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Resistance Mechanisms and Clinical Features of Fluconazole-Nonsusceptible *Candida tropicalis* Isolates Compared with Fluconazole-Less-Susceptible Isolates

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We investigated the azole resistance mechanisms and clinical features of fluconazole-nonsusceptible (FNS) isolates of *Candida tropicalis* recovered from Korean surveillance cultures in comparison with fluconazole-less-susceptible (FLS) isolates. Thirty-five clinical isolates of *C. tropicalis*, comprising 9 FNS (fluconazole MIC, 4 to 64 μg/ml), 12 FLS (MIC, 1 to 2 μg/ml), and 14 control (MIC, 0.125 to 0.5 μg/ml) isolates, were assessed. *CDR1*, *MDR1*, and *ERG11* expression was quantified, and the *ERG11* and *UPC2* genes were sequenced. Clinical features of 16 patients with FNS or FLS bloodstream isolates were analyzed. Both FNS and FLS isolates had >10-fold higher mean expression levels of *CDR1*, *MDR1*, and *ERG11* genes than control isolates (*P* values of <0.02 for all). When FNS and FLS isolates were compared, FNS isolates had 3.4-fold higher mean *ERG11* expression levels than FLS isolates (*P* = 0.004), but there were no differences in those of *CDR1* or *MDR1*. Of all 35 isolates, 4 (2 FNS and 2 FLS) and 28 (8 FNS, 11 FLS, and 9 control) isolates exhibited amino acid substitutions in Erg11p and Upc2p, respectively. Both FNS and FLS bloodstream isolates were associated with azole therapeutic failure (3/4 versus 4/7) or uncleared fungemia (4/6 versus 4/10), but FNS isolates were identified more frequently from patients with previous azole exposure (6/6 versus 3/10; *P* = 0.011) and immunosuppression (6/6 versus 3/10; *P* = 0.011). These results reveal that the majority of FNS *C. tropicalis* isolates show overexpression of *CDR1*, *MDR1*, and *ERG11* genes, and fungemia develops after azole exposure in patients with immunosuppression.

Candida tropicalis has become an important cause of bloodstream infections (BSIs) in seriously ill patients (1–3). Although *C. tropicalis* is usually susceptible to azole antifungals, 7 to 40% of clinical isolates have recently been reported to be resistant to azoles, particularly fluconazole (2–5). The mechanisms responsible for acquired azole resistance are well-characterized in *Candida albicans* and include mutations in the *ERG11* gene, which encodes the drug target enzyme (lanosterol 14α-demethylase), overexpression of *ERG11*, and overexpression of genes encoding efflux pumps (6). In *C. tropicalis*, overexpression of *ERG11* associated with missense mutations has been described as the most frequent azole resistance mechanism in clinical isolates (7, 8). However, the azole resistance mechanisms of *C. tropicalis* remain a matter of debate, and only five studies have been reported to date (7–11). In addition, there is a lack of substantial research on mutations in the transcription factor Upc2p of *C. tropicalis*, which can induce *ERG11* overexpression and contribute to the development of fluconazole resistance in *C. albicans* (12–14).

Fluconazole MIC distributions of wild-type *C. tropicalis* ranged from 0.125 to 64 μg/ml following 24 h of incubation using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) methods, and their modal MIC values were 0.125 to 0.5 μg/ml (6). Recently, the CLSI established a *C. tropicalis* species-specific clinical breakpoint for fluconazole (susceptible, MIC of ≤2 μg/ml) which is identical to the epidemiological cutoff (MIC, 2 μg/ml) (6, 15). In this study, we investigated the azole resistance mechanisms, genotypes, and clinical features of fluconazole-nonsusceptible (FNS) isolates (fluconazole MIC, ≥4

μg/ml) recovered from Korean surveillance cultures in comparison with fluconazole-less-susceptible (FLS) isolates (fluconazole MIC, 1 to 2 μg/ml) or fluconazole-susceptible control isolates (fluconazole MIC, 0.125 to 0.5 μg/ml). *CDR1*, *MDR1*, and *ERG11* expression levels in all isolates were quantified by real-time PCR, and *ERG11* sequencing and multilocus sequence typing (MLST) were performed. For the first time, we investigated the amino acid substitutions in Upc2p of these isolates and assessed their associations with azole resistance. In addition, the clinical features of *C. tropicalis* fungemia caused by FNS isolates were compared with those of FLS isolates.

MATERIALS AND METHODS

***C. tropicalis* isolates and antifungal susceptibility testing.** A total of 35 nonduplicate clinical isolates of *C. tropicalis*, which comprised 9 FNS (flu-

conazole MIC, 4 to 64 µg/ml), 12 FLS (fluconazole MIC, 1 to 2 µg/ml), and 14 control (fluconazole MIC, 0.125 to 0.5 µg/ml) isolates, were assessed. Twenty-one FNS and FLS isolates were recovered from eight Korean university hospitals during performance of nationwide surveillance in Korea (2003 to 2013) (16, 17) and were obtained from blood (*n* = 16), urine (*n* = 3), ear pus (*n* = 1), and nasal swab (*n* = 1). All 14 control isolates were obtained from blood cultures. *In vitro* antifungal susceptibility testing for fluconazole, voriconazole, itraconazole, posaconazole, amphotericin B, anidulafungin, and micafungin was performed on all isolates using the BMD method of the CLSI according to document M27-3; MIC values were determined after 24 h of incubation (18). The categorical antifungal MICs determined by the CLSI BMD method were determined based on the new species-specific CLSI clinical breakpoints (fluconazole, voriconazole, anidulafungin, and micafungin) or epidemiological cutoff values (ECVs; itraconazole, posaconazole, and amphotericin B) for *C. tropicalis* (15, 19). In addition, the fluconazole and voriconazole susceptibilities of all isolates were determined using the Vitek 2 system (AST-YS07 card; bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Quality control was performed by testing *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

Quantification of CDR1, MDR1, and ERG11 expression. Total RNA extraction and real-time reverse transcription-PCR (RT-PCR) for the evaluation of overexpression of *CDR1*, *MDR1*, and *ERG11* were performed using methods described previously (7, 8). RNA was extracted from each isolate using the RNeasy plant minikit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's recommendations. Total RNA was quantified using a Synergy H1 Microplate Reader (Biotek, Winooski, VT, USA) and reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Quantitative PCR of *ACT1*, *CDR1*, *MDR1*, and *ERG11* was performed in triplicate as technical replicates using the QuantiTect SYBR green PCR kit (Qiagen, Hilden, Germany) and a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The primers used for real-time RT-PCR were described previously (7, 8). Amplification conditions consisted of 10 min of denaturation at 95°C, followed by 40 cycles of 15 s at 95°C for denaturation, 11 s at 54°C for annealing, and 22 s at 72°C for elongation. The cycle threshold (C_T) value of the gene was normalized to that of *ACT1* (internal control) as a ΔC_T value. Relative gene expression ($\Delta\Delta C_T$) was calculated as the fold change in expression of the isolates compared to the mean expression values in 14 control strains with fluconazole MICs of 0.125 to 0.5 µg/ml (set to 1.0).

