTACGATCAAGGTGGTAATGGCAAT
	FKS2R[a]	PCR, sequencing	CACGCGGCCGCTTATTTTATAGTGGACCAGGTCTTT
	CgFKS2SQ1	sequencing	GCCGAGAACAACGAATTCTA
	CgFKS2SQ2	sequencing	AACCAACAAATTTGGTTTCCA
	CgFKS2SQ3	sequencing	TCACCATTATGCCAACAGAGA
	CgFKS2SQ4	sequencing	TGGCTAGTGCAATCCAGTTG
	CgFKS2SQ5	sequencing	CATCAATCGAAGATTGTTTTAGG
	CgFKS2SQ6	sequencing	TGCCAACTTTTACCGTCTTG
	CgFKS2SQ7	sequencing	TGGTTTCAATGCAAAGATTGG
	CgFKS2SQ8	sequencing	TCTGGTGTGCTTGGTGATGT
	CgFKS2SQ9	sequencing	TGATGTCTTGTATCCTATCGGTTG
	CgFKS2SQ10	sequencing	TTCCTGGGAAGATTTCTTCTTG
	CgFKS2SQ11	sequencing	TCACTAGAATGCTGGTTGGTGT
cdr1	CDR1F[b]	Detection of gene expression	TAGCACATCAACTACACGAACGT
	CDR1R[b]	Detection of gene expression	AGAGTGAACATTAAGGATGCCATG
cdr2	CDR2F[b]	Detection of gene expression	GTGCTTTATGAAGGCTACCAGATT
	CDR2R[b]	Detection of gene expression	TCTTAGGACAGAAGTAACCCATCT
snq2	SNQ2F[b]	Detection of gene expression	ACCATGTGTTCTGAATCAATCAAT
	SNQ2R[b]	Detection of gene expression	TCGACATCATTACAATACCAGAAA
erg11	ERG11F[b]	Detection of gene expression	ATTGGTGTCTTGATGGGTGGTC
	ERG11R[b]	Detection of gene expression	TCTTCTTGGACATCTGGTCTTTCA
ura3	URA3F[b]	Detection of gene expression	GAAAACCAATCTTTGTGCTTCTCT
	URA3R[b]	Detection of gene expression	CATGAGTCTTAAGCAAGCAAATGT

[a] Niimi et al., 2012 [19].
[b] Sanguinetti et al., 2005 [20].

real-time PCR with specific primers for the target genes (Table 2) [20]. The experiments were performed in triplicate using Power SYBR® Green PCR Master Mix and StepOnePlus™ Real-Time PCR System (both Applied Biosystems), as per manufacturer specifications. Expression levels of the target genes were evaluated with relative quantification using the comparative CT method, according to manufacturer guidelines. *URA3* was used as a reference gene for normalization of the target genes' expression levels in each strain. Results were presented as a fold expression relative to the expression levels identified in azole-susceptible *C. glabrata* ATCC 90030.

2.5. Statistical analysis

The quantitative real-time RT-PCR experiments were performed in triplicate for each isolate. All numerical data were presented as mean ± standard deviation. Data were analyzed using the unpaired two-tailed *t*-test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism (ver. 5.0; GraphPad, La Jolla, CA, USA).

3. Results

3.1. Species distribution of Candida bloodstream isolates recovered from 2008 to 2013

Our results revealed that the incidence of candidemia had increased from 2009 to 2012, ranging from 11 to 31 episodes per year; the incidence in 2008 was slightly higher than that in 2009. The prevalence of *C. albicans* was much lesser in 2009 than in the other years. Collectively, *C. albicans* was the most common isolate (45.9%), followed by C. *parapsilosis* (24.1%), *C. glabrata* (12.8%), and *C. tropicalis* (9.0%; Table 1).

