Mycobiology

Research Note

Azole Resistance Caused by Increased Drug Efflux in *Candida glabrata* Isolated from the Urinary Tract of a Dog with Diabetes Mellitus

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Abstract A yeast-like organism was isolated from a urine sample of a 6-year-old neutered male miniature poodle dog with urinary tract infection, diabetes ketoacidosis, and acute pancreatitis. We identified the yeast-like organism to be *Candida glabrata* and found that this fungus was highly resistant to azole antifungal drugs. To understand the mechanism of azole resistance in this isolate, the sequences and expression levels of the genes involved in drug resistance were analyzed. The results of our analysis showed that increased drug efflux, mediated by overexpression of ATP transporter genes *CDR1* and *PDH1*, is the main cause of azole resistance of the *C. glabrata* isolated here.

Keywords Azole resistance, *Candida glabrata*, Diabetes, Dog, Drug efflux, Urinary tract

A 6-year-old neutered male miniature poodle dog was presented to Haemaru Referral Animal Hospital with a history of polyuria, polydipsia, anorexia, vomiting, lethargy, and hematuria. A complete blood count, serum biochemistry, blood gas analysis, urinalysis, abdominal radiography, and abdominal ultrasonography were performed, and cystitis, diabetes ketoacidosis, and acute pancreatitis were diagnosed. In the cytologic examination of urine collected by bladder centesis, numerous yeast-like organisms were present extracellularly and within neutrophils, and this cystitis was suspected to be caused by a fungal infection. Alongside the standard therapy for diabetes ketoacidosis and acute pancreatitis, fluconazole (10 mg/kg orally every 12 hr) was administered for the treatment of cystitis. After 2 days of fluconazole therapy, hematuria and pyuria were persistently observed. Bilateral renal pelvis dilation and a unilateral renal parenchymal cyst filled with echogenic fluids were detected by abdominal ultrasonography. A significant number of the yeast-like organisms were observed in the cytologic examination of fluids from the renal pelvis and renal parenchymal cyst. A fungal culture of urine collected by bladder centesis was performed, and the yeast-like organism was isolated. Despite fluconazole administration for 7 days, the urine was positive for fungal culture, and pyelonephritis, perirenal inflammation, renal abscess, and cystitis had persisted severely, which raised the possibility that the yeast-like organism was azole resistant.

To determine the azole resistance of the yeast-like organism, we first performed molecular identification of the isolated cells, followed by an investigation of their antifungal sensitivity. Genomic DNA was extracted, and the D1/D2 region of the large subunit of 26S rRNA gene was amplified by PCR, using primers NL_1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL_4 (5′-GGTCCGTGTTTCAAGACGG-3′) [1]. The amplified PCR fragments were sequenced, and 593 nucleotides were determined. This sequence was subsequently used for database searching with the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) [2]. The results of the BLAST showed 99% identity to the type strain of *Candida glabrata* CBS 138, and as result, the fungal strain was identified as *C. glabrata*, and we named the strain HMR_001 (Supplementary Fig. 1). We next investigated the antifungal sensitivity of this strain to fluconazole and itraconazole,

Mycobiology 2017 December, **45**(4): 426-429
https://doi.org/10.5941/MYCO.2017.45.4.426
pISSN 1229-8093 • eISSN 2092-9323

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Received November 5, 2017
Revised November 23, 2017
Accepted November 23, 2017