Sequence analysis of ERG11 and UPC2. *C. tropicalis* genomic DNA was extracted as described previously (7) and was used as the template for amplification of the full-length *ERG11* and *UPC2* genes. Genomic DNA was extracted from each isolate using the DNeasy plant minikit (Qiagen Inc., Valencia, CA, USA) and used as the template for PCR amplification. The primers used for amplification and sequencing of *ERG11* were described previously (7). Amplification conditions consisted of 5 min of denaturation at 94°C, followed by 30 cycles of 40 s at 94°C for denaturation, 1 min at 50°C for annealing, and 50 s at 72°C for elongation, and a final elongation step of 10 min at 72°C. PCR products were purified using a PCR purification kit (GeneAll) according to the manufacturer's recommendations. PCR products were sequenced using the above-mentioned PCR primer pairs. Sequencing of both strands was performed using an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed using the MegAlign software package (DNAStar, Inc., Lasergene, Madison, WI, USA) and compared with those of *C. tropicalis* ERG11 ATCC 750 (GenBank accession number M23673). Newly designed primers were used for amplification and sequencing of *UPC2*: for amplification, 5′-CCACTACCATCACTATCATCAC-3′ (CtUPC2-F1) and 5′-CAATACCTATTGTGATTACTTC-3′ (CtUPC2-R3); for sequencing, 5′-TCAGAAGCTAATGGCCCA-3′ (CtUPC2-F5), 5′-GGATATTCATCTTGGCAGC-3′ (CtUPC2-R5), 5′-ACCCCTACATCCATTCCA-3′ (CtUPC2-F4), 5′-CACGCAATAATCG CAAGG-3′ (CtUPC2-R6), and 5′-CAATACCTATTGTGATTACTTC-3′ (CtUPC2-R3). Amplification conditions were 5 min of denaturation at

94°C, followed by 32 cycles of 30 s at 94°C for denaturation, 90 s at 56°C for annealing, and 3 min at 72°C for elongation, and a final elongation step of 10 min at 72°C. Purification and sequencing of PCR products were performed by the same method as that used for *ERG11* sequencing. Nucleotide sequences were analyzed using the MegAlign software package (DNAStar, Inc.) and compared with that of *C. tropicalis* (ATCC MYA-3404).

MLST analysis. The 35 *C. tropical* isolates were typed using an MLST scheme described previously (20). The internal regions of six housekeeping genes (*ICL1*, *MDR1 SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*) were sequenced. Sequence analysis of both strands was performed using an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Each strain was characterized as a diploid sequence type (DST), which was the combination of the genotypes of the six loci. MLST data have been deposited in the MLST database (http://pubmlst.org/).

Clinical data analysis. Clinical information of 16 fungemic patients from whom FNS and FLS BSI isolates of *C. tropicalis* were obtained was collected retrospectively and included patient demographics, clinical diagnosis, antifungal therapy, and outcome of fungemia (21). Therapeutic failure was defined as either the persistence of *Candida* in the bloodstream despite 3 days of antifungal therapy or development of breakthrough fungemia while receiving azole antifungal agents for 3 days (21). Previous use of azole antifungal agents was defined as administration within 3 months prior to the onset of fungemia. Immunosuppression was defined as either the presence of neutropenia (<500/mm³ absolute neutrophil count) or use of immunosuppressive therapy at the time of onset of fungemia. Clearance of fungemia was assessed at the end of therapy, and the outcome of the patients (survival or death) was assessed 30 days after the first positive blood culture result. This study was approved by the Institutional Review Board of Chonnam National University Hospital (IRB CNUH-2014-290). A waiver of the requirement for informed consent was granted given the retrospective nature of the project. Patient information was anonymized and deidentified prior to analysis, and no information that could lead to patient identification was used.

Statistical analysis. Comparison of relative gene expression was performed using the Mann-Whitney test. Data expressed as numbers or percentages were compared using the chi-squared or Fisher's exact test. Relationships between *CDR1*, *MDR*, and *ERG11* gene expression were examined using Spearman's correlation coefficient. All statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL), and a *P* value of <0.05 was considered to indicate significance.

RESULTS

Antifungal susceptibilities. Table 1 shows *CDR1*, *MDR1*, and *ERG11* expression levels, *ERG11* and *UPC2* sequences, and MLSTs of 35 clinical isolates of *C. tropicalis*. Of the 35 isolates, 9 (isolates 1 to 9) were determined to be FNS, including 6 fluconazole-resistant (MIC, ≥8 µg/ml) isolates and 3 fluconazole-susceptible dose-dependent (SDD; MIC, 4 µg/ml) isolates by the 24-h CLSI method, while 12 (isolates 10 to 21) were FLS (MIC, 1 to 2 µg/ml) isolates and 14 (isolates 22 to 35) were control (MIC, 0.125 to 0.5 µg/ml) isolates. Of the nine FNS isolates, three were resistant to voriconazole (voriconazole MIC, ≥1 µg/ml), and the remaining six exhibited dose-dependent susceptibility (SDD voriconazole MIC, 0.25 to 0.5 µg/ml) to voriconazole. However, 11 of 12 FLS isolates and all 14 control isolates were susceptible to voriconazole. Fluconazole and voriconazole MICs obtained by Vitek-2 yeast susceptibility testing were 1-fold lower than those determined by the CLSI method, but the essential agreements (within one dilution) of the CLSI for both fluconazole and voriconazole testing were 97%. Itraconazole MICs for FNS, FLS, and control isolates ranged from 0.5 to 2 µg/ml, 0.125 to 0.5 µg/ml, and 0.03 to 0.25 µg/ml, respectively, and posaconazole MICs ranged from 0.25 to 1 µg/ml, 0.06 to 1 µg/ml, and 0.03 to 0.25 µg/ml, respec-

TABLE 1 Molecular characterization of 35 clinical isolates of *Candida tropicalis*