3.2. Antifungal susceptibility testing

Table 3 summarizes the MIC distribution, MIC_{50}, and MIC_{90} of the antifungal agents tested for the predominant *Candida* spp. The MIC_{90}s of voriconazole against *C. albicans* (0.0078 μg/mL), *C. parapsilosis* (0.0313 μg/mL), and *C. tropicalis* (0.0313 μg/mL) were the lowest among the agents tested. In contrast, the MIC_{90} of voriconazole against C. *glabrata* (0.5 μg/mL) was higher than those of micafungin (0.0625 μg/mL) and caspofungin (0.125 μg/mL). The MIC_{90}s of amphotericin B were not markedly different (MIC_{90}s, 2–4 μg/mL) among *Candida* spp. Of the 133 isolates, two strains were identified as drug-resistant. One was a fluconazole-resistant (MIC, 64 μg/mL) *C. glabrata* strain, and the other was a micafungin-resistant (MIC, 0.5 μg/mL) *C. glabrata* strain (Table 4). Additionally, we evaluated the *in vitro* antifungal activity of T-2307 that has a novel mechanism of action against drug-resistant *C. glabrata* isolated in this study (Fig. 1) [16,21]. The MICs of T-2307 against fluconazole- and micafungin-resistant *C. glabrata* strains were 0.0039 and 0.0078 μg/mL, respectively (Table 4).

3.3. FKS mutations in the micafungin-resistant strain

The patient from whom the micafungin-resistant strain was isolated had been treated twice with micafungin for a few weeks at

Table 3
MIC distributions of antifungals determined by the broth microdilution method for 133 *Candida* isolates.

Species and antifungal agent (no. of isolates)	No. of isolates with MIC (μg/mL) of[a]:																		MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
	0.001	0.002	0.0039	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	>64		
***C. albicans* (61)**																				
Fluconazole							5	42	14										0.125	0.25
Itraconazole						11	33	12	4	1									0.0625	0.125
Voriconazole	1	6	39	15															0.0039	0.0078
Amphotericin B										1	24	35	1						2	2
Caspofungin				1		1	21	37	1										0.125	0.125
Micafungin					1	29	31												0.0625	0.0625
***C. parapsilosis* (32)**																				
Fluconazole								1	6	16	6	3							0.5	1
Itraconazole							7	17	8										0.125	0.25
Voriconazole		1	2	12	10	5	1	1											0.0156	0.0313
Amphotericin B											1	31							2	2
Caspofungin									5	26	1								0.5	0.5
Micafungin										14	14	4							1	2
***C. glabrata* (17)**																				
Fluconazole											1		9	4	2		**1**		4	16
Itraconazole									3	5	7	1		1[b]					1	2
Voriconazole						2	2	8	3	1		1							0.125	0.5
Amphotericin B											2	14	1						2	2
Caspofungin							1	15	1										0.125	0.125
Micafungin						13	3			**1**									0.0313	0.0625
***C. tropicalis* (12)**																				
Fluconazole								3	6	3									0.25	0.5
Itraconazole								4	5	2	1								0.25	0.5
Voriconazole				2	6	3		1											0.0156	0.0313
Amphotericin B											1	9	2						2	4
Caspofungin		1				1	6	4											0.0625	0.125
Micafungin						6	5	1											0.0313	0.0625
Other *Candida* spp. (11)																				
Fluconazole										1	2	3		3		1		1	2	32
Itraconazole								3		5	1	1		1[b]					0.5	2
Voriconazole			1			4		4	1				1						0.125	0.25
Amphotericin B											2	8	1						2	2
Caspofungin									8	3									0.25	0.5
Micafungin							1	1		4	2	3							0.5	2
All organisms (133)																				
Fluconazole							5	46	26	20	9	6	9	7	2	1	1	1	0.25	4
Itraconazole						11	40	36	20	13	9	2		2[b]					0.125	0.5
Voriconazole	1	7	42	29	16	14	3	14	4	1		1	1						0.0078	0.125
Amphotericin B										1	30	97	5						2	2
Caspofungin		1		1		2	28	56	15	29	1								0.125	0.5
Micafungin					1	48	40	2		19	16	7							0.0625	1

[a] Boldface values indicate isolates with MICs above Clinical and Laboratory Standards Institute (CLSI) resistance breakpoints [18].
[b] The MIC values are >4 μg/mL.

Table 4
In vitro activity of antifungal agents against *Candida* bloodstream isolates resistant to fluconazole or micafungin.

Organism	MIC (μg/mL)						
	T-2307	Fluconazole	Itraconazole	Voriconazole	Amphotericin B	Caspofungin	Micafungin
C. glabrata	0.0078	8	0.5	0.125	2	0.125	0.5
C. glabrata	0.0039	64	>4	2	2	0.125	0.0625

the time of strain isolation. *C. glabrata* strains with *FKS* mutations are recovered almost exclusively from patients with prior echinocandin exposure [11]. Therefore, we searched for amino acid substitutions in *FKS1* and *FKS2* of micafungin-resistant *C. glabrata* and identified 12 mutations in *FKS1* and one in *FKS2* (Fig. 2). Of these, a single mutation in *FKS1* hotspot 2, V1342I, was newly identified. This strain was susceptible to the other antifungal agents including caspofungin (Table 4).

