Research of Mrr1, Cap1 and MDR1 in *Candida albicans* resistant to azole medications

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Abstract. The aim of the present study was to investigate the association between Mrr1, adenylyl cyclase-associated protein 1 (Cap1) and multi-drug resistance gene 1 (MDR1), and to assess the mutations in Mrr1 and Cap1 in azole-resistant *Candida albicans* strains. The study isolated 68 *C. albicans* strains from patients with vulvovaginal candidiasis. Drug susceptibility testing was conducted to characterize the resistance profile of these strains to fluconazole, itraconazole and voriconazole. Polymerase chain reaction (PCR) amplification was performed for Cap1 and Mrr1, and the PCR products were sequenced to identify any mutations. Reverse transcription-quantitative PCR was performed to measure Cap1, Mrr1 and MDR1 mRNA in *C. albicans* strains. The results of the present study indicated S381N, P311S and A390T missense mutations in Cap1 and T917M, T923I, N937K, E1020Q, F1032L and S1037L missense mutations in Mrr1 in azole-resistant *C. albicans* strains. Fluconazole-resistant strains had significantly elevated Cap1 and MDR1 mRNA levels compared with fluconazole-sensitive strains (P<0.01). The mRNA levels of Cap1, Mrr1 and MDR1 were significantly increased in the strains resistant to all three of fluconazole, itraconazole and voriconazole compared with strains sensitive to the three agents (P<0.001, P=0.037 and P<0.001, respectively). Cap1 expression was positively correlated with MDR1 expression in fluconazole-resistant strains (P<0.05). No significant correlation was observed between Cap1, Mrr1 and MDR1 in the strains resistant to fluconazole, itraconazole or voriconazole. The results of the present study suggested that fluconazole resistance may involve MDR1 overexpression mediated by Cap1 overexpression. Cross-resistance between fluconazole, itraconazole and voriconazole may be associated with mutations in Cap1 and Mrr1, rather than their overexpression. In addition, the present study also revealed two novel mutations in Mrr1; T917M and T923I. These findings may provide a basis for elucidating the molecular mechanisms of and improving therapeutic treatments to tackle azole resistance.

Introduction

Vulvovaginal candidiasis (VVC) is the second leading cause of vaginitis, accounting for 15-25% of vaginitis cases worldwide (1,2). A large number of heterogeneous species of *Candida* may cause VVC, and the *Candida albicans* strain is a primary pathological factor (3). Azole antifungal drugs, including fluconazole, itraconazole and voriconazole, are widely used to treat patients with VVC (4-6). However, *C. albicans* strains that are resistant to azole antifungal drugs have developed, which makes eradicating *C. albicans* a difficult task and increases the risk of VVC recurrence (7). Therefore, investigating the molecular mechanisms underlying azole resistance is of great importance to improve therapeutic treatments for patients with VVC.

Concerns surrounding azole-resistant *C. albicans* strains have led researchers to investigate the underlying molecular mechanisms (8). It has been previously demonstrated that altered expression of drug efflux pumps [cerebellar degeneration related protein (CDR)1, CDR2 and multi-drug resistance gene 1 (MDR1)] and ergosterol biosynthesis (ERG) genes, as well as mutations in ERG11, are associated with the resistance mechanisms (9). A study by Schubert *et al* (10) reported that Mrr1 and adenylyl cyclase-associated protein 1 (Cap1), zinc cluster transcription factors, have synergistic roles in inducing MDR1 expression in wild type *C. albicans* strains, whereas gain-of-function mutations in either Mrr1 or Cap1 function independently to mediate MDR1 overexpression and drug resistance. Furthermore, it has been reported that Spt-Ada-Gcn5-acetyltransferase complex subunit Ada2 promotes Cap1-mediated upregulation of the multidrug efflux pump MDR1 in fluconazole-resistant *C. albicans* (11). Additionally, a study by Sasse *et al* (12) demonstrated that two polymorphic promoter alleles in MDR1 may be associated with the development of fluconazole resistance. A study by Wang *et al* (13) identified mutations in the EGR11 gene in azole-resistant *C. albicans*. However, the mutations in Mrr1 and Cap1, which are implicated in the development of resistance to azoles, have not been fully elucidated.

