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Mechanisms of azole resistance among clinical isolates of *Candida glabrata* in Poland

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Candida glabrata is currently ranked as the second most frequently isolated aetiological agent of human fungal infections, next only to *Candida albicans*. In comparison with *C. albicans*, *C. glabrata* shows lower susceptibility to azoles, the most common agents used in treatment of fungal infections. Interestingly, the mechanisms of resistance to azole agents in *C. albicans* have been much better investigated than those in *C. glabrata*. The aim of the presented study was to determine the mechanisms of resistance to azoles in 81 *C. glabrata* clinical isolates from three different hospitals in Poland. The investigation was carried out with a Sensititre Yeast One test and revealed that 18 strains were resistant to fluconazole, and 15 were cross-resistant to all other azoles tested (voriconazole, posaconazole and itraconazole). One isolate resistant to fluconazole was cross-resistant to voriconazole, and resistance to voriconazole only was observed in six other isolates. All strains were found to be susceptible to echinocandins and amphotericin B, and five were classified as resistant to 5-fluorocytosine. The sequence of the *ERG11* gene encoding lanosterol 14-α demethylase (the molecular target of azoles) of 41 isolates, including all strains resistant to fluconazole and three resistant only to voriconazole, was determined, and no amino acid substitutions were found. Real-time PCR studies revealed that 13 of 15 azole-resistant strains showed upregulation of the *CDR1* gene encoding the efflux pump. No upregulation of expression of the *CDR2* or *ERG11* gene was observed.

INTRODUCTION

The genus *Candida* comprises over 150 heterogeneous species (Lass-Flörl, 2009), at least 17 of which are known to be aetiological agents of human and animal infections (Silva *et al.*, 2012). The incidence of fungal infections caused by *Candida* spp. has increased dramatically during the last three decades, due mainly to the rise in number of immunocompromised patients (Arendrup, 2010; Pfaller & Diekema, 2010). Currently, candidiasis represents the fourth leading cause of nosocomial infections (8–10 %), and mortality due to systemic candidiasis remains high, ranging from 15 to 35 % depending on the infecting *Candida* sp. (Pfaller & Diekema, 2007). Moreover, the annual treatment costs exceed US$10 billion in the USA alone (Jabra-Rizk, 2006). In most cases, *Candida* infections are derived from the individual's own endogenous reservoir when the host presents certain risk factors such as immunosuppressive and cytotoxic therapies, treatment with broad-spectrum antibacterial antibiotics, AIDS, diabetes and drug abuse (Pfaller & Diekema, 2007). *Candida albicans* is still classified as the most common fungal

One supplementary figure and one table are available with the online Supplementary Material.

pathogen in humans, although recently a shift towards systemic infections by non-*albicans Candida* spp. has been reported (Papon *et al.*, 2013). In a tertiary paediatric hospital in Poland, the prevalence of non-*albicans* species increased from 12.5 % in 2000 to 70 % in 2010 (Dzierżanowska-Fangrat *et al.*, 2014). It has been observed worldwide that one of most frequently isolated non-*albicans* species is *Candida glabrata* (Papon *et al.*, 2013; Rodrigues *et al.*, 2014). This species lacks a number of the virulence factors allied to other *Candida* spp. pathogenicity, such as hyphal growth, the ability to secrete proteases and efficient adhesion to the surface of host tissue (Kaur *et al.*, 2005; Rodrigues *et al.*, 2014). It is believed that the increase in the number of *C. glabrata* infections is mainly a consequence of its intrinsically low susceptibility to azoles, agents used commonly for the treatment and prevention of fungal infections (Tscherner *et al.*, 2011). Similar to some other species in the genus *Candida* (especially *C. albicans*), *C. glabrata* belongs to the natural microbiota of the oral cavity, gastrointestinal and vaginal tracts of humans (Rodrigues *et al.*, 2014). Elimination of other yeast species from the host organism through azole abuse leads to selection and overgrowth of resistant strains of *C. glabrata*, causing different clinical manifestations, starting from minor infections of mucosal membranes to life-threatening invasive infections (Chavanet *et al.*, 1994).

