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Molecular and Clinical Features of Fluconazole Non-susceptible *Candida albicans* Bloodstream Isolates Recovered in Korean Multicenter Surveillance Studies

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Acquired fluconazole resistance (FR) in bloodstream infection (BSI) isolates of *Candida albicans* is rare. We investigated the FR mechanisms and clinical features of 14 fluconazole non-susceptible (FNS; FR and fluconazole-susceptible dose-dependent) BSI isolates of *C. albicans* recovered from Korean multicenter surveillance studies during 2006–2021. Mutations causing amino acid substitutions (AASs) in the drug-target gene *ERG11* and the FR-associated transcription factor genes *TAC1, MRR1*, and *UPC2* of the 14 FNS isolates were compared with those of 12 fluconazole-susceptible isolates. Of the 14 FNS isolates, eight and seven had Erg11p (K143R, F145L, or G464S) and Tac1p (T225A, R673L, A736T, or A736V) AASs, respectively, which were previously described in FR isolates. Novel Erg11p, Tac1p, and Mrr1p AASs were observed in two, four, and one FNS isolates, respectively. Combined Erg11p and Tac1p AASs were observed in seven FNS isolates. None of the FR-associated Upc2p AASs were detected. Of the 14 patients, only one had previous azole exposure, and the 30-day mortality rate was 57.1% (8/14). Our data show that Erg11p and Tac1p AASs are likely to contribute to FR in *C. albicans* BSI isolates in Korea and that most FNS *C. albicans* BSIs develop without azole exposure.

Key Words: Amino acid substitution, Azoles, *Candida albicans*, *ERG11*, Fluconazole, *MRR1*, Mutation, Sepsis, *TAC1, UPC2*

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Candida albicans, a predominant human fungal pathogen, causes both mucosal and bloodstream infections (BSIs), and fluconazole is one of the most widely prescribed antifungal agents used to treat these infections [1, 2]. Acquired fluconazole resistance (FR) in *C. albicans* has been reported at its highest frequency in HIV-infected patients with oropharyngeal candidiasis as well as in patients with recurrent vaginal candidiasis [2, 3]. The long-term use of fluconazole for prophylaxis or treatment of mucosal *C. albicans* infections can lead to selective pressure, resulting in the emergence of acquired FR in *C. albicans* [2, 3]. The main mechanisms responsible for acquired FR in *C. albicans* from mucosal infections include mutations or overexpression of *ERG11*, which encodes an enzyme targeting the drug (lanosterol 14α-demethylase), and the overexpression of genes encoding efflux pumps (*CDR1, CDR2*, and *MDR1*) [2, 4, 5]. The overexpression of FR-associated genes occurs mainly due to gain-of-function (GoF) mutations in the transcription factor-encoding genes *TAC1* (involved in *CDR1* and *CDR2* regulation), *MRR1* (involved in *MDR1* regulation), and *UPC2* (involved in *ERG11* regulation) [2, 5-7].

In contrast to mucosal isolates with FR rates of 12%–22%, the rates of FR among BSI isolates of *C. albicans* are low (0.06%–2.3%); this could be partly because of the relatively short-term use of antifungal agents for the treatment of candidemia [2, 8-10]. Among 2,712 *C. albicans* BSI isolates obtained from Korean multicenter surveillance studies during 2006–2021, only 14 (0.5%) were determined to be fluconazole non-susceptible (FNS; minimum inhibitory concentration [MIC] ≥4 μg/mL). To date, only few studies have characterized the molecular and clinical features of BSI isolates of *C. albicans* with acquired FR [8]. Therefore, we investigated the gene mutations causing amino acid substitutions (AASs) in *ERG11, TAC1, MRR1*, and *UPC2*; their genotypic relationships; and the clinical features of FNS BSI isolates of *C. albicans* that were submitted to Chonnam National University Hospital from Korean multicenter surveillance studies over 15 years (2006–2021).