3.4. Expression levels of ABC transporter genes in the azole-resistant strain

The patient from whom the fluconazole-resistant strain was isolated had been treated with fluconazole for 2 weeks before strain isolation. The major mechanism of acquired azole resistance in *C. glabrata* is the induction of efflux pumps, which are encoded by the ABC transporter genes (*CDR1*, *CDR2*, and *SNQ2*) and MDR [12,13]. Upregulation of the *CDR* genes confers resistance to almost all azoles, whereas MDR-encoded efflux pumps have a narrow spectrum specific for fluconazole [13]. The fluconazole-resistant *C. glabrata* isolated in our study was less susceptible to itraconazole and voriconazole compared with other *C. glabrata* isolates (Tables 3 and 4). Thus, we focused on the upregulation of the ABC transporter genes as a mechanism contributing to azole resistance in the isolated strains. The expression levels of *CgCDR1*, *CgCDR2*, and *CgSNQ2* in the azole-resistant isolate were analyzed using quantitative real-time RT-PCR [20] and were significantly increased by 41.9-, 6.1-, and 2.8-fold, respectively, compared with the

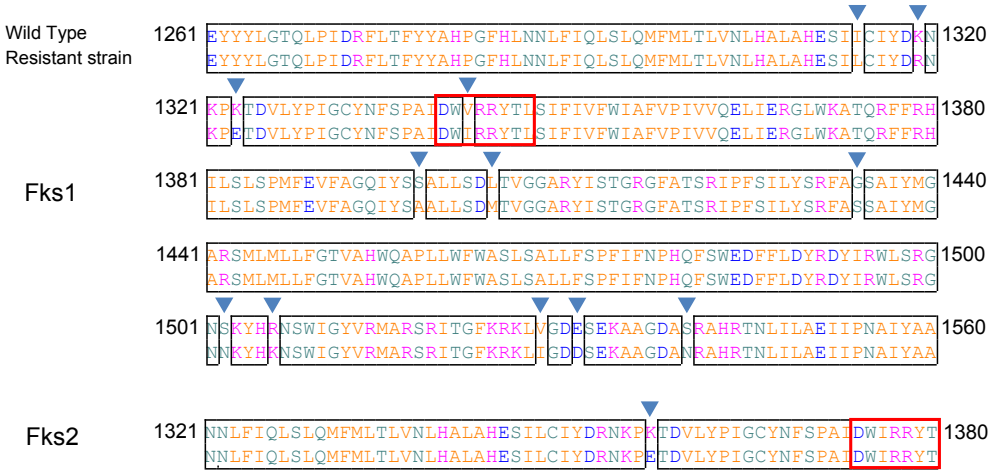

Fig. 2. Alignment of the deduced *FKS1* and *FKS2* amino acid sequences from *C. glabrata* CBS138 and micafungin-resistant *C. glabrata* strain using GENETYX 12.0 (Genetyx Co., Ltd., Tokyo, Japan). The red rectangular boxes indicate *FKS* hotspot regions. The blue triangles above the amino acid sequences indicate substituted amino acid positions in *FKS*.

susceptible control isolate ATCC90030 (Fig. 3). *CgERG11* expresses the azole target enzyme, and its expression did not vary between the susceptible and resistant strains.

4. Discussion

In this study, we evaluated the susceptibility of commercially available antifungal agents against 133 *Candida* bloodstream isolates and found one fluconazole-resistant and one micafungin-resistant *C. glabrata* isolates. *C. albicans* was the most common isolate, followed by *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*. The species distribution of *Candida* bloodstream isolates was in accordance with the Japanese and global trends; however, the prevalence of C. *guilliermondii* and *C. metapsilosis* was high [22–24].