Isolate (patient) no.	Source	Isolation (yr)	Antifungal agent MIC[a] (µg/ml)						Gene expression[b]			Amino acid substitution(s)[c]		MLST[d] (DST)
			FLU		VOR		ITR (CLSI)	POS (CLSI)	CDR1	MDR1	ERG11	Erg11p	Upc2p	
			CLSI	Vitek 2	CLSI	Vitek 2								
Fluconazole nonsusceptible														
1	Blood	2013	32	≥64	0.5	0.25	1	0.5	13.9	23.3	6.6	None	<u>A251T, Q289L</u>	232
2	Blood	2008	16	8	1	0.5	2	0.5	16.2	10.5	23.1	None	A297S,T393I, Δ(301-304), Q320QPP	399
3	Blood	2009	16	4	0.5	0.25	1	0.25	49.2	3.8	24.8	None	None	169
4	Blood	2003	8	8	0.5	0.25	1	0.25	5.6	17	75.6	None	A251T, G392E	395
5	Blood	2007	4	4	0.5	0.25	1	0.25	11.6	11.8	28.4	<u>L333I</u>	G392E	398
6	Blood	2011	4	2	0.25	≤0.12	1	0.5	8.1	18.1	56.7	None	A251T, Q289L, G392E	402
7	Ear pus	2006	64	≥64	2	1	2	1	5.9	27.1	27.6	<u>G464S</u>	A251T, Q289L	396
8	Urine	2012	16	8	2	0.5	1	0.25	85.5	2.2	25.8	None	A251T, Q289L, L343F	188
9	Urine	2012	4	2	0.25	≤0.12	0.5	0.25	13.3	12.3	64	None	A251T, Q289L, G392E	404
Fluconazole less susceptible														
10	Blood	2013	2	2	0.03	≤0.12	0.5	0.06	41.8	30.4	5.2	None	S187L	392
11	Blood	2006	2	≤1	0.125	≤0.12	0.25	0.125	7.3	9.5	14.5	None	G392E	401
12	Blood	2012	2	≤1	0.125	≤0.12	0.125	0.25	4.7	9.1	8.5	G464S	A251T, Q289L, G392E	393
13	Blood	2012	2	≤1	0.03	≤0.12	0.5	0.25	3.9	10.6	3.1	None	A251T, Q289L, G392E	405
14	Blood	2012	1	≤1	0.25	≤0.12	0.5	1	24.4	13.5	2.9	None	<u>A251T, Q289L</u>	232
15	Blood	2007	1	≤1	0.125	≤0.12	0.5	0.125	17.7	14.6	8	None	A251T, Q289L	232
16	Blood	2013	1	≤1	0.06	≤0.12	0.125	0.25	15.7	28	28.7	None	A251T, Q289L, G392E	395
17	Blood	2013	1	≤1	0.06	≤0.12	0.5	0.25	4.3	15.7	16.5	None	A251T, Q289L, G392E	359
18	Blood	2012	1	≤1	0.06	≤0.12	0.25	0.125	18.5	12.2	6.9	None	G392E	394
19	Blood	2006	1	≤1	0.06	≤0.12	0.5	0.125	10.2	17.2	16	None	A251T, Q289L	400
20	Nasal swab	2012	2	2	0.125	≤0.12	0.5	0.25	9.1	13.6	15.7	K344N, V362 M	None	403
21	Urine	2012	2	≤1	0.06	≤0.12	0.5	0.25	5.6	10.2	5.7	None	A251T, Q289L	232
Fluconazole susceptible (control)														
22	Blood	2012	0.125	≤1	0.03	≤0.12	0.03	0.03	6.4	15.5	7.8	None	G392E	448
23	Blood	2012	0.25	≤1	0.06	≤0.12	0.25	0.25	1.7	0.3	0.3	None	A251T, Q289L, S702L	385
24	Blood	2013	0.25	≤1	0.03	≤0.12	0.03	0.03	2.1	9.5	4.6	None	<u>A251T, Q289L, G392E</u>	395
25	Blood	2012	0.5	≤1	0.03	≤0.12	0.06	0.06	0.2	0.8	0.8	None	None	417
26	Blood	2012	0.25	≤1	0.03	≤0.12	0.125	0.125	1.3	3.1	2.6	None	None	401
27	Blood	2012	0.25	≤1	0.03	≤0.12	0.125	0.06	0.5	0.5	0.5	None	None	401
28	Blood	2012	0.25	≤1	0.03	≤0.12	0.06	0.03	0.2	0.5	0.5	None	None	110
29	Blood	2012	0.125	≤1	0.06	≤0.12	0.125	0.125	2.1	4.5	16.6	None	None	426
30	Blood	2012	0.125	≤1	0.03	≤0.12	0.25	0.125	0.7	9.1	10	None	A251T, Q289L	400
31	Blood	2013	0.125	≤1	0.03	≤0.12	0.06	0.03	1.4	0.9	3.9	None	A251T, A263T	NA[e]
32	Blood	2012	0.25	≤1	0.06	≤0.12	0.25	0.06	0.5	0.5	2	None	A251T, <u>Q289L</u>	232
33	Blood	2012	0.125	≤1	0.03	≤0.12	0.06	0.06	0.6	0.5	0.1	None	A251T, Q289L	124
34	Blood	2012	0.125	≤1	0.03	≤0.12	0.125	0.03	8.4	17.4	17.8	None	A251T, Q289L, G392E	395
35	Blood	2012	0.125	≤1	0.03	≤0.12	0.06	0.03	2.5	8.4	0.2	None	A251T, <u>Q289L</u>, G392E	431

[a] Antifungal MICs were determined by the 24-h CLSI M27-A3 broth microdilution method and the Vitek 2 yeast susceptibility system (AST-YS07; bioMérieux, Marcy L'Etoile, France). ITR, itraconazole; FLU, fluconazole; POS, posaconazole; VOR, voriconazole.

[b] Quantification was performed using real-time RT-PCR. Values are averages from three independent experiments and represent increases in gene expression levels relative to the mean expression levels in 14 control strains (isolates 22 to 35) with fluconazole MICs of 0.125 to 0.5 µg/ml (set to 1.0).

[c] Homozygote alleles are underlined, while heterozygote alleles are not underlined.

[d] By multilocus strain typing (MLST), each strain was characterized as a diploid sequence type (DST) using the combination of the genotypes of the six genes (*ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*) in the MLST database (http://pubmlst.org/).