We assessed 26 BSI isolates of *C. albicans*: 11 FR (MIC ≥8 μg/mL), three fluconazole-susceptible dose-dependent (F-SDD; MIC, 4 μg/mL), and 12 control fluconazole-susceptible (FS; MIC, 0.25–0.5 μg/mL) isolates. All isolates were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker Biotyper library v. 4.0; Bruker Daltonics GmbH, Bremen, Germany) or by sequencing the D1/D2 domains of the 26S rRNA gene [11]. *In vitro* antifungal susceptibility testing was performed with the Sensititre Yeast One system (Thermo Fisher Scientific Inc., Cleveland, OH, USA). Sequence analyses of *ERG11, TAC1, MRR1*, and *UPC2* were conducted as described previously [6, 12, 13]. All isolates were genotyped using multi-locus sequence typing (MLST); each strain was assigned a diploid sequence type (DST) reflecting the combination of the genotypes of seven genes in the MLST database (https://pubmlst.org/organisms/candida-albicans), and a dendrogram was constructed [14]. Clinical information for all 14 patients with FNS *C. albicans* BSI isolates was collected retrospectively [15]. This study was approved by the Institutional Review Board of Chonnam National University Hospital, Gwangju, Korea (approval No. CNUH-2014-290) that also waived the requirement for informed consent.

Table 1 shows the results of antifungal susceptibility testing and *ERG11, TAC1, MRR1*, and *UPC2* sequencing of all 14 FNS (11 FR [R1–R11] and 3 F-SDD [D1–D3]) and 12 FS (S1–S12) *C. albicans* BSI isolates. No isolate was found to be resistant to amphotericin B or the three echinocandins. The sequencing results of all 14 FNS *C. albicans* isolates were compared with those of 12 FS control isolates and previously reported data [2, 6, 7, 16-20]; five Erg11p, seven Tac1p, and one Mrr1p AASs were found in only the FNS isolates. Of the five Erg11p AASs that were found in only the FNS isolates, two (R264T and D428N) were potentially novel; three AAS (K143R, F145L, and G464S) that were found in eight FNS isolates were confirmed to cause FR through *in vitro* experiments [16]. Of the seven AASs in Tac1p that were not present in the FS isolates, three (T225A, A736T, and A736V) AASs that were found in six FNS isolates were previously described as GoF mutations [2]; another (R673L) AAS found in one FNS isolate was previously described in azole-resistant isolates [2, 18-20]. The remaining three (Y269H, L744I, and N972K) Tac1p AASs from four FNS isolates were potentially novel, although N972K occurred at a position already described in FR isolates [19]. Only one substitution in Mrr1p (N33S), which was found in one FNS isolate, was not described previously [2, 7]. Although it is unclear whether the newly identified AASs in this study result in FR, the Erg11p, Tac1p, Mrr1p, and Upc2p AASs that were either novel or described in azole-resistant isolates were found in 8 (57.1%), 11 (78.6%), 1 (7.1%), and 0 (0%) isolates, respectively. A previous study characterized azole resistance mechanisms in five invasive FNS *C. albicans* isolates that had been collected in 29 countries in 2014 and 2015. The authors found that *MDR1* overexpression (three isolates) was more common than *CDR2* expression (one isolate) or *ERG11* mutation (one isolate) [10]. In contrast, our results suggested that *ERG11* mutations and *CDR* overexpression are likely the dominant mechanisms of FR in *C. albicans* BSI isolates from Korean hospitals.