In the present study, 68 *C. albicans* strains were isolated from patients with VVC and the resistance profile of these strains to fluconazole, itraconazole and voriconazole was characterized. Mutations in Mrr1 and Cap1 were also investigated in these *C. albicans* strains. The mRNA levels of Mrr1, Cap1 and MDR1 were compared between azole-resistant and azole-sensitive strains, and the associations between Mrr1, Cap1 and MDR1 expression were analyzed in fluconazole-, itraconazole- and/or voriconazole-resistant strains. The results of the present study may provide an insight into the development of azole resistance.

Materials and methods

Strains and growth conditions. In the present study, 68 *C. albicans* strains isolated from the vaginal secretions of 68 patients with VVC (mean age, 34.5±8.9 years; range, 20-55 years) were used. Patients were recruited between March and November 2014 at the Skin Venereal Division of the Second Clinical Medical College of Shanxi Medical University (Taiyuan, China). The present study was approved by the Ethics Committee of Shanxi Medical University and all patients provided informed consent. The reference *C. albicans* strain, ATCC11006, was purchased from the Fungi and Fungal Disease Research Center of Peking University (Beijing, China). All strains were stored as frozen stocks in 15% glycerol at -80°C in microbiological laboratories. The strains were routinely grown on Sabouraud's agar plates (bioMérieux, Marcy-l'Étoile, France).

Drug susceptibility testing. The minimal inhibitory concentrations of fluconazole, itraconazole and voriconazole were detected using the broth microdilution method provided by the Clinical and Laboratory Standards Institute (CLSI) standard M27-A3 (14). To determine the susceptibilities of the strains to each drug (fluconazole, itraconazole or voriconazole), two-fold serial dilutions of each drug were prepared in RPMI medium (Saipuruisi Co., Ltd., Beijing, China) from 64 to 0.125 μg/ml. Susceptibility tests were conducted using the microdilution method, as previously described by Ruhnke *et al* (15).

An aliquot of 100 μl of each dilution was added into each well of a 96-well plate, with 100 μl of RPMI serving as a control. The isolated strains were sub-cultured on Sabouraud's agar plates at 35°C for 24 h, twice. Each isolate was suspended in sterile distilled water and then vortexed at 1,000 x g for 15 sec at room temperature. The turbidity of the inoculum suspension was detected by electronic turbidimeter (ATB1525; bioMérieux) with a wavelength of 530 nm and adjusted to a McFarland standard of 0.5, ~1-5x10^6 colony forming units/ml using a bioMérieux DensiChek instrument (bioMérieux). The inoculum suspension was then diluted by 500-fold and an aliquot of 100 μl was added into each well of the 96-well plate (except the blank controls) and incubated at 37°C. After 24 and 48 h, the 96-well plates were read visually. Results were analyzed according to the CLSI standard: Fluconazole (sensitive, ≤4 mg/ml; susceptible dose dependent, 8-16 mg/l; resistant, ≥32 mg/l), itraconazole (sensitive, ≤0.125 mg/ml; susceptible dose dependent, 0.25-0.5 mg/l; resistant, ≥1 mg/l) and voriconazole (sensitive, ≤1 mg/ml; susceptible dose dependent, 2 mg/l; resistant, ≥4 mg/l) (14).

Isolation of genomic DNA and polymerase chain reaction (PCR) amplification. Genomic DNA was isolated from *C. albicans* strains using a Yeast DNAiso kit (Takara Bio, Inc., Otsu, Japan), as previously described (16). Fungal primers were as follows: forward primer (ITS86), 5'-GTGAATCAT CGAATCTTTGAAC-3' and reverse primer (ITS4), 5'-TCC TCCGCTTATTGATATGC-3'. PCR was performed using a Perkin-Elmer 4800 thermal cycler (Perkin Elmer, Inc., Waltham, MA, USA) with 35 cycles of pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 8 min. The PCR products were stored at -4°C. The PCR mixture included 25 μl MasterMix (TIANGEN, Beijing, China), 1 μl ITS4 primer, 1 μl ITS86 primer, 5 μl cDNA and 18 μl RNase-free water. The PCR products were separated by electrophoresis and analyzed using a Bio-Rad GelDocXR imaging system with QuantityOne version 4.6 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