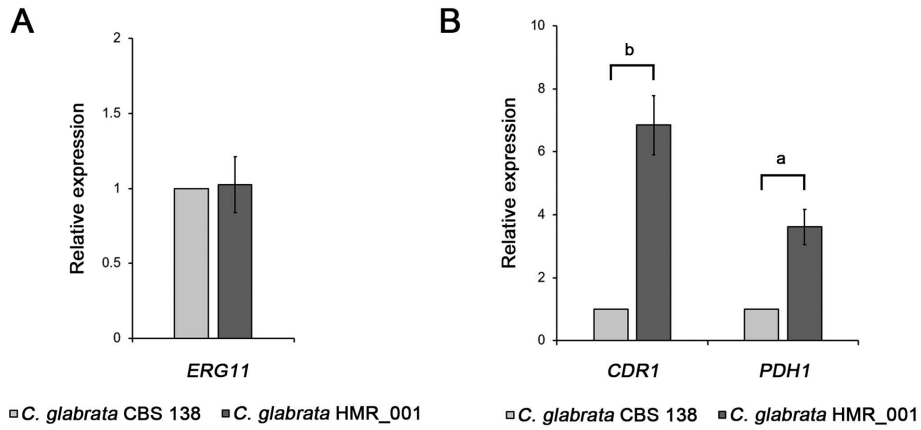

Fig. 1. Comparison of the relative expression of the genes related to azole resistance. Transcript levels of *ERG11* (A), and *CDR1* and *PDH1* (B) in *Candida glabrata* HMR_001 were evaluated and compared with those of the reference strain *C. glabrata* CBS 138, using quantitative real-time PCR. Values represent averages from 3 replicates and error bars indicate standard deviations ($^{a}p < 0.002$, $^{b}p < 0.0005$).

which are the most commonly used azole antifungal drugs to treat superficial and invasive mycoses; they inhibit fungal 14-a-sterol demethylase (Erg11) required for ergosterol biosynthesis [3]. Additionally, amphotericin B, caspofungin, and anidulafungin were included for the sensitivity assay. Amphotericin B is a polyene drug, which binds to ergosterol directly and induces the formation of channels in the fungal membrane, leading to the loss of intracellular ions, causing cell death [4]. The echinocandins, which include caspofungin, micafungin, and anidulafungin, are non-competitive inhibitors for β(1,3)-glucan synthase, which is required for glucan synthesis, an essential component of the fungal cell wall [5]. Sensitivities to the antifungal drugs were determined by Clinical and Laboratory Standards Institute (CLSI) broth microdilution method M27-A2 [6], and *C. glabrata* CBS 138 and *Candida albicans* SC5314 were included as references. The results of the antifungal sensitivity assay showed that the minimal inhibitory concentration (MIC) of *C. glabrata* HMR_001 of itraconazole and fluconazole were 0.5 and 16 mg/mL respectively, which are 8- and 32-folds higher than that for *C. glabrata* CBS 138, respectively (Table 1). In contrast, there was no difference in the MICs of amphotericin B, caspofungin acetate, and anidulafungin between *C. glabrata* CBS 138 and *C. glabrata* HMR_001, which suggested that the drug resistance of the isolated fungus was specific to azole antifungals and that amphotericin B, caspofungin or echinocandin could be an alternative to azole drugs.

In contrast to the azole resistance, results suggested that caspofungin was effective against *C. glabrata* HMR_001. We therefore replaced fluconazole with caspofungin (1 mg/kg diluted in 0.2 mg/mL intravenously for 1 hr every 24 hr) at day 7 of treatment. Clinical signs were improved, but the renal abscess and moderate to severe renal/perirenal inflammation persisted, and repeated cultures of urine samples were positive for fungi during the caspofungin administration, which continued for 14 days. Caspofungin was subsequently switched to micafungin (5.4 mg/kg diluted in 0.5 mg/mL intravenously for 1.5 hr every 24 hr), the dose of which was determined by extrapolation from the human dose [7]. The renal abscess and renal/perirenal inflammation improved, but repeated cultures of urine samples were positive for fungi, despite micafungin administration continuing for 16 days. The dose of micafungin was increased to 8 mg/kg, and culture of urine sample was negative for fungi 6 days after administering the increased dose. Micafungin administration was maintained for 10 days after the first negative fungal culture. Urine samples, performed monthly, have remained

Table 1. Antifungal sensitivity of *Candida* strains

	MIC (μg/mL)			
	Range	*C. glabrata* HMR_001	*C. glabrata* CBS 138	*C. albicans* SC5314[a]
Itraconazole	0.002–2	0.5	0.063	0.031
Fluconazole	0.063–64	16	0.5	0.5
Amphotericin B	0.016–16	2	2	2
Caspofungin	0.004–4	1	1	0.125–0.5
Anidulafungin	0.004–4	0.031	0.031–0.063	0.004

MIC, minimum inhibitory concentration.
[a]*C. albicans* SC5314 was included as a reference.

negative for more than 6 months. The dog's diabetes mellitus has been stably controlled, and there have been no specific clinical signs for urinary tract infection since.

