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Differential expression of the *Candida glabrata CgRTA1* and Cg*RSB1* genes in response to various stress conditions

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ABSTRACT

Candida glabrata, a human opportunistic pathogen is characterized by intrinsic, low susceptibility to fluconazole and a high capacity for acquiring high-level azole resistance. This is related to the elevated expression of genes belonging to the CgPdr1-governed regulon, comprising numerous genes, of which the multidrug ABC transporter-encoding *CgCDR1*, *CgCDR2*, *CgSNQ2* are the best characterized. The function of certain PDR loci, such as Cg*RTA1* and Cg*RSB1* is poorly understood. These are homologs of *ScRTA1* and *ScRSB1* from *Saccharomyces cerevisiae*, members of the LTE family of plasma membrane proteins characteristic of fungi. While overproduced, they are involved in tolerance to 7-aminocholesterol or phytosphingosine, respectively. In this report we shed light on the differential regulation of *CgRTA1* and *CgRSB1* in *C. glabrata*. *CgRTA1* expression positively correlated with intrinsic azole tolerance in clinical isolates. In contrast to *CgRSB1*, a high induction of *CgRTA1* was observed upon fluconazole exposure, which was accompanied by a parallel up-regulation of its transcriptional activator *CgPDR1*. Hypoxia or presence of ketoconazole, both leading to ergosterol depletion, resulted in increased level of *CgRTA1* transcript, whereas *CgRSB1* was highly responsive to mitochondrial dysfunction. On the other hand, the expression of *CgRTA1* was suppressed during growth in pseudohyphae formation promoting media. Our results are the first report linking the divergent regulation of LTE family members and azole sensitivity in *C. glabrata*.

1. Introduction

Fungal infections caused by *Candida glabrata* are on the rise [1]. *C. glabrata* colonizes as a commensal in more than 80% of healthy individuals. Weakening of the immune system may turn this species into opportunistic pathogen. Progressive emergence of candidemia caused by *C. glabrata* correlates with increasing population of immunocompromised individuals (surgical patients, chemotherapy receiving patients, elderly). *C. glabrata* exhibits enormous flexibility and easily adapts to occupied niches. In contrast to *C. albicans*, it is recognized as an intrinsically azole-tolerant species and is about eight times more resistant to the widely used antifungal drug fluconazole [2,3]. It can also easily acquire azole resistance during drug exposure [4–6].

The mechanisms of intrinsic as well as acquired azole resistance in *C. glabrata* rely mainly on the overproduction of the ABC type transporters CgCdr1p, CgCdr2p, CgSnq2p extruding drugs out of the cell [4–7]. Expression of the ABC pumps coding genes is regulated by CgPdr1p, comprising the function of both orthologous ScPdr1p and ScPdr3p transcription factors found in *S. cerevisiae*. Wide genome analyses of matched pairs of azole-resistant and azole-sensitive *C. glabrata* clinical isolates deciphered a global picture of the network of genes involved in drug resistance development and highlighted several weakly characterized loci, including *CgRSB1* and *CgRTA1* [8,9]. *CgRTA1* exhibits homology to *ScRTA1*, known as the sole plasma membrane located multicopy suppressor of 7-aminocholesterol toxicity in *S. cerevisiae* [10,11]. *CgRTA1* is one of the members of the *PDR1* regulon in *C. glabrata* [8,9].

To shed more light on the regulation of *CgRTA1* expression and its role in drug resistance, its expression pattern was analyzed along with that of *CgRSB1* in several clinical isolates characterized by various levels of azole susceptibility. The effect of various stress conditions and influence of mitochondrial status on the transcript level of both genes was also investigated.

Abbreviations: ABC, ATP-binding cassette; EtBr, ethidium bromide; PDRE, pleiotropic drug-response element; 7-ACH, 7-aminocholesterol; UTR, untranslated; FCZ, fluconazole; CTZ, clotrimazole; KTZ, ketoconzole.

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2. Materials and methods

2.1. Strains and culture conditions

C. glabrata clinical isolates used in this study (Table 1) were recovered from patients in the Laboratory of Molecular Diagnostics "Bio-Genetik" (Wroclaw, Poland) or generously obtained from from J. Subik and D. Sanglard [12–14]. Strains were cultivated in complete YPD media (2% peptone, 1% yeast extract and 2% glucose). For the analysis of various stress conditions, strains were grown in YPD containing appropriate azole drug or 10% FCS, or in VS medium [15]. For testing hypoxic conditions, cells were grown in preconditioned VS medium in the hypoxic chamber (BBL™ GasPack™ Anaerobic System). Petite mutants were generated by incubation of the cells in liquid YPD media containing 25 μg/ml of ethidium bromide for 4 h. The petite phenotype was verified on YPGE solid medium (2% ethanol, 2% glycerol, 1% yeast extract and 2% agar).