[e] NA, MLST data not available. The genotypes of five genes, *ICL1*, *MDR1*, *SAPT2*, *XYR1*, and *ZWF1a*, were 1, 110, 39, 3, and 7, respectively, but the genotype of *SAPT4* was not obtained due to repeated sequencing failures.
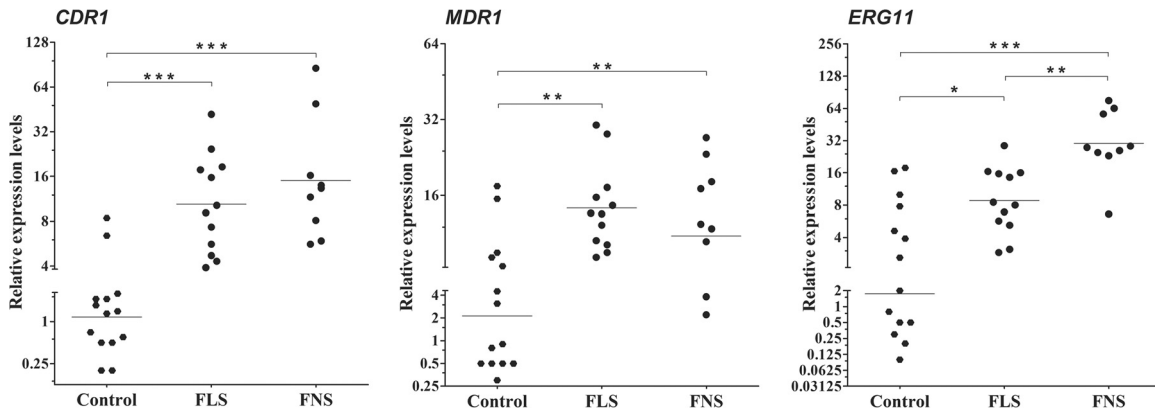
FIG 1 Relative expression levels of *CDR1*, *MDR1*, and *ERG11* in 9 fluconazole-nonsusceptible isolates (FNS; MIC, ≥4 μg/ml), 12 fluconazole-less-susceptible isolates (FLS; MIC, 1 to 2 μg/ml), and 14 control strains (control; MIC, 0.125 to 0.5 μg/ml) of *Candida tropicalis*. The relative expression level of each gene is calculated relative to the means from 14 control strains, which was set to 1.0. Filled circles represent individual isolates of *C. tropicalis*; horizontal bars show the mean gene expression levels of each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tively. When applying species-specific ECVs, eight (all FNS) and 17 (9 FNS, 7 FLS, and 1 control) isolates of the 35 were categorized as non-wild-type isolates for itraconazole (>0.5 μg/ml) and posaconazole (>0.125 μg/ml), respectively. The MIC ranges of amphotericin B, anidulafungin, and micafungin for all 35 isolates were 0.25 to 1 μg/ml (all, wild-type), 0.03 to 0.125 μg/ml (all, susceptible), and 0.03 to 0.125 μg/ml (all, susceptible), respectively, by the CLSI method.

Expression of *CDR1*, *MDR1*, and *ERG11*. Using quantitative RT-PCR, most control strains exhibited basal levels of *CDR1*, *MDR1*, or *ERG11* expression, with some fluctuations, but there were two outliers (isolates 22 and 34) that showed overexpression of both *CDR1* (>5-fold compared with the mean of all controls) and *MDR1* (>15-fold compared with the mean of all controls) (Fig. 1). Nine FNS isolates had 23.3-, 14.0-, and 37.0-fold higher mean expression levels of the *CDR1*, *MDR1*, and *ERG11* genes than the 14 control isolates (*P* values of 0.0003, 0.0073, and 0.0002, respectively). In addition, 12 FLS isolates also had 13.6-, 15.4-, and 11.0-fold higher expression levels of *CDR1*, *MDR1*, and *ERG11* genes than control isolates (*P* values of 0.0001, 0.0011, and 0.0193, respectively). There were no differences in the mean expression levels of *CDR1* or *MDR1* between FNS and FLS isolates, but the mean *ERG11* expression levels were significantly (3.4-fold) higher in FNS isolates ($P = 0.004$). Moreover, *ERG11* overexpression (>20-fold compared with controls) was more frequent in FNS than FLS isolates (FNS versus FLS, 8/9 versus 1/12; $P < 0.0001$).

According to Spearman's correlation analysis, there was no significant correlation between *CDR1* and *MDR1* expression when all 35 isolates were assessed ($r^2 = 0.0159$, $P = 0.4698$); however, there was a significant correlation between *CDR1* and *MDR1* expression when 12 FLS isolates were assessed ($r^2 = 0.4871$, $P = 0.0116$), while the expression of *CDR1* was negatively correlated with that of *MDR1* when 9 FNS isolates were assessed ($r^2 = 0.5878$, $P = 0.0159$) (Fig. 2). Also, there was no significant correlation between *ERG11* and *CDR1* or *MDR1* for all FNS and FLS isolates.

Amino acid substitutions in Erg11p and Upc2p. The *ERG11* sequence analysis revealed that only 4 (two each of FNS and FLS) of 35 isolates of *C. tropicalis* exhibited amino acid substitutions in Erg11p. Homozygous L333I (isolate 5) and G464S (isolate 7) substitutions were present in two FNS isolates, while heterozygous G464S (isolate 12) and both K344N and V362M (isolate 20) substitutions were detected in two FLS isolates.

By *UPC2* gene sequencing, one to four amino acid substitutions were found in 28 isolates, comprising 8 FNS, 11 FLS, and 9 control isolates. Notably, a BSI isolate (isolate 2), which showed 23.1-fold overexpression of *ERG11* and a fluconazole MIC of 16 μg/ml, exhibited the following heterozygous amino acid substitutions: deletion of four amino acids, AQSP, at residues 301 to 304 (Δ301-304) and insertion of two proline residues at amino acid position 320 (Q320PPQ), in addition to A297S and T393I. Also, seven distinct amino acid substitutions in *UPC2* (S187L, A251T, A263T, Q289L, L343F, G392E, and S702L) were found in 27 iso-
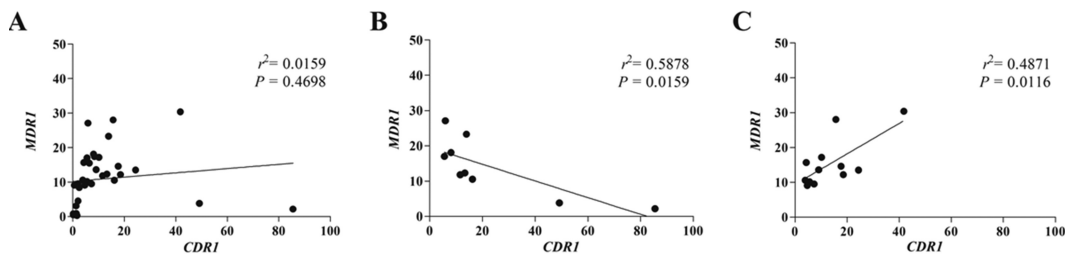