The MIC_{50}/MIC_{90} values of the antifungal agents against *Candida* bloodstream isolates in a national surveillance in 2002 and at one institution from 2007 to 2011 were not markedly different; however, those of azole against *C. tropicalis* were higher than those at our hospital [23,25]. This national surveillance was firstly performed after micafungin was approved. In other words, azoles may have been used more often before [23]. Furthermore, Katsuragi et al. demonstrated that the low susceptibility of *C. tropicalis* to azoles was due to the high frequency of fluconazole use at their facility [25]. Although there are no epidemics of nosocomial infection caused by echinocandin-resistant strains in Japan, the susceptibility of micafungin against *C. albicans* appears to be decreasing, as described by the results of our study and those of previous studies [23,25]. Additionally, in North America, the susceptibilities of micafungin against *C. albicans*, *C. glabrata*, and *C. tropicalis* in bloodstream infections were decreased after the clinical introduction of micafungin [24]. These reports have demonstrated that clinical use of azoles or echinocandins could gradually induce resistance to each class of antifungal agents; therefore, a novel class of antifungal agents is urgently required. We evaluated the *in vitro* antifungal activity of T-2307 against drug-resistant *C. glabrata* isolates. T-2307 exhibited potent antifungal activity against drug-resistant *C. glabrata* strains. In a previous report, the MIC_{50}/MIC_{90} of T-2307 against clinical isolates of *C. glabrata*, whose MIC_{90}s to fluconazole and micafungin were within the susceptible ranges, were equivalent to the MICs of T-2307 against drug-resistant *C. glabrata* isolates obtained in our study [16]. Furthermore, the MICs of fluconazole-resistant *C. albicans* strains to T-2307 were equivalent to the MIC_{50} of clinical isolates of fluconazole-susceptible *C. albicans* strains to T-2307 [16]. These results demonstrated that the *in vitro* activity of T-2307 against *Candida* spp. was not affected by the susceptibilities to other agents. T-2307 exhibited potent *in vivo* activity against *Candida spp.* including fluconazole- or caspofungin-resistant strains

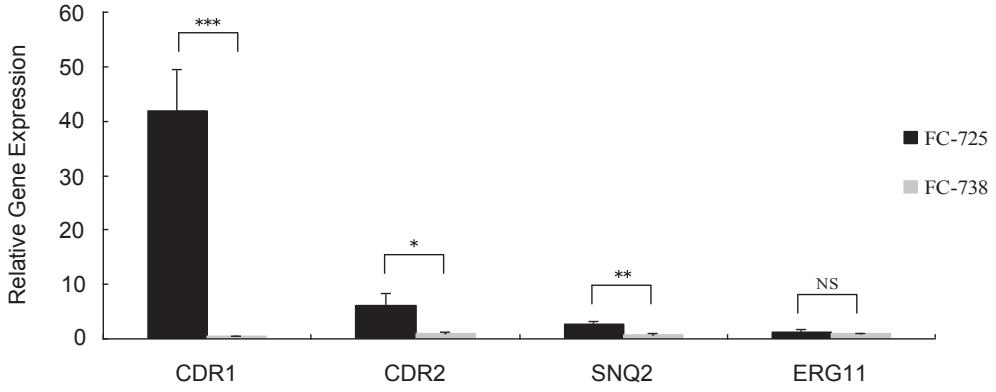

Fig. 3. Expression levels of fluconazole resistance-related genes. The fold changes are determined from the mean normalized expression relative to that of *C. glabrata* ATCC 90030. FC-725 is a fluconazole-resistant (64 μg/mL) *C. glabrata* strain. FC-738 is the *C. glabrata* strain most susceptible to fluconazole (1 μg/mL). All error bars represent the mean ± standard deviation from triplicate tests. NS: not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

[16,26–28]. The MICs of T-2307 against at least *C. glabrata* strains were similar to those in our study [26,27], suggesting that T-2307 could be a promising therapeutic option for treating bloodstream infections caused by *Candida* spp. including drug-resistant strains.