Of the 14 FNS isolates, 12 exhibited weak FR (MICs: 4–16

Table 1. Comparison of azole antifungal susceptibility testing and sequencing of azole-resistant related genes between fluconazole non-susceptible and fluconazole susceptible bloodstream isolates of *Candida albicans*

Isolate No.	MIC (μg/mL)*	Erg11p AAS found in†		Tac1p AAS found in†		Mrr1p AAS found in†		Upc2p AAS found in†	
	FLC/VOR/ITRA/POSA	FNS isolates only‡	Both FNS and FS isolates	FNS isolates only‡	Both FNS and FS isolates‖	FNS isolates only	Both FNS and FS isolates‖	FNS isolates only	Both FNS and FS isolates
R1	>256/>8/>16/>8	None	D116E, K128T	L744I§	N772K, S935L	None	L171P, L248V, V341E	None	None
R2	>256/>8/>16/>8	**K143R**	D116E, K128T	None	I558V, N772K	None	-	None	I142S
R3	16/0.12/0.25/0.25	**K143R**	D116E, K128T	**A736T**	N772K, S935L	None	L171P, L248V, V341E	None	None
R4	16/0.12/0.25/0.25	**K143R**	D116E, K128T	**A736T**	N772K, S935L	None	L171P, L248V, V341E	None	None
R5	16/0.12/0.25/0.25	**K143R**	D116E, K128T	**A736T**	N772K, S935L	None	N159H, I160T, A162P, L171P, L248V, V341E	None	None
R6	16/0.5/0.5/0.5	R264T§, **G464S**	D116E, K128T	N972K§	N772K, S935L	None	V27I¶, L171P, L248V, V341E	None	None
R7	8/0.12/0.25/0.25	None	D116E, E266D, V488I	**R673L**	I895T, N896S	None	L171P, V341E	None	None
R8	8/0.12/0.06/0.03	None	D116E, E266D, V488I	None	I895T, N896S	None	L171P, V341E	None	None
R9	8/0.12/0.25/0.25	**F145L**	K342R¶	Y269H§	N896S	None	L171P, V341E	None	I142S
R10	8/0.12/0.25/0.25	None	D116E, K128T	**A736V**	N772K, S935L	None	N159H, I160T, A162P, L171P, L248V, V341E	None	None
R11	8/0.03/0.12/0.06	**K143R**	D116E, K128T	**A736T**	N772K, S935L	None	N159H, I160T, A162P, L171P, L248V, V341E	None	None
D1	4/0.12/0.25/0.25	**F145L**, D428N§	K342R¶	Y269H§	N896S	N33S§	L171P, V341E	None	I142S, P299L
D2	4/0.015/0.06/0.03	None	E266D, V437I	None	K87N, M170I, N174D, F189S	None	L171P, L248K, V341E	None	I142S
D3	4/0.06/0.25/0.25	None	E266D, V488I	**T225A**	K87N, M170I, N174D, F189S, N772K, N896S	None	-	None	I142S, S190N, S228N
S1	0.5/0.03/0.03/0.015	None	D153E	None	I895T, N896S	None	L171P	None	R68K, I142S, S228N
S2	0.5/0.03/0.06/0.03	None	E266D, V488I	None	N896S	None	S16I, T73K, L171P	None	None
S3	0.25/0.008/0.015/0.015	None	D116E, D153E	None	I895T, N896S	None	L171P	None	R68K, I142S, S228N
S4	0.25/0.015/0.03/0.015	None	D116E, D153E	None	I895T, N896S	None	L171P	None	I142S, S228N
S5	0.25/0.008/0.03/0.03	None	E266D, V437I	None	K87N, A90T, M170I, N174D, F189S, F222L	None	P19L, G75R, N937K, F1032L	None	R68K, I142S, S190N, S228N
S6	0.25/0.008/0.03/0.015	None	E266D, V437I	None	M170I, N174D, F189S	None	N937K, F1032L	None	R68K, I142S, S190N, S228N
S7	0.25/0.008/0.03/0.015	None	E266D, V437I	None	A90T	None	N937K, F1032L	None	R68K, I142S, S190N, S228N
S8	0.25/0.015/0.03/0.03	None	D116E, D153E	None	I895T, N896S	None	-	None	R68K, I142S, S288N, P299L, A300P
S9	0.25/0.015/0.03/0.03	None	D116E, K128T	None	N772K	None	L171P, L248V, V341E	None	None
S10	0.25/0.008/0.015/0.015	None	E266D, V488I	None	N772K, N896S	None	L171P	None	None
S11	0.25/0.008/0.015/0.03	None	E266D, V488I	None	N896S	None	L171P, S219F	None	None
S12	0.25/0.008/0.03/0.015	None	D116E	None	I895T, N896S	None	L171P	None	I142S, S228N