FIG 2 Correlation between *CDR1* and *MDR1* expression in clinical isolates of *C. tropicalis*. (A) No significant correlation between *CDR1* and *MDR1* expression was observed when all 35 isolates were assessed. However, the expression level of *CDR1* showed a negative correlation with that of *MDR1* when 9 fluconazole-nonsusceptible isolates were assessed ($r^2 = 0.5878$, $P = 0.0159$) (B) and a positive correlation with that of *MDR1* when 12 fluconazole-less-susceptible isolates were assessed ($r^2 = 0.4871$, $P = 0.0116$) (C).
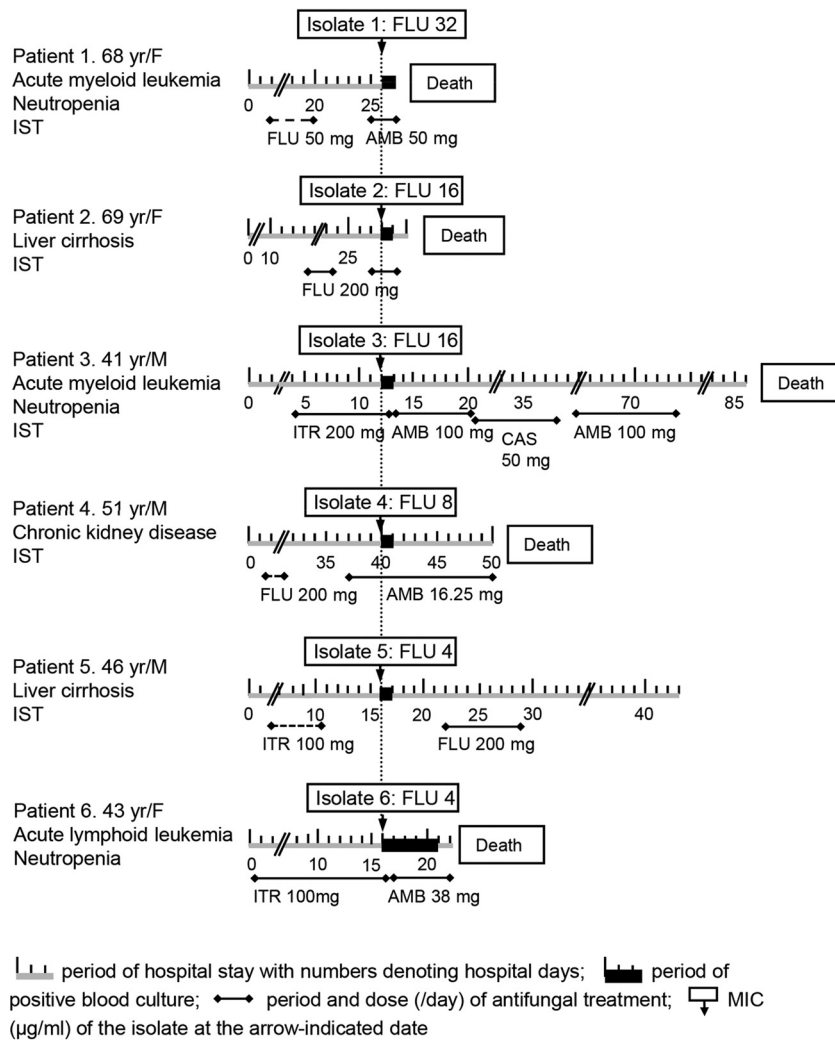
FIG 3 Treatment regimens, fluconazole MICs of *C. tropicalis* isolates, and outcomes of six fungemia patients (patients 1 to 6) with fluconazole-nonsusceptible (fluconazole MIC, ≥4 µg/ml) isolates of *C. tropicalis* (isolates 1 to 6). Table 1 provides detailed information on each isolate. Previous antifungal exposure within 3 months was identified in all six patients with FNS isolates. Three patients (patients 2, 3, and 6) developed breakthrough fungemia during azole therapy. AMB, amphotericin B; FLU, fluconazole; IST, immunosuppressive therapy at the time of onset of fungemia; ITR, itraconazole; CAS, caspofungin; F, female; M, male.

lates. Of the seven amino acid substitutions in Upc2p, three (A251T, Q289L, and G392E) were present irrespective of MIC distribution, and the homozygous variant of Q289L was found in five (one FNS, one FLS, and three control) isolates. While two amino acid substitutions (A263T and S702L) were detected only in control strains, two amino acid substitutions, L343F and S187L, were found in one FNS (isolate 8) isolate and one FLS (isolate 10) isolate, respectively.

MLST analysis. By MLST, the nine FNS isolates were found to have nine distinct DST types. Of the 35 total isolates, 24 isolates yielded new (unpublished) allele numbers, and the following 18 new DSTs were identified: 359, 392-396, 398-405, 417, 426, 431, and 448. Overall, all 35 isolates yielded 25 distinct DSTs, while 4 DSTs were shared by 14 isolates (40%): DST 232, DST 395, DST 400, and DST 401 were shared by five (one FNS, three FLS, and one control) isolates, four (one FNS, one FLS, and two control) isolates, two (one FLS and one control) isolates, and three (one FLS and two control) isolates, respectively.

Clinical characteristics of fungemia. The clinical course and

antifungal therapy of the 16 patients with FNS (patients 1 to 6) and FLS (patients 10 to 19) BSI isolates are summarized in Fig. 3 and 4, respectively. All 16 fungemic patients (15 adult and 1 pediatric patient) had severe underlying diseases. Three (50%) FNS and five (50%) FLS BSI isolates were recovered from patients with malignancy. Twelve patients were using central venous catheters at the time of onset of fungemia, but the results of the catheter tip culture were negative for *C. tropicalis* in all patients, with the exception of one (patient 18). Of four patients with FNS isolates (patients 2, 3, 5, and 6) who received >3 days of azole therapy, three (patients 2, 3, and 6) developed breakthrough fungemia during azole therapy. Patient 2 developed breakthrough fungemia during intravenous fluconazole therapy (200 mg/day) for 2 days. He had received intravenous fluconazole therapy (200 mg/day) for 10 days, which stopped 10 days before fungemia onset. Patients 3 and 6 developed breakthrough fungemia while receiving oral itraconazole therapy for 10 days (200 mg/day) and 16 days (100 mg/day), respectively. All patients with FNS isolates had a fatal outcome except for one, patient 5, whose isolate showed a fluconazole MIC of 4 µg/ml and