The most important factors affecting the low level of susceptibility of *C. glabrata* isolates to azoles are similar to those found in *C. albicans* and include overexpression of genes encoding the most important ABC drug transporters, namely Cdr1p and Pdh1p (known also as Cdr2p) (Rodrigues *et al.*, 2014; Sanglard *et al.*, 1999, 2001; Sanguinetti *et al.*, 2005), as well as upregulation of genes involved in the biosynthesis of ergosterol, especially *ERG11* (Marichal *et al.*, 1997; Rodrigues *et al.*, 2014). Interestingly, in contrast to *C. albicans*, most of the investigations carried out in different regions of the world have revealed that point mutations within the *ERG11* gene of azole-resistant *C. glabrata* isolates are rarely identified or are not present at all (Berila & Subik 2010; Berila *et al.*, 2009; Sanguinetti *et al.*, 2005). There also exist many other possible, not fully recognized mechanisms affecting *C. glabrata* resistance to azoles, such as mitochondrial dysfunction (Ferrari *et al.*, 2011) and calcium signalling (Kaur *et al.*, 2004). However, they do not seem to be as important as overexpression of drug efflux proteins or enzymes involved in ergosterol synthesis (Rodrigues *et al.*, 2014).

The aim of the presented study was to analyse the mechanisms of resistance to azole antifungal agents in 81 *C. glabrata* clinical isolates from three hospitals in Poland. To the best of our knowledge, this is one of the largest populations studied so far.

METHODS

Strains and susceptibility testing. A total of 81 *C. glabrata* were isolated from a variety of clinical specimens: urine, faeces, blood, fluid from the peritoneal cavity, stoma, bronchopulmonary lavage fluid, and swabs of the mouth, throat and anus from patients at three Polish hospitals: the Children's Memorial Health Institute in Warsaw (n=17 isolates, 21 %, collected in 2011 and 2012), the Medical University of Gdansk (n=54 isolates, 66.7 %, collected in 2011 and 2012) and the Pomeranian Medical University in Szczecin (n=10 isolates, 12.3 %, collected in 2008). In the Medical University of Gdansk, superficial infections are treated with azoles, and invasive candidosis with azoles and echinocandins. In the period of collection of the strains from Warsaw and Szczecin, only azoles were used for eradication of infections.

Species identification was carried out using three different methods: growth on Chromagar *Candida* Lab-agar plates (Biocorp), a *Candida* API test (bioMérieux) and species-specific PCR. All tested strains were cultured on a chromogenic medium and plates were incubated at 37 °C for 24–8 h. *C. glabrata* isolates were determined by growth as violet colonies. The *Candida* API test allows identification based on the sugar assimilation patterns, as this species is known to ferment only glucose and trehalose. Molecular identification was carried out according to the method developed by Olchawa *et al.* (2013). The species-specific PCR products (423 bp) were detected on 1.5 % agarose gel with the addition of ethidium bromide.

Susceptibility to antifungal agents was determined using the commercially available Sensititre Yeast One test (TREK Diagnostic Systems). This test enables analysis of the antifungal activity of the nine most common antimycotics, over the range of concentrations indicated (μg ml^{-1}): micafungin, 0.008–8; caspofungin, 0.008–8; 5-fluorocytosine, 0.06–64; posaconazole, 0.008–8; voriconazole, 0.008–8; itraconazole, 0.015–16; fluconazole, 0.012–256; anidulafungin, 0.015–8; and amphotericin B, 0.12–8. The detailed instructions for the test, as well as interpretation of obtained results, have been presented by us previously (Szweda *et al.*, 2014).