PCR amplification and sequencing of Cap1 and Mrr1. Genomic DNA was isolated from *C. albicans* strains using a Yeast DNAiso Kit (Takara Bio, Inc.), as described previously (16). Primers were designed by Wuhan GeneCreate Biological Engineering Co., Ltd., (Beijing, China) and used for the amplification of Cap1 and Mrr1. Primer sequences were as follows: Cap1, forward 5'-CCAGTGCCCAAATTTAAA CGTTCAGGT-3' and reverse 5'-TTAATGTTTTATACTTCG CTCTAGTAATTGATTCAC-3'; and Mrr1, forward 5'-GCT CTTATTATTCGAGTGAATATGAGC-3' and reverse 5'-TCT CCTCAGTTCTGGTCGTGG-3'. PCR was performed using a Perkin-Elmer 4800 thermal cycler, with 30 cycles of pre-denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 10 min. The PCR mixture was comprised of 2 μl DNA, 2 μl forward primer, 2 μl reverse primer, 25 μl Etaq Mix (Tiangen Biotech Co., Ltd., Beijing, China) and 19 μl RNase-free water. Following separation, the PCR products were purified and sequenced by Wuhan GeneCreate Biological Engineering Co., Ltd. The sequences obtained were aligned to the known sequences in the GenBank database (ncbi.nlm.nih.gov/) using DNASTAR Lasergene v. 7.1 software (DNASTAR, Madison, WI, USA) for detection of gene locus mutations.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from *C. albicans* strains using RNAiso Reagent (Takara Bio, Inc.), as described previously (16). The RNA obtained was quantified in an Eppendorf biophotometer D30 (Eppendorf, Hamburg, Germany) and reverse transcribed into cDNA using a PrimeScript™ One Step RT-PCR Kit (cat. no. RR055A/B; Takara Bio, Inc.) The final (10 μl) reaction system included 2 μl 5x PrimeScript RT Master Mix, 7 μl RNase-Free water and 1 μl RNA at 37°C for 15 min and followed by 65°C for 15 sec. PCR was performed using a real-time Light Cycler system (Roche Diagnostics, Basel, Switzerland) using SYBR Premix Ex Taq II (Takara Bio, Inc.), with 40 cycles of pre-denaturation at 94°C for 30 min, denaturation at 94°C for 10 sec, annealing and extension at 56°C for 34 sec, and a final extension at 72°C for 8 min. Primer

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Primers	Direction	Sequence (5'-3')	Length, bp
ACT1	Forward	ACTACCATGTTCCCAGGTATTG	122
	Reverse	CCACCAATCCAGACAGAGTATT	
Cap1	Forward	CTGGTGGTAGCGATTTTCTGG	258
	Reverse	GTTGTTGTTGTTGATGCTGGTG	
Mrrl	Forward	AACGCTGGTTATGGGTGA	230
	Reverse	TTTGCTGTTGGGCTTCTT	
MDR1	Forward	TGCCATTGTCGGTGGTATCT	249
	Reverse	GGAGCACCAAATAATGGGAAC	

ACT1, actinin α1; Cap1, adenylyl cyclase-associated protein 1; MDR1, multi-drug resistance gene 1.

sequences are presented in Table I. ACT1 was used as an internal reference. *C. albicans* strain ATCC11006 was used as a control group. Relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Statistical analysis. Data were expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences between two groups were analyzed using Student's t-tests. Associations between two genes were analyzed using Pearson correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Drug susceptibility testing. Drug susceptibility testing revealed that, of the 68 strains isolated, 33 were sensitive to fluconazole and 22 were resistant to fluconazole, with a fluconazole resistance rate of 36.72%. A total of 30 strains were sensitive to itraconazole, 12 were intermediate and 26 strains were itraconazole-resistant, with an itraconazole resistance rate of 38.71%. The present study identified 34 voriconazole-sensitive strains, 29 voriconazole-resistant strains and 5 voriconazole-intermediate strains, with a voriconazole resistance rate of 42.65%. Notably, a total of 13 strains were resistant to fluconazole, itraconazole and voriconazole and a total of 12 strains were sensitive to all three agents. The cross-resistance rate between fluconazole, itraconazole and voriconazole was 19.12%. The reference strain (ATCC11006) was sensitive to all three agents.

Identification of mutations in Cap1 and Mrr1. Upon sequencing the Cap1 gene, an S381N missense mutation was identified in 1 fluconazole-resistant strain and 2 fluconazole-sensitive strains. A P311S missense mutation was discovered in 2 itraconazole-resistant strains, 2 itraconazole-sensitive strains and 1 strain resistant to both fluconazole and itraconazole. Additionally, 1 strain sensitive to all three drugs and 2 strains resistant to all three drugs had A390T missense mutations (Table II and Fig. 1). No mutation was detected in the reference strain.