Patient 10. 65 yr/F
Hemoperitoneum
Isolate 10: FLU 2
0 15 20 30 35 60 65
FLU 400 mg

Patient 11. 72 yr/M
Gastric cancer
Isolate 11: FLU 2
0 30 35 40 45 50 55 70 100
FLU 200 mg 400 mg

Patient 12. 72 yr/M
Aplastic anemia
Isolate 12: FLU 2
Death
0 50 55
FLU 400 mg

Patient 13. 83 yr/F
Cerebral infarction
Isolate 13: FLU 2
0 5 10
FLU 400 mg 100 mg

Patient 14. 76 yr/F
Guilan-Barre synd.
Isolate 14: FLU 1
Death
0 10 15
FLU 400 mg

Patient 15. 70 yr/M
Lung cancer
Isolate 15: FLU 1
0 10 15 20 25 30
FLU 400 mg

Patient 16. 64 yr/M
Enterocutaneous fistula
Isolate 16: FLU 1
0 45 50 55 60 80
FLU 400 mg

Patient 17. 48 yr/M
Hepatoma
IST
Isolate 17: FLU 1
Death
0 10 15 20 25 30
FLU 100 mg

Patient 18. 45 yr/F
Acute promyelocytic
leukemia
Neutropenia
IST
Isolate 18: FLU 1
Death
0 15 20
AMB 47 mg

Patient 19. 9 yr/M
Osteomalacia
Neutropenia
IST
Isolate 19: FLU 1
0 20 25 30 55 190
ITR 30 mg AMB 13 mg

period of hospital stay with numbers denoting hospital days; period of
positive blood culture; period and dose (/day) of antifungal treatment; MIC
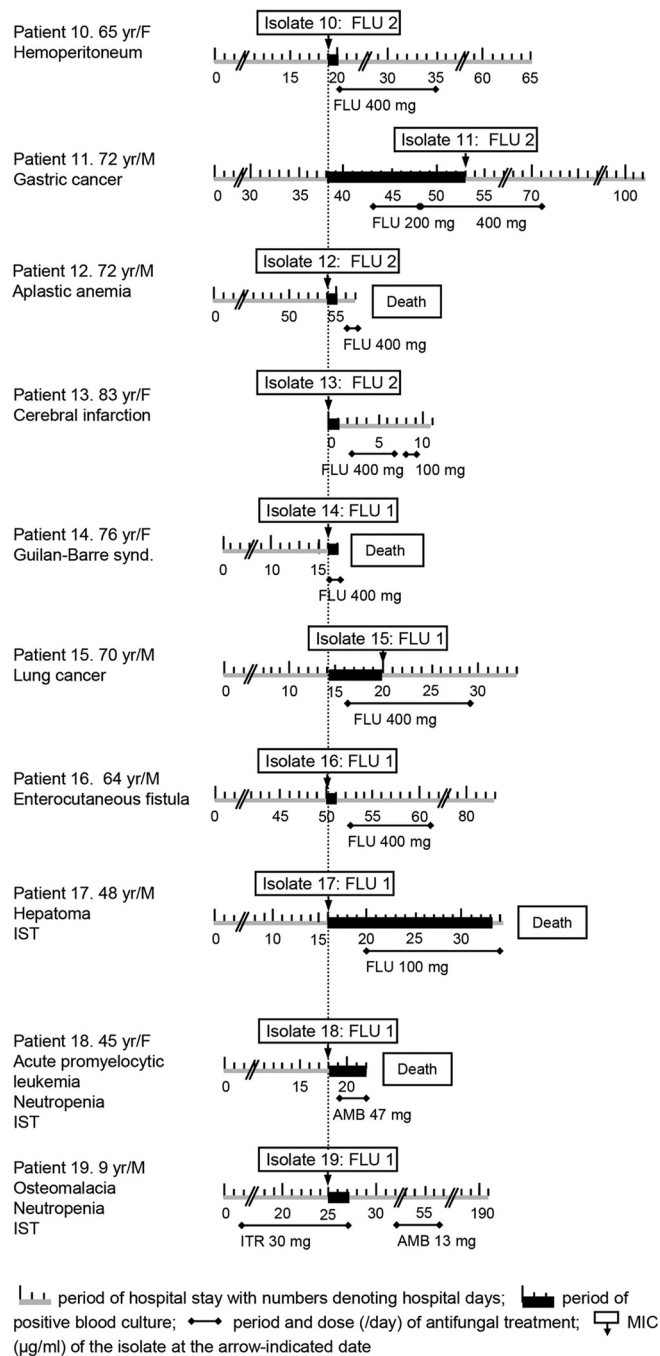(μg/ml) of the isolate at the arrow-indicated date

FIG 4 Treatment regimens, fluconazole MICs of *C. tropicalis* isolates, and
outcomes of 10 fungemia patients (patients 10 to 19) with fluconazole-less-
susceptible (fluconazole MIC, 1 to 2 μg/ml) isolates of *C. tropicalis* (isolates 10
to 19). Table 1 provides detailed information on each isolate. Of seven patients
(patients 10, 11, 13, 15, 16, 17, and 19) who received azole therapy, one (patient
19) developed breakthrough fungemia and three (patients 11, 15, and 17)
showed persistent fungemia despite 3 days of azole therapy. AMB, amphoter-
icin B; FLU, fluconazole; IST, immunosuppressive therapy at the time of onset
of fungemia; ITR, itraconazole; F, female; M, male; synd., syndrome.

negative after intravenous fluconazole therapy in three patients (400 mg/day for 16 days in patient 10, 400 mg/day for 6 days and then changed to 100 mg for 2 days in patient 13, and 400 mg/day for 9 days in patient 16). However, 4 (57%) showed azole thera-peutic failure: one patient (patient 19) developed breakthrough fungemia during oral itraconazole therapy (30 mg/day, 19 days), and the remaining three patients (patients 11, 15, and 17) showed persistent candidemia despite 3 days of azole therapy. Patients 11 and 15 received 32 days (200 to 400 mg/day) and 13 days (400 mg/day) of intravenous fluconazole therapy, respectively, and both patients completely recovered, but patient 17 died after 15 days of intravenous fluconazole therapy (100 mg/day).