Gene expression analysis. The yeast isolates were grown on Sabouraud agar plates for 18–20 h at 30 °C. A small amount of biomass from single colonies of each tested strain was suspended in a volume of 4 ml Sabouraud broth and incubated with continuous shaking for about 5 h at 30 °C to achieve an OD_{660} of 0.6. The yeast cells were then harvested by centrifugation (4200 *g* for 5 min) and mRNA was isolated using a Total RNA Mini Plus Concentrator kit (A&A Biotechnology) with the acid phenol method according to the manufacturer's protocol. The isolated RNA was purified with DNase. The reaction mixture was composed of 10 μl sterile RNase-free water, 7 μl isolated RNA solution, 2 μl 10 × Reaction Buffer, 1 μl DNase (10 U μl^{-1}) (all from A&A Biotechnology) and was incubated at 30 °C for 30 min, and again purified with a Total RNA Mini Plus Concentrator kit. The concentration was determined using a NanoDrop1000 (Thermo Scientific), which showed that double purification resulted in a reduction in efficiency but was necessary to obtain a totally pure mRNA solution that was not contaminated with DNA. Immediately after obtaining the pure mRNA, a reverse transcription reaction with a TranScriba kit (A&A Biotechnology) was carried out. The reaction was done in two steps. In the first, 140 ng isolated RNA was incubated with 1 of oligo(dT)$_{18}$ solution (100 μM) and RNase-free water to a volume of 10 μl at 65 °C for 5 min. The following reagents were then added: 4 μl 5 × Reaction Buffer, 2 μl dNTP Mix solution (2.5 mM each) and 4 μl TranScriba Reverse Transcriptase (20 U μl^{-1}), and the final mixture was incubated at 41 °C for 60 min. The reaction was terminated by heating the sample at 70 °C for 5 min. Quantitative analysis of expression of the *ERG11*, *CDR1*, *CDR2* and reference *URA3* genes was performed by real-time PCR with a LightCycler Nano PCR Real-Time System (Roche). Primers and probes were as designed by Sanguinetti *et al.* (2005) and were synthesized by Sigma (Table 1). Real-time PCR was performed with a 20 μl volume containing the following reagents: 10 μl RealTime 2 × HS-PCR Master Mix Probe (*Taq* DNA polymerase 0.1 U μl^{-1}, 2 × Reaction Buffer, 10 mM $MgCl_2$ and 0.5 mM each dNTP (A&A

Table 1. Primers and fluorescent probes used for real-time PCR (Sanguinetti *et al.*, 2005)

6-FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine.

Gene (GenBank accession no.)	Primer or probe	Sequence(5′→3′)	Location within gene (nt)
CDR1 (AF109723)	CDR1a	TAGCACATCAACTACACGAACGT	4500–4522
	CDR1b	AGAGTGAACATTAAGGATGCCATG	4647–4670
	CDR1pr	6FAM-TGCTGCTGCTTCTGCCACCTGGTT-TAMRA	4621–4644
CDR2 (AF251023)	CDR2a	GTGCTTTATGAAGGCTACCAGATT	164–187
	CDR2b	TCTTAGGACAGAAGTAACCCATCT	251–274
	CDR2pr	6FAM-TACCTTTGCGTGCTGGGCGTCACC-TAMRA	217–240
ERG11 (L40389)	ERGa	ATTGGTGTCTTGATGGGTGGTC	928–949
	ERGb	TCTTCTTGGACATCTGGTCTTTCA	1019–1042
	ERGpr	6FAM-ACTTCCGCTGCTACCTCCGCTTGG-TAMRA	955–978
URA3 (L13661)	URAa	GAAAACCAATCTTTGTGCTTCTCT	168–191
	URAb	CATGAGTCTTAAGCAAGCAAATGT	268–291
	URApr	6FAM-ACGTCACCACCACCAGCGAATTGT-TAMRA	194–217