The Mrr1 gene was sequenced successfully in 63 *C. albicans* strains. An E1020Q missense mutation was detected in 45 *C. albicans* strains and the reference strain (Table III and Fig. 2). One strain resistant to fluconazole, itraconazole and voriconazole was revealed to have five missense mutations: T917M, T923I, E1020Q, F1032L and S1037L. A total of 4 itraconazole-resistant strains had six missense mutations: T917M, T923I, N937K, E1020Q, F1032L and S1037L. One fluconazole-resistant strain had four missense mutations: N937K, E1020Q, F1032L and S1037L. A total of 2 fluconazole-sensitive strains had two missense mutations: N937K and E1020Q. The E1020Q missense mutation was identified in 9 fluconazole-sensitive strains.

Comparison of mRNA levels of Cap1, Mrr1and MDR1 between different strains. Fluconazole-resistant strains had significantly elevated mRNA levels of Cap1 and MDR1 compared with fluconazole-sensitive strains (P<0.01; Table IV). No significant difference was observed in the mRNA expression of Mrr1 between the fluconazole-resistant strains and fluconazole-sensitive strains (Table IV). The itraconazole-resistant strains had significantly increased levels of Cap1 and MRR1 mRNA compared with the itraconazole-sensitive strains (P=0.038 and P=0.034, respectively; Table IV). Levels of Cap1, Mrr1 and MDR1 mRNA were not significantly different between the voriconazole-resistant and voriconazole-sensitive strains (Table IV). Furthermore, the levels of Cap1, Mrr1 and MDR1 mRNA were significantly increased in the strains resistant to fluconazole, itraconazole and voriconazole, compared with strains sensitive to the three agents (P<0.001, P=0.037 and P<0.001, respectively; Table IV).

Associations between Cap1, Mrr1 and MDR1. Pearson correlation analysis was applied to investigate the associations between levels of Cap1, Mrr1 and MDR1 mRNA (Table V). In fluconazole-resistant strains, itraconazole-resistant strains and voriconazole-resistant strains, Cap1 expression was significantly positively correlated with MDR1 expression (r=0.414, r=0.511 and r=0.413, respectively; P<0.05). No significant correlation was observed between Mrr1 and MDR1, or between Cap1 and Mrr1. However, in the strains resistant to fluconazole, itraconazole and voriconazole, no significant correlations were observed between Cap1, Mrr1 or MDR1 expression.

Table II. Adenylyl cyclase-associated protein 1 mutations in 61 *Candida albicans* strains.

Strain	Resistance	Mutation locus	Amino acid change
CA10	R	T907C/ G1169A /T1171A/C1371-/A1372G	A390T
CA14	FCAR	T906C/ G1142A /A1212G	S381N
CA30	S	T906C/ C931T /C972T	P311S
CA32	S	T906C/ G1168A /T1170A	A390T
CA33	S	T906C/ G1168A /T1170A	A390T
CA36	S	C931T /C972T/A1212G	P311S
CA51	ITRR	C932T /C973T	P311S
CA52	ITRR	C931T /C972T/A1212G	P311S
CA53	ITR/VRCR	T907C/ C932T /C973T	P311S
CA63	ITRR	C931T /C972T	P311S
CA67	VRCR	C972T/A1212G/ G1442A	G481E

Boxes indicate missense mutations. R, resistance to fluconazole, itraconazole and voriconazole; FCAR, resistance to fluconazole only; S, sensitivity to fluconazole, itraconazole and voriconazole; ITRR, resistance to itraconazole only; VRCR, resistance to voriconazole only.
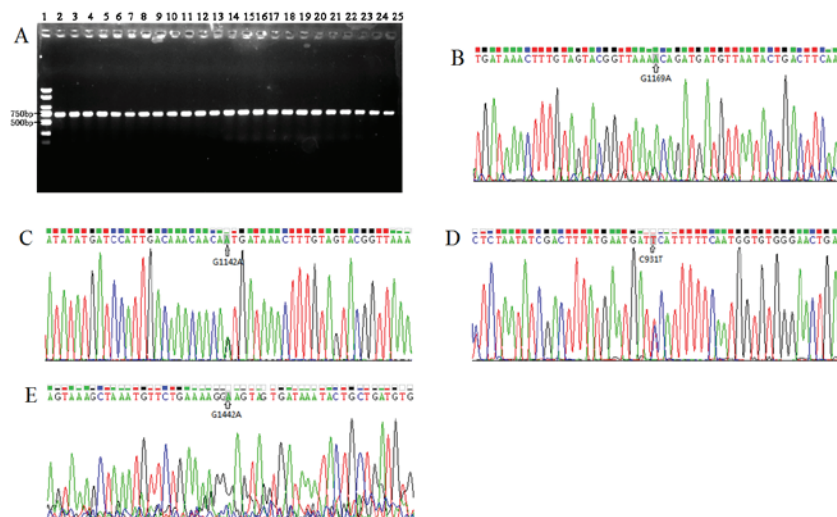