Several mechanisms of azole resistance of *Candida* species have been previously reported. They include (1) inactivation of Erg3 resulting in the utilization of alternative sterols for synthesis of the fungal membrane, (2) utilizing exogenously supplied sterols to compensate for inhibition of endogenous sterol production by an azole drug, (3) increased expression of ATP-binding cassette (ABC) transporters and major facilitator superfamily transporters, (4) increased expression of Erg11, (5) nonsynonymous mutations in *ERG11* resulting in reduced affinity for azole binding, and (6) aneuploidy that may contribute to adaptation of the fungal cell to the environment containing an azole drug [3, 8]. Among the resistance mechanisms, overexpression of ABC efflux pumps has been mainly implicated in azole resistance of *C. glabrata*, while altered expression levels of Erg11 or a mutation within the *ERG11* gene are comparatively rare. No study has reported aneuploidy-mediated azole resistance of *C. glabrata*.

To understand the mechanism of azole resistance of *C. glabrata* HMR_001, we analyzed the nucleotide sequences of *ERG11* to find the presence of any mutations. Genomic DNA of *C. glabrata* HMR_001 was extracted using the method previously described [9], and the coding region of *ERG11* was amplified with a proof reading *pfu* DNA polymerase (Bioneer, Deajeon, Korea) and primers, CAGL_ERG11_F1 (GGCCTTTGCTCAGCACAGTGA) and CAGL_ERG11_R1 (CGTGTTTCTTTAGGCTTACCAAGG), which were designed using sequence information (sequence ID CAGL0E04334g) from the *Candida* genome database (CGD, http://www.candidagenome.org/). An amplified PCR product was sequenced using primers CAGL_ERG11_pro_F (ATTCCACCTCGAAGAACCCG), CAGL_ERG11_ORF_F (CGAAAACAACTCCGGTATCGTC), CAGL_ERG11_ter_R (GCCCTCTAAACGAAACAACCAG), CAGL_ERG11_ORF_F2 (GTCTACTTGGGTCCAAAGGGT), and CAGL_ERG11_ORF_R (GGCAAGTATGGAGAGGAAACAC), and the obtained sequence of *ERG11* from *C. glabrata* HMR_001 was compared with the sequence of the same gene of the type strain *C. glabrata* CBS 138. A total of 6 mutations, C678T, T768C, T834C, A1023G, T1275C, and T1557A, were found in *ERG11* of *C. glabrata* HMR_001 compared to that of *C. glabrata* CBS 138 (Supplementary Fig. 2). However, none were nonsynonymous mutation, suggesting that the influence of altered nucleotide sequences in azole resistance of *C. glabrata* HMR_001 was unlikely. Furthermore, we compared transcript levels of *ERG11* between *C. glabrata* HMR_001 and *C. glabrata* CBS 138 by quantitative real-time PCR (qRT-PCR) to investigate whether expression of the gene is altered in the resistant fungus. The results of qRT-PCR showed that transcript levels of *ERG11* in *C. glabrata* HMR_001 were similar to that in *C. glabrata* CBS 138 (Fig. 1A). Our results agreed with the fact that a mutation or increased expression of *ERG11* is rarely observed from clinically isolated azole resistant *C. glabrata* [8].