Biotechnology), 1 µl each primer solution (10 µM), 0.5 µl probe solution (10 µM), total cDNA sample (1 µl) and distilled water to a final volume of 20 µl. Standard curves for evaluation of real-time PCR efficiency were prepared in triplicate. Twofold serial dilutions of a mixture of the cDNA of all strains tested were used as a template for standard curves in the case of all tested genes. Amplification conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s and 15 s of primer annealing (at 55 °C for *URA3*, 59 °C for *ERG11* and *CDR1*, and 62 °C for *CDR2*), with a final elongation step at 72 °C for 15 s. The temperature transition rate was set at 20 °C s^{-1}. Genes for gene expression analysis were amplified under the same conditions as for the standard curves, with 7 ng cDNA as template. The experiments were repeated in duplicate for each specimen.

Acquisition of fluorescence signals from up to 32 samples was carried out at the end of every annealing/extension step for 100 ms.

Quantitative analysis of the level of expression of the investigated genes was carried out using the $2^{-\Delta\Delta C_T}$ method, also known as Livak's method (Livak & Schmittgen, 2001). This method allows determination of relative differences in the expression levels of analysed target genes between the investigated strains and reference strain, also called the calibrator. For our research, the Gd6 strain from the Medical University of Gdansk, isolated from throat swab, was selected as the calibrator. The *C. glabrata* Gd6 strain is recognized as susceptible-dose dependent (S-DD) with a relatively low MIC value for fluconazole of 8 µg ml^{-1} and is sensitive to other tested azoles: posaconazole, voriconazole and itraconazole. The level of expression of target genes in a particular strain was determined based on comparison of the threshold cycle (C_T) values of amplification of the gene of interest and the reference gene (internal control). As a reference gene for this research, the *URA3* gene, one of the housekeeping genes of *C. glabrata*, which is expressed in cells in a constitutive manner, was used.

Livak's equation value of parameter $R=1$ indicates that the level of target gene expression in the investigated sample (strain) and calibrator are the same. A value >1 indicates a higher level of expression of the tested gene in the cells of the investigated strain in comparison with the cells of the calibrator, whereas a significant increase in the level of the gene expression is considered to have occurred when the value of the parameter R is >2.

According to CLSI (2012) guidelines, all strains tested by real-time PCR ($n=71$) could be divided into two groups: resistant to fluconazole (MIC $\geq$64 µg ml^{-1}; $n=15$) and S-DD (MIC $\leq$32 µg ml^{-1}; $n=56$) based on the Sensititre Yeast One test. A real-time PCR was carried out in duplicate for all samples so there were also two R values for each sample. Using the GraphPad Prism 5 program, a statistical analysis was performed and the non-parametric Mann–Whitney U test with a 0.05 level of significance was chosen for comparison of two populations of the R values. A value of $P<0.05$ indicated that the compared populations were significantly different.

***ERG11* gene sequence analysis.** The *ERG11* gene was amplified using the set of primers: forward 5′-ATGTCCACTGAAAACACTTC-3′ and reverse 5′-CTAGTACTTTTGTTCTGGATGTC-3′, designed on the basis of the sequence encoding the *ERG11* gene of *C. glabrata* CBS 138 (GenBank accession no. XM_445876). PCR was performed in a 50 µl volume containing the following reagents: 25 µl 2× PCR Mix Plus High GC (A&A Biotechnology), 17 µl nuclease-free water, 2.5 µl each primer solution (10 µM) and 3 µl isolated DNA solution. Amplification conditions were as follows: 4 min of initial denaturation at 94 °C, followed by 32 cycles of 60 s of denaturation at 94 °C, 30 s of annealing at 57 °C and 90 s of primer extension at 72 °C, with 10 min of final extension at 72 °C. The PCR products (1602 bp) were detected on 1.5 % agarose gel stained with ethidium bromide. The products of amplification were then purified according to the A&A Biotechnology protocol enclosed with the Clean up AX kit. Sequencing was carried out by Macrogen (The Netherlands). Analysis of sequences was performed using MEGA 6.1 software, and statistical analysis was carried out with the GraphPad Prism 5.0 program.