Figure 1. Detection of missense mutations in Cap1 in *Candida albicans* strains resistant to azoles. (A) Gel image of polymerase chain reaction products of Cap1: Lane 1, marker; lane 2, reference *C. albicans* strain ATCC11006 (700 bp); lane 3-25, *C. albicans* strains isolated from patients with vulvovaginal candidiasis (700 bp). (B) G1169A, (C) G1142A, (D) C931T and (E) G1442A missense mutations detected in Cap1. Cap1, adenylyl cyclase-associated protein 1.

Discussion

VVC affects up to 75% of women during their lifetime (18). *C. albicans* strains in the vaginal mucosa account for the majority of VVC cases (18). In the present study, 68 *C. albicans* strains were isolated from the vaginal secretions of patients with VVC. Of these 68 strains, 22 (36.72%) were resistant to fluconazole, 26 (38.71%) were resistant to itraconazole and 29 (42.65%) were resistant to voriconazole. The present study characterized the resistance profile of all isolated *C. albicans* strains.

Previous studies have demonstrated that efflux pumps (CDR1, CDR2 and MDR1) are important molecular mechanisms responsible for the development of resistance to fluconazole by

actively transporting the drug out of the cell (19,20). Mrr1, a zinc cluster transcription factor, is not only indispensable for the constitutive overexpression of MDR1, but also for chemical-induced expression (21). A study by Mogavero *et al* (22) reported that active Cap1, a bZip transcription factor, causes overexpression of the MDR1 efflux pump, resulting in resistance to pharmacological agents. In the present study, the mRNA levels of Cap1 and MDR1 were demonstrated to be significantly elevated in the fluconazole-resistant compared with the fluconazole-sensitive strains. Furthermore, Cap1 expression was positively correlated with MDR1 expression in *C. albicans* strains resistant to fluconazole. These findings indicated that overexpression of Cap1, rather than Mrr1, may lead to MDR1 overexpression and the development of

Table III. Mrr1 mutations in 63 *Candida albicans* strains.

Strains	Resistance	Mutation locus	Amino acid change
CA10	R	G2676A/G2691A/C2715A/T2724C/ **C2750T** / **C2768T** / G3058C / T3096A /G3108A/ C3110T C3110T/T3117A	T917M/T923I/E1020Q/ F1032L/S1037L
CA30	S	G2577A/A2589G/C2595T/C2625T/ C2811G /A3024G/G3058C	N937K
CA37	S	G2577A/A2589G/C2595T/C2625T/ C2811G /A3024G/ G3058C	N937K/E1020Q
CA42	VRCR	C2625T/ C2811G /A3024G/ G3058C / T3096A /G3108A/ C311OT /T3117A	N937K/E1020Q/F1032L/ S1037L
CA50	ITRR	T2529C/C2538T/C2595T/C2700T/ **C2750T** / **C2768T** / G2865A/C2871T/T2880C/ G3058C	T917M/T923I/E1020Q
CA51	ITRR	G2577A/A2589G/C2595T/C2625T/ C2811G /A3024G/ G3058C	N937K/E1020Q
CA53	ITR/VRCR	G2577A/A2589G/C2595T/C2625T/C2811G/A3024G/ G3058C/T3096A/G3108A/C3110T/T3117A	N937K/E1020Q/F1032L/ S1037L
CA62	ITRR	C2529T/G2691A/C2700T/T2724C/ **C2768T** /G2865A/ T2880C/ G3058C	T923I/E1020Q
CA63	ITRR	G2577A/A2589G/C2595T/C2625T/ C2811G /A3024G/ G3058C /T3096A/ T3096A / C3110T /T3117A	N937K/E1020Q/F1032L/ S1037L