*Antifungal MICs were determined using the Sensititre Yeast One system (Thermo Fisher Scientific Inc., Cleveland, OH, USA); †The sequences of the isolates were compared and analyzed based on the reference sequences for *ERG11* (GenBank accession No. X13296), *TAC1* (GenBank accession No. DQ393587), *MRR1* (GenBank accession no. XM711520), and *UPC2* (GenBank accession No. EU583451) from *C. albicans* [6]; homozygous alleles are underlined; ‡AASs that were previously detected in fluconazole-resistant *C. albicans* isolates are shown in bold; §New AASs (Erg11p R264T and D428N AASs, Tac1p Y269H, N744I, and N972K, and Mrr1p N33S) that were found in the FNS isolates of *C. albicans* in this study have been deposited into GenBank with accession numbers OQ161592, OQ161593, OQ161595, OQ383350, OQ161594, and OQ161598, respectively; ‖Eight common Tac1p AASs (F104V, S199N, R206H, V207A, N396S, D776N, E829Q, and L941P) and one Mrr1p ASS (E1020Q) that were found in almost all (≥23) isolates were excluded; ¶Erg11p K342R AAS and Mrr1p V27I AAS were reported previously in FS isolates [7, 17].

Abbreviations: MIC, minimal inhibitory concentration; AAS, amino acid substitution; FLC, fluconazole; VOR, voriconazole; ITRA, itraconazole; POSA, posaconazole; FNS, fluconazole non-susceptible; FS, fluconazole-susceptible.

mg/L) without voriconazole resistance, whereas the remaining two isolates (R1 and R2) showed high MICs for fluconazole (>256 mg/L) and voriconazole (>8 mg/L). Isolate R1 harbored L744I in Tac1p (new AAS), which might be the major contributor to *CDR*-mediated azole resistance; isolate R2 harbored Erg11p K143R. Of the five Erg11p K143R isolates with variable MICs for fluconazole (8–>256 mg/L), isolate R2 showed the highest fluconazole MIC; however, it did not show FR-specific Tac1p, Mrr1p, or Upc2p AASs. Two FNS isolates (R8 and D2) did not show any of the FR-associated AASs that were evaluated in this study. *CDR1/CDR2* and *MDR1* overexpression can be explained by *TAC1* and *MRR1* GoF mutations, but *ERG11* overexpression is not always associated with *UPC2* GoF mutations, suggesting the existence of additional regulators [21]. Thus, these isolates might have other resistance mechanisms, such as the overexpression of *ERG11*, which could not be detected in this study.

The MLST results for the 14 FNS isolates showed that nine isolates had different DSTs, whereas three and two isolates belonged to the DSTs 1179 and 1539, respectively (Fig. 1). Three DST 1179 isolates (R3–R5) were isolated at three different hospitals but had the same AASs in Erg11p (K143R) and Tac1p (A736T). Two isolates of DST 1539 (R9 and D1) were also isolated at different hospitals, and they had similar Erg11p AASs (F145L and F145L+D428N) and the same Tac1p (Y269H) AAS. *TAC1* is located on the left arm of chromosome 5, where *ERG11* is also located, and a combination of *TAC1* and *ERG11* point mutations has been suggested to contribute to an increased MIC for fluconazole among azole-resistant isolates [17]. Overall, 50.0% (7/14) of the FNS isolates showed combined Erg11p and Tac1p AASs in this study.