We examined the micafungin-resistant but caspofungin-susceptible *C. glabrata* strain with 13 amino acid substitutions in *FKS1* and *FKS2*, including a novel substitution V1342I in *FKS1* hotspot 2. Although mutation in hotspot 1 is considered the major mechanism for echinocandin resistance [9,29,30], *C. glabrata* isolates susceptible to micafungin and anidulafungin but intermediately resistant to caspofungin were reported to harbor hotspot 2 mutation or non-hotspot mutation [29], which might have caused micafungin resistance in our study, too. Although minor mutations were present in our isolate, the patient had been treated twice with micafungin for a few weeks at the time of strain isolation and died after the isolation. The patients from whom *C. glabrata* strains with these *FKS* mutations were detected may be at risk for breakthrough infections despite the necessity of further investigation about the clinical impact of the *FKS* mutations. We also identified an azole-resistant *C. glabrata* strain in which ABC transporter genes are highly expressed. The patient from whom this azole-resistant strain was isolated had been treated with fluconazole for 2 weeks. Azole resistance with the upregulation of ABC transporter genes was observed in a clinical isolate after exposure to fluconazole for only 2 weeks. Although the resistance rate of *C. glabrata* to azoles or echinocandins in our study was 11.8%, drug-resistant *C. glabrata* strains could have caused clonal spread in our hospital [31]. In fact, echinocandin resistance has been increasing, particularly among fluconazole-resistant *C. glabrata* isolates in North America [9,32]. Thus, we should effectively monitor resistance trends so as to not increase drug-resistant strains and extend their resistance spectrum.

This study has some limitations for generalization. Firstly, the sample size of isolates, particularly those of *C. tropicalis* strains, was small. Secondly, the *FKS* mutational pattern of the micafungin-resistant strain was novel. Finally, this study was performed at a single institution. Therefore, further studies are required to survey the resistance rate of *Candida* bloodstream isolates to antifungal agents, contribution of *FKS* mutations to micafungin resistance and their clinical outcomes, and resistance trends at other hospitals for the optimal use of antifungal agents, including a novel class of antifungal agents such as T-2307.

In conclusion, collectively, drug-resistant *Candida* spp. were still rare from 2008 to 2013 at our hospital. Of the strains tested, only two strains of *C. glabrata* were resistant to azoles or echinocandins, which upregulated the ABC transporter genes or had novel *FKS* mutations, respectively.