Overall, the rate of azole therapeutic failure was similar in patients with FNS and FLS isolates (FNS versus FLS, 3/4 [75%] versus 4/7 [57%], respectively). Regarding other clinical characteristics of *C. tropicalis* fungemia, FNS BSI isolates were more frequently iso-lated from patients with immunosuppression (presence of neu-tropenia or use of immunosuppressive therapy) than FLS BSI iso-lates (6/6 and 3/10, respectively; $P = 0.011$). In addition, FNS isolates were more frequently associated with previous use of azole agents than FLS isolates (6/6 or 100% versus 3/10 or 30%; $P = 0.011$). FNS isolates from fungemic patients were more frequently associated with a fatal outcome (FNS versus FLS, 4/6 or 67% ver-sus 4/10 or 40%, respectively) and uncleared fungemia (4/6 or 67% versus 4/10 or 40%, respectively) than FLS isolates, although the difference was not significant.

DISCUSSION

The upregulation of *CDR1* and *MDR1*, which encode efflux pumps, has been reported to contribute to the azole resistance of clinical isolates of *C. albicans* or *C. glabrata* (6, 22), and Barchiesi et al. revealed the upregulation of *CDR1* and *MDR1* in an experi-mentally induced fluconazole-resistant *C. tropicalis* strain (11). However, Jiang et al. reported that *ERG11* expression levels of 31 azole-resistant isolates were higher than those of 21 azole-suscep-tible isolates, but the *CDR1* and *MDR1* expression levels were not significantly different (8). In contrast to the four studies of azole resistance mechanisms involving only one or two isolates of *C. tropicalis* (7, 9–11), Jiang et al. investigated 52 clinical isolates of *C. tropicalis* from sputum, urine, or stool cultures (8). However, we found that almost all (19/21) azole-susceptible isolates tested in that study had fluconazole MICs of ≥1 μg/ml; therefore, these would have been classified as FLS in the present study. In the present study, there were no differences in the mean expression levels of *CDR1* or *MDR1* between FNS and FLS isolates, but the mean *ERG11* expression levels were significantly (3.4-fold) higher in FNS isolates, which is in agreement with Jiang et al. However, compared with those of the 14 control strains (fluconazole MIC, 0.125 to 0.5 μg/ml), both FNS and FLS isolates showed signifi-cantly higher mean expression levels (>10-fold) of *CDR1* and *MDR*, as well as *ERG11*.

Based on the unresolved problems related to the reproducibil-ity of caspofungin MICs observed in multicenter evaluations, test-ing caspofungin for *in vitro* susceptibility currently is not recom-mended (23). Instead, testing anidulafungin or micafungin is recommended. All 35 isolates in this study were susceptible to both anidulafungin and micafungin by the CLSI method. All FNS isolates were resistant or SDD to voriconazole, while almost all FLS and all 14 control isolates were susceptible to voriconazole. In addition, FNS isolates showed higher MICs for both itraconazole
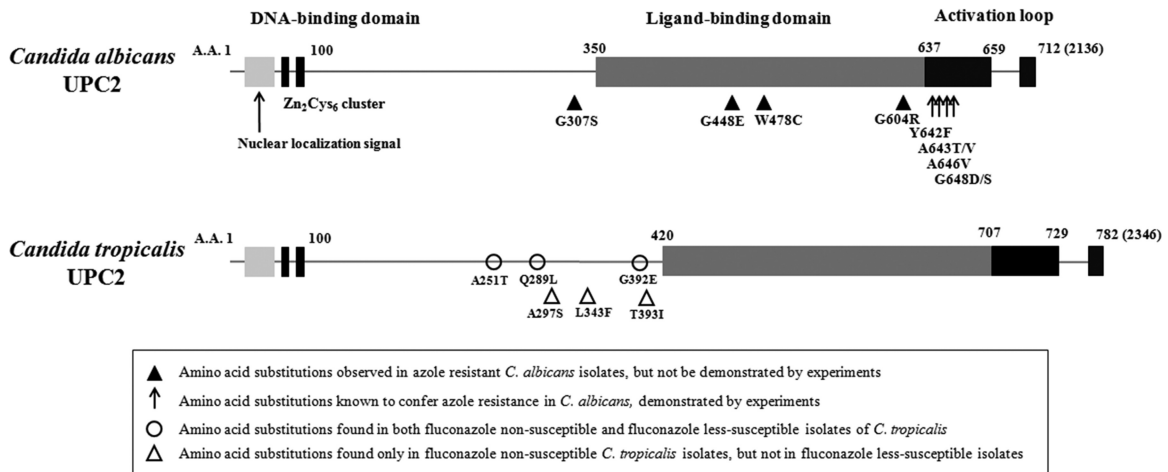
who improved after 8 days of intravenous fluconazole (200 mg/day).

Of the 10 patients with FLS isolates, 7 (patients 10, 11, 13, 15, 16, 17, and 19) received azole therapy. Blood cultures became

FIG 5 Amino acid substitutions in Upc2p known to confer azole resistance in *C. albicans* compared to those in *C. tropicalis* in this study. While many gain-of-function mutations (e.g., Y642F, A643T, A643V, A646V, G648D, or G648S) in azole-resistant clinical isolates of *C. albicans* are located in the C-terminal activation loop of Upc2p, six amino acid substitutions in Upc2p in fluconazole-nonsusceptible isolates of *C. tropicalis* in the present study are located upstream of the C-terminal ligand-binding domain of Upc2p.

and posaconazole than FLS or control isolates, and of the nine FNS isolates, eight and nine were categorized as non-wild-type isolates (i.e., exceeding the ECVs) for itraconazole and posaconazole, respectively.

In the present study, convergent overexpression of *CDR1* and *MDR1* was observed among the FLS isolates as well as in two control strains (isolates 22 and 34), but it was not observed among the FNS isolates. These findings suggest that *CDR1* and *MDR1* expression patterns differ between the FNS and FLS or control isolates and that the *CDR1* or *MDR1* genes are differentially expressed in FNS isolates. Notably, most FNS (89%) isolates showed >20-fold *ERG11* overexpression compared with the controls, which was significantly higher than that of the FLS isolates. The study by Jiang et al. also indicated that *C. tropicalis* isolates resistant to all three azole antifungals (fluconazole, itraconazole, and voriconazole) had a higher level of *ERG11* expression than did isolates resistant to fluconazole or itraconazole only (8). These results prompted us to speculate that induction of the efflux pumps encoded by the *MDR* or *CDR* genes is the initial step in increasing fluconazole MICs to 1 to 2 μg/ml (to FLS isolates) in *C. tropicalis*, and that overexpression of *ERG11* further contributes to the development of fluconazole and voriconazole resistance in FNS isolates.