The clinical features of all 14 patients are summarized in Table 2. All 14 patients were adults with various underlying diseases, but no patient was infected with HIV. Previous amphotericin B (two patients) or fluconazole (one patient) exposure was identified in only three patients, indicating that almost all (92.9%) FNS *C. albicans* BSI isolates were from patients not previously ex-

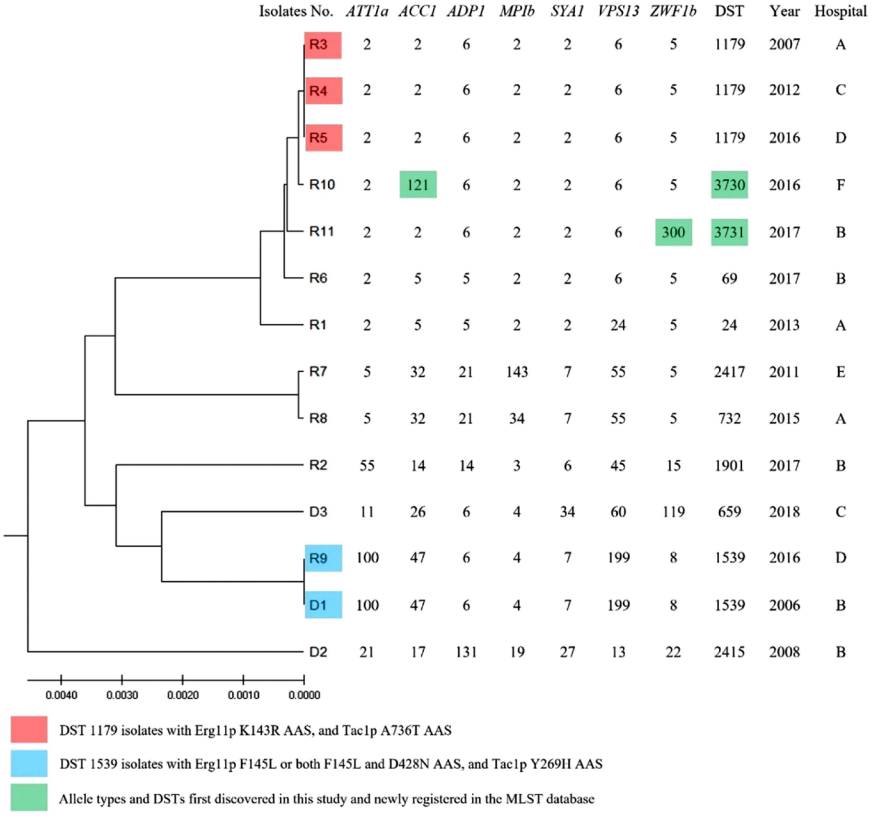

Fig. 1. Dendrogram based on a combination of seven housekeeping genes (*AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13,* and *ZWF1b*) of 14 fluconazole non-susceptible *Candida albicans* isolates, constructed based on the UPGMA using MEGA 11 software [14]. Three isolates of DST 1179 (R3–R5) are more closely related to four FNS isolates (R1, R6, R10, and R11), all of which share the same Erg11p (D116E and K128T), Tac1p (N772K and S935L), and Mrr1p (L171P, L248V, and V341E) AASs. See Table 1 for detailed information regarding each isolate.
Abbreviations: UPGMA, unweighted pair group method with arithmetic averages; DST, diploid sequence type; MLST, multi-locus sequence typing; AAS, amino acid substitution; FNS, fluconazole non-susceptible.