PDR5 is the gene encoding the ABC transporter and known to be involved in azole resistance of *Saccharomyces cerevisiae*. *C. glabrata* possesses orthologs of *S. cerevisiae PDR5*, *CDR1* (CAGL0M01760g), and *PDH1* (CAGL0F02717g), which are also known to be associated with fungal resistance to azoles [10, 11]. We therefore investigated transcript levels of *CDR1* and *PDH1* in *C. glabrata* HMR_001 by qRT-PCR and compared them with those of the reference strain *C. glabrata* CBS 138. The results revealed that transcript levels of *CDR1* and *PDH1* were 6.84- and 3.61-folds up-regulated, respectively, in *C. glabrata* HMR_001 compared to the *C. glabrata* CBS 138 strain, suggesting that overexpression of these ABC transporters, *CDR1* in particular, may be the main cause of azole resistance of this fungus (Fig. 1B).

Increased expression of ABC transporters is correlated with enhanced drug efflux in the azole-resistant isolates. Monitoring the intracellular accumulation of rhodamine 6G has been used to estimate drug efflux in pathogenic fungi including *C. albicans* and *C. glabrata* [10, 12, 13]. We therefore analyzed accumulation of rhodamine 6G in *C. glabrata* HMR_001 and compared it to that of the *C. glabrata* CBS 138 strain by measuring intracellular fluorescence of rhodamine 6G at 535 nm with a FACScan flow cytometer (BD Bioscience, San Jose, CA, USA) every 2 min for a 10-min period. The results of our analysis revealed that at 2 min, rhodamine 6G had accumulated 3.61-fold less in *C.*

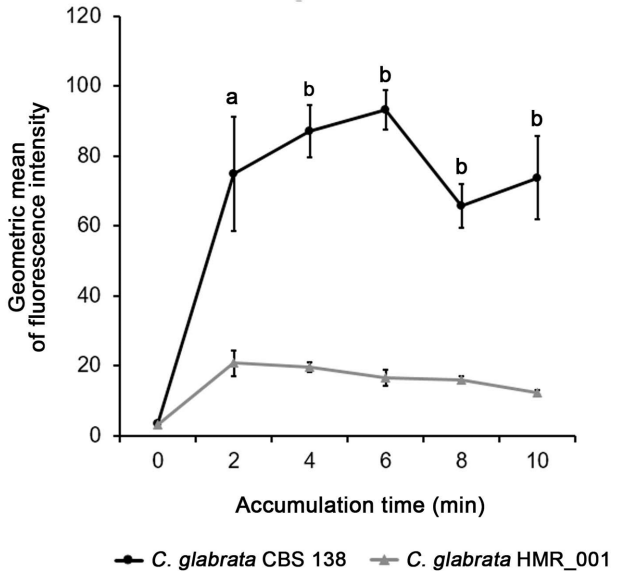

Fig. 2. Comparison of intracellular accumulation of rhodamine 6G between strains. Accumulation of rhodamine 6G (R6G) in *Candida glabrata* HMR_001 was evaluated and compared with those of the reference strain *C. glabrata* CBS 138, and the results were numerically presented as the geometric means of fluorescence intensity. Values represent averages from 3 replicates and error bars indicate standard deviations ($^{a}p = 0.005$, $^{b}p < 0.001$).

glabrata HMR_001 than in *C. glabrata* CBS 138, and this reduced accumulation of the compound in the resistant fungus remained afterwards (Fig. 2). Reduced intracellular accumulation of rhodamine 6G in *C. glabrata* HMR_001 suggested that drug efflux is activated more in the resistant fungus than in *C. glabrata* CBS 138, which also supports our observation of the increased transcript levels of ABC transporter genes *CDR1* and *PDH1* in the fungus. Taken together, we concluded that increased drug efflux mediated by overexpression of ATP transporter genes *CDR1* and *PDH1* is the main cause of the azole resistance of *C. glabrata* HMR_001.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including two figures can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-45-426-s001.pdf.

ACKNOWLEDGEMENTS

This research was supported by the Chung-Ang University Research Scholarship Grants in 2016 (MK), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1D1A1B03931890).

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