The missense mutation (A393T) of *ERG11*, which might lead to decreased binding affinity for azoles, was first documented in an azole-resistant clinical isolate of *C. tropicalis* (7). Forastiero et al. reported the G464S substitution in a fluconazole-resistant *C. tropicalis* isolate (10), and we found the G464S substitution in two isolates with fluconazole MICs of 64 and 2 μg/ml, although whether this substitution was responsible for the azole resistance is unclear. Of the four *ERG11* missense mutations (L333I, G464S, K344N, and V362M) of *C. tropicalis* isolates detected in this study, only one change, G464S, was found to be located in three previously defined hot spot regions of *C. albicans*, corresponding to amino acids 105 to 165, 266 to 287, and 405 to 488 (24). In the study by Jiang et al., *ERG11* missense mutations were found in 8 FNS (fluconazole MIC, 4 to 64 μg/ml) and 4 FLS (fluconazole MIC, 2 μg/ml) isolates of *C. tropicalis*, but all 12 isolates showed

the same *ERG11* missense mutation (Y132F and S145F) (8). On the other hand, we did not find the Y132F and S145F substitutions in our isolates. Because increased resistance of *C. tropicalis* to fluconazole and outbreaks of infection caused by fluconazole-resistant *C. tropicalis* have been recognized in some hospitals (5, 25, 26), *C. tropicalis* isolates with the *ERG11* mutation should be genotyped to investigate the transmission of particular clones. Although overexpression of *ERG11* associated with missense mutations has been described as the mechanism of azole resistance in clinical isolates of *C. tropicalis* (7, 8), in the present study, only two FNS isolates harbored *ERG11* mutations, suggesting that gene overexpression, rather than *ERG11* mutation, is the main mechanism of azole resistance in *C. tropicalis* isolates from Korean hospitals. However, the contribution of *ERG11* mutations to the development of fluconazole resistance remains a possibility, as no changes in *ERG11* were found in the 14 control isolates.

Gain-of-function mutations in *UPC2* can lead to *ERG11* overexpression and contribute to fluconazole resistance in clinical *C. albicans* isolates (12–14). This is, to our knowledge, the first description of *UPC2* variations in fluconazole-resistant *C. tropicalis* isolates. Of the nine FNS isolates, seven (isolates 1 and 4 to 9) isolates exhibited one or more of the three amino acid substitutions (A251T, Q289L, and G392E); however, all three were also found in control isolates. Two fluconazole-resistant isolates (isolates 2 and 8) harbored mutations, including A297S, T393I, Δ301-304, Q320PPQ, or L343F, which were not found in FLS or control isolates, but whether these substitutions result in azole resistance is unclear. Upc2 is involved in sensing the ergosterol level in the cell through its C-terminal ligand-binding domain, which contains a glycine-rich activation loop at the extreme C terminus (27). Many gain-of function mutations in the C-terminal activation loop of Upc2p (e.g., Y642F, A643T, A643V, A646V, G648D, or G648S) have been reported to contribute to the fluconazole resistance of clinical *C. albicans* (12–14). However, six amino acid substitutions in Upc2p (A251T, Q289L, A297S, L343F, G392E, and T393I) in FNS isolates in the present study are located upstream of the C-terminal ligand-binding domain of Upc2p isolates (Fig. 5). Thus, these results suggest that mutations upstream

of the C-terminal ligand-binding domain of Upc2p in *C. tropicalis* do not individually lead to decreased susceptibility to azole drugs, although further genome-wide transcriptional analyses are needed.

Previous azole exposure is an important risk factor for fungemia caused by fluconazole-resistant *Candida* isolates (28). However, the azole resistance of some clinical isolates of *C. tropicalis* may not be associated with previous exposure to azole agents (3). In the present study, previous fluconazole exposure was identified in all six fungemic patients with FNS isolates. These findings, together with the fact that all FNS isolates exhibited unique DST types, suggest that *C. tropicalis* FNS isolates in Korea have arisen sporadically due to selective pressure imposed by prior azole therapy.

C. tropicalis fungemia caused by FNS and FLS strains is uncommon in Korea (16). Among 565 *C. tropicalis* BSI isolates from Korean surveillance cultures between 2003 and 2013, only 6 (1.1%) displayed fluconazole MIC values of ≥ 4 μg/ml, and 10 (1.8%) displayed fluconazole MIC values of 1 to 2 μg/ml by the 24-h CLSI method. This is, to our knowledge, the first report on the molecular and clinical features of *C. tropicalis* FNS BSI isolates in comparison with those of FLS BSI isolates. Compared with FLS isolates, FNS BSI isolates were significantly more frequently associated with previous azole exposure or immunosuppression. However, there were no significant differences in the rates of azole therapeutic failure (3/4 versus 4/7) or uncleared fungemia (4/6 versus 4/10). Alternatively, although only 30% (3/10) of FLS BSI isolates were associated with previous azole exposure, two FLS isolates were obtained from two patients receiving azole therapy for *C. tropicalis* fungemia. This suggests that FLS isolates of *C. tropicalis* can be generated during treatment of *C. tropicalis* fungemia. In addition, of seven fungemic patients with FLS isolates who received >3 days of azole therapy, four (57%) showed therapeutic failure, suggesting that greater clinical attention should be paid to *C. tropicalis* isolates with reduced susceptibility to fluconazole (i.e., MIC of 1 to 2 μg/ml).

In summary, by comparing FLS and control isolates, we clarified the molecular and clinical features of FNS *C. tropicalis* clinical isolates recovered from Korean hospitals. First, a few FNS and FLS isolates showed amino acid substitutions in Erg11p, but most exhibited overexpression of *CDR1*, *MDR1*, and *ERG11* compared to control isolates, and FNS isolates showed greater *ERG11* overexpression than FLS or control isolates. This suggests that overexpression of all three genes contributes to the development of fluconazole and voriconazole resistance in FNS isolates from Korean hospitals. Second, amino acid substitutions in Upc2p were frequently found among FNS, FLS, and even control isolates, but none were likely to be located in the C-terminal activation loop of Upc2p, suggesting that they do not contribute to the constitutive activation of Upc2p in these *C. tropicalis* isolates. Third, FNS BSI isolates were found more frequently in patients with immunosuppression or those with previous azole exposure than FLS isolates, but some FLS BSI isolates can be obtained during therapy for fungemia and are associated with azole therapeutic failure. Therefore, further investigation is needed to determine the mechanisms causing *ERG11* expression and the clinical and molecular consequences of the stepwise changes that result in the transformation of FLS to FNS isolates of *C. tropicalis*.

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