Table 2. Clinical features of 14 patients with fluconazole non-susceptible bloodstream isolates of *Candida albicans*

Isolate No.	Age (yr)/ sex	Diagnosis	Prior antifungal exposure	Immuno-suppression	CVC	Duration of fungemia (days)	Antifungal treatment	Patient outcome (days)*
R1	30/F	Acute myeloid leukemia	Yes (AMB)	Yes	Yes	5	AMB, ANI	Death (6)
R2	63/F	Pancreatic cancer	No	Yes	Yes	1	CAS	Death (52)
R3	52/M	Diabetes mellitus	No	No	No	10	AMB	Improved
R4	75/M	COPD	No	No	No	3	FLC	Death (19)
R5	49/F	Breast cancer	No	No	Yes	1	None	Death (2)
R6	52/M	T/NK-cell lymphoma	No	No	Yes	5	CAS	Death (7)
R7	74/M	Diabetes mellitus	No	No	Yes	1	None	Death (1)
R8	74/F	Chronic myeloid leukemia	Yes (FLC)	Yes	No	2	CAS	Death (4)
R9	62/M	Rheumatoid arthritis	No	No	Yes	4	MICA	Improved
R10	79/F	Traumatic subdural hemorrhage	No	No	Yes	1	FLC	Improved
R11	80/M	Spinal abscess	No	No	Yes	2	CAS	Improved
D1	79/M	Diabetes mellitus	Yes (AMB)	No	Yes	8	FLC	Death (15)
D2	43/M	Down syndrome	No	Yes	Yes	6	AMB	Improved
D3	67/F	Fulminant myocarditis, COPD	No	No	Yes	1	None	Death (3)

*Time to death after the first positive culture.
Abbreviations: F, female; M, male; COPD, chronic obstructive pulmonary disease; AMB, amphotericin B; FLC, fluconazole; CVC, central venous catheter; ANI, anidulafungin; CAS, caspofungin; MICA, micafungin.

posed to azole. Among the 14 patients with FNS BSIs, eight had a fatal outcome within 30 days, three (R5, R7, and D3) died without receiving antifungal therapy, two (R4 and D1) died despite fluconazole therapy, and three (R1, R6, and R8) with a hematological malignancy died despite >3 days of echinocandin or amphotericin B therapy. The overall 30-day mortality rate of the patients was 57.1% (8/14), which was higher than the mean 30-day mortality rate (36.4%, 123/338) of patients with *C. albicans* BSIs reported at 11 Korean hospitals from 2017 to 2018, although the difference was not significant [15].

Given the marked genetic diversity among Korean *C. albicans* BSI isolates in our previous MLST study [14], it is interesting that in the present study, five FNS isolates shared two DSTs (1179 and 1539). Additionally, the dendrogram obtained through MLST testing revealed that three isolates of DST 1179 (isolates R3–R5) were more closely related to four FNS isolates (R1, R6, R10, and R11) (Fig. 1). However, there was no time- or location-based clustering of these isolates, which excludes the potential of cross-transmission in the hospitals. Alternatively, some patients could accidentally acquire clonal FNS isolates of *C. albicans* already present in the environment of healthcare settings in Korea, leading to the development of healthcare-associated BSIs; further studies are needed to confirm this possibility.

In summary, our results showed that most FNS *C. albicans* BSI isolates from Korean hospitals harbor mutations in *ERG11* or *TAC1* and that fungemia can develop without azole exposure. This is the first study to describe both the molecular and clinical features of FNS BSI isolates of *C. albicans* obtained from candidemia surveillance studies.

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AUTHOR CONTRIBUTIONS

Shin JH designed the study; Choi MJ and Byun SA performed the laboratory measurements and molecular studies; Kim MN, Lee WG, Lee J, Yong D, Chang CL, Won EJ, and Kim SH collected the clinical isolates and data; Shin JH, Kwon YJ, and Lee SY wrote the preliminary manuscript; Shin JH, Kwon YJ, and Lee SY analyzed the data; Shin JH revised the manuscript; Kim MN, Lee WG, Lee J, Chang CL, Won EJ, and Kim SH provided valuable comments and recommendations. All authors revised and accepted the final version of the manuscript.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article are reported.

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