2.2. Growth inhibition assays

Resistance to azoles (fluconazole – FCZ, voriconazole – VOR, ketoconazole – KCZ and clotrimazole – CTZ) was verified by the use of previously described microdilution tests [16] according to M27-A2 standard guidelines [17]. Cells were grown in RPMI 1640 MOPS buffered medium. Growth was monitored after 48 h by OD_{600} measurements in the Asys HiTech microplate reader and visual inspection. The lowest drug concentration inhibiting growth in 80% of that of drug-free control was defined as minimal inhibitory concentration (MIC_{80}). Susceptibility to 7-aminocholesterol was determined as described previously [11].

2.3. Quantitative real-time PCR

Overnight cultures were diluted in appropriate medium and incubated for 2 h at 30 °C. For the analysis of drug challenge, exponentially growing cells were split and the same amount of cells were re-grown for 2 h in YPD without drug or YPD containing FCZ or KTZ. Total RNA was isolated by the hot-phenol method [18]. Prior to RT-PCR experiments, total RNA was treated with DNase I (Sigma) and purified on silica containing resin (Qiagen). First strand cDNAs were synthesized from 1.25 μg total RNA using iScript Synthesis kit (Bio-Rad). Independent amplifications were performed on the same cDNA for the gene of interest and the *CgTUB1* reference gene using SYBR Green PCR mix (Bio-Rad) on the iQ5 apparatus (Bio-Rad). Fold change of the analyzed transcript was calculated by the Pfaffl method [19]. The following primers were used in qPCR: CgRTA1f 5′-GCATAGACAGTTCCTGACGCAAT-3′,
CgRTA1r 5′-TTAACGGTATATGGGCAGCAAAT-3′,
CgPDR1f 5′- GGAATGGTGACTCGGAAGAA-3′,
CgPDR1r 5′-ATGGCGTCAATGGATGATTT-3′,
CgRSB1f 5′-CATTGTTCGTTGTTGCTACCGTC-3′,
CgRSB1r 5′-CCAGTGTCACCATCAGGGAATCG-3′,
CgTUB1f 5′-AGAGAAGAATTTCCTGATAGAATGATGG-3′,
CgTUB1r 5′-ACAGACAGAGTGGCGTTATATGG-3′.

3. Results and discussion

3.1. Azole susceptibility of clinical isolates

Recent reports indicate that spontaneous gain-of-function mutations in transcriptional regulator CgPdr1p contribute to azole resistance in clinical isolates [8,9,20]. In addition, several CgPdr1p-governed target genes were shown to be connected with enhanced virulence in animal models [21]. Thus, the PDR network is very likely to facilitate adaptation to the host and to sustain chemical stress. CgPdr1p targets comprise among other two weakly characterized loci, classified by homology as *CgRTA1* and *CgRSB1* in *Candida* Genome Database [8,9]. To shed some light on the regulation of *CgRTA1* expression and its contribution to the development of drug resistance, its expression pattern was analyzed along with that of *CgRSB1* in several clinical isolates characterized by various levels of azole susceptibility. Twenty clinical isolates were recovered from patients (source: Laboratory of Molecular Diagnostics "Bio-Genetik", Wroclaw, isolates MD1 to MD8) or generously obtained from J. Subik and D. Sanglard (strains JS1 to JS30, DSY 92 to DSY 565). Screening for azole resistance of all isolates revealed nine azole-resistant strains with MIC_{80} for fluconazole (FCZ) equal to 128 μg/

Table 1
Drug susceptibility of *C. glabrata* clinical isolates used in this study.

Isolate	MIC_{80} (μg/ml)				Site of isolation or reference
	FCZ	CTZ	KTZ	VOR	
MD 1	16	>2.7	1	0.05	Stool
MD 2	32	0.67	1	0.05	Blood
MD 3	16	0.58	1	0.05	Vagina
MD 4	32	>2.7	1	0.05	Vagina
MD 5	32	2.7	0.5	0.05	Vagina
MD 6	128	5.4	4	>14	Oral cavity
MD 7	32	>2.7	>4	0.05	Oral cavity
MD 8	8	2.7	1	0.05	Oral cavity
JS1	128[a]	>8	8	32[a]	[12]
JS3	128[a]	>4	4	>32[a]	[12]
JS7	128[a]	8	8	32[a]	[12]
JS20	128[a]	>8	8	0.75[a]	[12]
JS21	128[a]	>8	4	>32[a]	[12]
JS22	128[a]	>8	8	>32[a]	[12]
JS27	128[a]	8	8	>32[a]	[12]
JS28	8[a]	2	1	0.38[a]	[12]
JS29	8[a]	4	1	0.38[a]	[12]
JS30	8[a]	1	1	1[a]	[12]
DSY92	1[a]	0.125	0.125	0.05	[13]
DSY94	1[a]	0.125	0.125	0.05	[13]
DSY562	8[a]	4	1	0.2	[13]
DSY565	128[a]	8	4	1.6	[13]
ATCC 90030	64	4	8	0.05	[14]

MIC_{80}, minimal inhibitory concentration was defined as the lowest azole concentration that reduced growth to 80% of that of drug free control.
[a] MIC for FCZ and VOR were in agreement with previously published values [12,13].