Boxes indicate missense mutations. Mutations marked in bold are novel mutations detected in the present study. R, resistance to fluconazole, itraconazole and voriconazole; S, sensitivity to fluconazole, itraconazole and voriconazole; ITRR, resistance to itraconazole only; VRCR, resistance to voriconazole only.
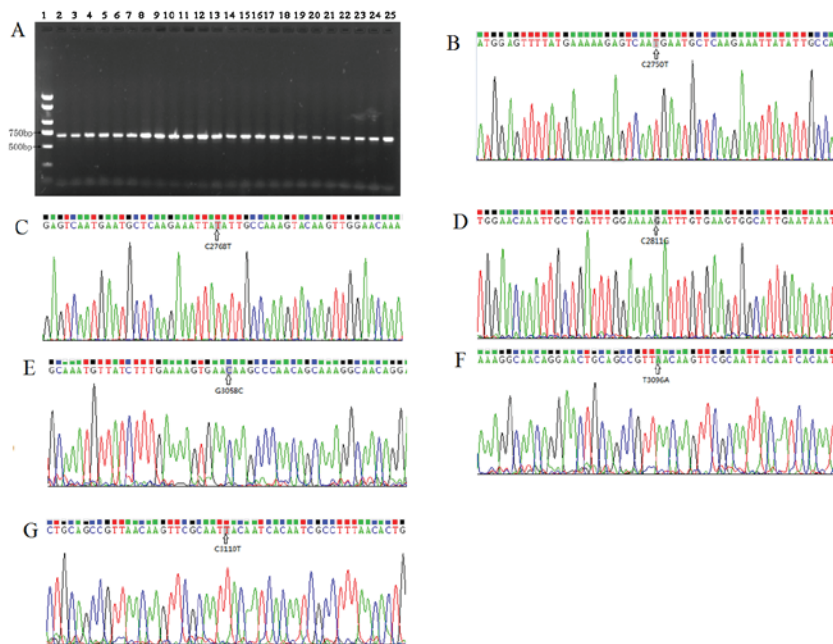

Figure 2. Detection of missense mutations in Mrr1 in *Candida albicans* strains resistant to azoles. (A) Gel image of polymerase chain reaction products of Mrr1. Lane 1, marker; lane 2, reference *C. albicans* strain ATCC11006 (659 bp); lane 3-25, *C. albicans* strains isolated from patients with vulvovaginal candidiasis (659 bp). (B) C2750T, (C) C2768T, (D) C2811 G, (E) G3058C, (F) T3096A and (G) C3110T missense mutations detected in Mrr1.

fluconazole resistance. Levels of Cap1 and Mrr1 mRNA were increased in itraconazole-resistant strains but not in voriconazole-resistant strains. However, no significant difference was observed in the expression of MDR1 efflux pumps between the itraconazole-resistant and itraconazole-sensitive strains or between the voriconazole-resistant and voriconazole-sensitive

Table IV. Cap1, Mrr1 and CDR1 mRNA levels.

Drug	Gene	Group	No. strains	Relative mRNA expression level	t	P-value
FCA	Cap1	Resistant	25	5.43±2.21	9.331	<0.001
		Sensitive	33	1.26±0.43		
	Mrr1	Resistant	25	1.13±0.45	0.233	0.817
		Sensitive	33	1.10±0.50		
	MDR1	Resistant	25	2.10±0.57	4.710	<0.001
		Sensitive	33	1.25±0.75		
ITR	Cap1	Resistant	26	3.43±2.50	2.128	0.038
		Sensitive	30	2.07±2.31		
	Mrr1	Resistant	26	1.29±0.45	2.173	0.034
		Sensitive	30	1.02±0.46		
	MDR1	Resistant	26	1.59±0.81	0.415	0.680
		Sensitive	30	1.50±0.74		
VRC	Cap1	Resistant	29	3.10±2.39	0.665	0.508
		Sensitive	34	2.68±2.56		
	Mrr1	Resistant	29	1.15±0.40	0.686	0.495
		Sensitive	34	1.07±0.51		
	MDR1	Resistant	29	1.73±0.73	1.530	0.131
		Sensitive	34	1.43±0.81		
FCA, ITR and VRC	Cap1	Resistant	13	5.10±2.26	6.497	<0.001
		Sensitive	12	1.00±0.22		
	Mrr1	Resistant	13	1.25±0.44	2.211	0.037
		Sensitive	12	0.85±0.44		
	MDR1	Resistant	13	2.05±0.60	4.356	<0.001
		Sensitive	12	1.00±0.59		

Cap1, adenylyl cyclase-associated protein 1; MDR1, multi-drug resistance gene 1; FCA, fluconazole; ITR, itraconazole; VRC, voriconazole.