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Conflicts of interest

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References

[1] Kullberg BJ, Sobel JD, Ruhnke M, Pappas PG, Viscoli C, Rex JH, et al. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. Lancet 2005;366:1435–42.
[2] Flevari A, Theodorakopoulou M, Velegraki A, Armaganidis A, Dimopoulos G. Treatment of invasive candidiasis in the elderly: a review. Clin Interv Aging 2013;8:1199–208.
[3] Asmundsdottir LR, Erlendsdottir H, Gottfredsson M. Nationwide study of candidemia, antifungal use, and antifungal drug resistance in Iceland, 2000 to 2011. J Clin Microbiol 2013;51:841–8.
[4] Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, Lockhart SR, et al. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008-2011. Clin Infect Dis 2012;55:1352–61.
[5] Playford EG, Nimmo GR, Tilse M, Sorrell TC. Increasing incidence of candidaemia: long-term epidemiological trends, Queensland, Australia, 1999-2008. J Hosp Infect 2010;76:46–51.
[6] Yang ZT, Wu L, Liu XY, Zhou M, Li J, Wu JY, et al. Epidemiology, species distribution and outcome of nosocomial *Candida* spp. bloodstream infection in Shanghai. BMC Infect Dis 2014;14:241.
[7] Yamaguchi H, Nishiyama Y, Uchida K, Takahashi C, Kawashima C, Hayashi M, et al. Nationwide survey of antifungal drug susceptibility of clinical fungal isolates in Japan for the Japan Antifungal Surveillance Program (JASP), 2005. J Jpn Soc Clin Microbiol 2009;19:128–41 [in Japanese].
[8] Zaidi KU, Mani A, Parmar R, Thawani V. Antifungal susceptibility pattern of *Candida albicans* in human infections. Open Biol Sci J 2018;4:1–6.
[9] Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R, et al. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of *FKS* mutations and elevated minimum inhibitory concentrations. Clin Infect Dis 2013;56:1724–32.
[10] Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, Garey KW. FKS mutant *Candida glabrata*: risk factors and outcomes in patients with candidemia. Clin Infect Dis 2014;59:819–25.
[11] Shields RK, Nguyen MH, Clancy CJ. Clinical perspectives on echinocandin resistance among *Candida* species. Curr Opin Infect Dis 2015;28:514–22.
[12] Rodrigues CF, Rodrigues ME, Silva S, Henriques M. *Candida glabrata* biofilms: how far have we come? J Fungi (Basel) 2017;3:11.
[13] Kanafani ZA, Perfect JR. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 2008;46:120–8.
[14] Ferrari S, Sanguinetti M, Torelli R, Posteraro B, Sanglard D. Contribution of *CgPDR1*-regulated genes in enhanced virulence of azole-resistant *Candida glabrata*. PloS One 2011;6:e17589.
[15] McCarthy MW, Kontoyiannis DP, Cornely OA, Perfect JR, Walsh TJ. Novel agents and drug targets to meet the challenges of resistant fungi. J Infect Dis 2017;216(Suppl 3):S474–83.
[16] Mitsuyama J, Nomura N, Hashimoto K, Yamada E, Nishikawa H, Kaeriyama M, et al. *In vitro* and *in vivo* antifungal activities of T-2307, a novel arylamidine. Antimicrob Agents Chemother 2008;52:1318–24.
[17] Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd ed. M27-A3. Wayne: Clinical and Laboratory Standards Institute; 2008.
[18] Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts, 4th informational supplement. M27-S4. Wayne: Clinical and Laboratory Standards Institute; 2012.
[19] Niimi K, Woods MA, Maki K, Nakayama H, Hatakenaka K, Chibana H, et al. Reconstitution of high-level micafungin resistance detected in a clinical isolate of *Candida glabrata* identifies functional homozygosity in glucan synthase gene expression. J Antimicrob Chemother 2012;67:1666–76.
[20] Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. Antimicrob Agents Chemother 2005;49:668–79.
[21] Shibata T, Takahashi T, Yamada E, Kimura A, Nishikawa H, Hayakawa H, et al. T-2307 causes collapse of mitochondrial membrane potential in yeast. Antimicrob Agents Chemother 2012;56:5892–7.
[22] Kakeya H, Yamada K, Kaneko Y, Yanagihara K, Tateda K, Maesaki S, et al. National Trends in the distribution of *Candida* species causing candidemia in Japan from 2003 to 2014. Med Mycol J 2018;59:E19–22.
[23] Takakura S, Fujihara N, Saito T, Kudo T, Iinuma Y, Ichiyama S. National surveillance of species distribution in blood isolates of *Candida* species in Japan and their susceptibility to six antifungal agents including voriconazole and micafungin. J Antimicrob Chemother 2004;53:283–9.
[24] Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. Geographic variations in species distribution and echinocandin and azole antifungal resistance rates among *Candida* bloodstream infection isolates: report from the SENTRY Antimicrobial Surveillance Program (2008 to 2009). J Clin Microbiol 2011;49:396–9.
[25] Katsuragi S, Sata M, Kobayashi Y, Miyoshi T, Yamashita Y, Neki R, et al. Antifungal susceptibility of *Candida* isolates at one institution. Med Mycol J 2014;55:E1–7.
[26] Yamada E, Nishikawa H, Nomura N, Mitsuyama J. T-2307 shows efficacy in a murine model of *Candida glabrata* infection despite *in vitro* trailing growth phenomena. Antimicrob Agents Chemother 2010;54:3630–4.

[27] Wiederhold NP, Najvar LK, Fothergill AW, Bocanegra R, Olivo M, McCarthy DI, et al. The novel arylamidine T-2307 demonstrates *in vitro* and *in vivo* activity against echinocandin-resistant *Candida glabrata*. J Antimicrob Chemother 2016;71:692–5.

[28] Wiederhold NP, Najvar LK, Fothergill AW, Bocanegra R, Olivo M, McCarthy DI, et al. The novel arylamidine T-2307 maintains *in vitro* and *in vivo* activity against echinocandin-resistant *Candida albicans*. Antimicrob Agents Chemother 2015;59:1341–3.

[29] Castanheira M, Woosley LN, Messer SA, Diekema DJ, Jones RN, Pfaller MA. Frequency of *fks* mutations among *Candida glabrata* isolates from a 10-year global collection of bloodstream infection isolates. Antimicrob Agents Chemother 2014;58:577–80.

[30] Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, Farley MM, et al. Role of *FKS* mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother 2014;58:4690–6.

[31] Goemaere B, Lagrou K, Spriet I, Hendrickx M, Becker P. Clonal spread in *Candida glabrata* bloodstream isolates and fluconazole resistance affected by prolonged exposure: a 12-year single-center study in Belgium. Antimicrob Agents Chemother 2018;62, e00591-18.

[32] Pfaller MA, Castanheira M, Lockhart SR, Ahlquist AM, Messer SA, Jones RN. Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of *Candida glabrata*. J Clin Microbiol 2012;50:1199–203.