Table V. Associations between mRNA levels of Cap1, Mrr1 and MDR1.

Strains	Gene	Correlation efficient (r)	P-value
FCA-resistant strains	Cap1 and MDR1	0.414	0.039
	Mrr1 and MDR1	0.146	0.486
	Cap1 and Mrr1	0.288	0.163
ITR-resistant strains	Cap1 and MDR1	0.511	0.008
	Mrr1 and MDR1	0.035	0.864
	Cap1 and Mrr1	-0.013	0.948
VRC-resistant strains	Cap1 and MDR1	0.413	0.026
	Mrr1 and MDR1	0.033	0.863
	Cap1 and Mrr1	0.233	0.225
Strains resistant to FCA, ITR and VRC	Cap1 and MDR1	0.173	0.571
	Mrr1 and MDR1	-0.091	0.766
	Cap1 and Mrr1	0.025	0.936

Cap1, adenylyl cyclase-associated protein 1; MDR1, multi-drug resistance gene 1; FCA, fluconazole; ITR, itraconazole; VRC, voriconazole.

strains. This suggests that itraconazole resistance may involve Cap1 and Mrr1 overexpression, but not MDR1 overexpression. Voriconazole resistance may not be associated with Cap1, Mrr1 or MDR1; it may be attributed to other molecular mechanisms, such as mutations in Cap1 and Mrr1, and altered expression of ERG genes or mutations in ERG11 (23,24). Furthermore, the

results of the present study indicated significant increases in the levels of Cap1, Mrr1 and MDR1 mRNA in strains resistant to all three azoles compared with sensitive strains; however, no correlation was identified between Cap1, Mrr1 or MDR1. These observations indicated that MDR1 overexpression may be responsible for the cross-resistance between fluconazole, itraconazole and voriconazole, and may not be regulated by Cap1 and Mrr1 overexpression, rather by mutations in Cap1 and Mrr1.

Several previous studies have demonstrated that gain-of-function mutations in Mrr1 or Cap1 promote MDR1 overexpression, thus contributing to drug resistance (25-27). Nonetheless, a study by Morschhäuser *et al* (28) suggested that P683S and G997V mutations in Mrr1 did not influence the susceptibility of *C. albicans* strains to multiple drugs, including fluconazole. In the present study, sequencing analysis of Cap1 revealed an S381N missense mutation in 1 fluconazole-resistant strain, a P311S missense mutation in 2 itraconazole-resistant strains and 1 strain resistant to both fluconazole and itraconazole, and an A390T missense mutation in 2 strains resistant to all three azoles. The sequencing analysis of Mrr1 revealed four missense mutations in 1 fluconazole-resistant strain, six missense mutations in 4 itraconazole-resistant strains, and five missense mutations in a strain resistant to fluconazole, itraconazole and voriconazole. A study by Morio *et al* (29) previously identified five gain-of-function mutations: R557K, K844E, K884E, N937K, F1032L and S1037L. To the best of our knowledge, the T917M and T923I mutations identified in the present study have not been reported before. Further studies are required to determine whether these missense mutations are associated with drug resistance in *C. albicans* strains. The present study included only 68 *C. albicans* strains isolated from vaginal secretions of patients with VVC. A larger number of *C. albicans* strains is necessary to validate the findings of the present study.

In conclusion, the results of the present study suggested that MDR1 overexpression regulated by overexpression of Cap1 may be associated with the development of fluconazole resistance. MDR1 overexpression associated with cross-resistance between fluconazole, itraconazole and voriconazole may be correlated with mutations in Cap1 and Mrr1, as opposed to Cap1 and Mrr1 overexpression. Two novel missense mutations (T917M and T923I) of Mrr1 in azole-resistant *C. albicans* strains were identified in the present study; however, further studies with a larger sample size are required to verify these findings.

Acknowledgements

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