RESULTS

Species identification and analysis of antifungal resistance

The results of our tests performed by the three methods – yeast growth on a Chromagar *Candida* Lab-agar plate, an API Candida test and PCR – confirmed that the studied population of 81 clinical isolates consisted exclusively of *C. glabrata* strains. Analysis of the antifungal resistance of 65 of the isolates has been presented in our previous work (Szweda *et al.*, 2014), and here we expanded the examined group by adding 16 additional isolates. The strains were categorized as follows: 18 were resistant (MIC $\geq$64 µg ml^{-1}) and 63 were S-DD to fluconazole (MIC $\leq$32 µg ml^{-1}). However, for almost all strains tested, except one

Table 2. Azole susceptibility of 81 *C. glabrata* isolates received from three Polish hospitals

FZ, fluconazole; VOR, voriconazole; PZ, posaconazole; IZ, itraconazole.

Origin	Isolate designation	MIC (μg ml^{-1}) and susceptibility category							
		FZ		PZ		VOR		IZ	
Gdansk	6†	8	S-DD	0.5	WT	0.25	WT	0.5	WT
	9*†	16	S-DD	2	WT	0.25	WT	0.5	WT
	19†	16	S-DD	2	WT	0.5	WT	0.5	WT
	40†	16	S-DD	2	WT	0.25	WT	0.5	WT
	74†	8	S-DD	1	WT	0.25	WT	0.5	WT
	75*†	8	S-DD	1	WT	0.25	WT	0.5	WT
	82*†	16	S-DD	1	WT	1	Non-WT	1	WT
	99†	32	S-DD	2	WT	1	Non-WT	1	WT
	116†	32	S-DD	2	WT	0.5	WT	1	WT
	122†	32	S-DD	2	WT	0.5	WT	1	WT
	127†	16	S-DD	2	WT	0.5	WT	1	WT
	133†	32	S-DD	2	WT	0.5	WT	1	WT
	139*†	64	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	140†	8	S-DD	1	WT	0.25	WT	0.5	WT
	141†	16	S-DD	2	WT	0.5	WT	1	WT
	143†	16	S-DD	2	WT	0.5	WT	2	WT
	144*†	32	S-DD	2	WT	0.25	WT	1	WT
	151*†	32	S-DD	2	WT	0.5	WT	1	WT
	154†	16	S-DD	2	WT	0.5	WT	1	WT
	165†	32	S-DD	2	WT	0.5	WT	0.5	WT
	176†	16	S-DD	1	WT	0.25	WT	0.5	WT
	182*†	16	S-DD	2	WT	0.5	WT	1	WT
	183†	16	S-DD	1	WT	0.5	WT	1	WT
	184†	32	S-DD	2	WT	1	Non-WT	1	WT
	186*	16	S-DD	1	WT	0.25	WT	0.5	WT
	198*†	32	S-DD	2	WT	1	Non-WT	1	WT
	200*†	128	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	205†	8	S-DD	0.5	WT	0.12	WT	0.5	WT
	208*†	32	S-DD	2	WT	0.5	WT	1	WT
	209†	16	S-DD	1	WT	0.25	WT	0.5	WT
	211*†	128	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	215†	32	S-DD	2	WT	1	Non-WT	2	WT
	226*†	8	S-DD	1	WT	0.5	WT	1	WT
	227*†	32	S-DD	2	WT	0.25	WT	1	WT
	231†	8	S-DD	1	WT	0.25	WT	0.5	WT
	238†	16	S-DD	2	WT	0.25	WT	0.5	WT
	240†	16	S-DD	1	WT	0.25	WT	0.5	WT
	244†	16	S-DD	1	WT	0.25	WT	0.5	WT
	246*†	16	S-DD	2	WT	0.5	WT	1	WT
	247†	16	S-DD	1	WT	0.25	WT	0.5	WT
	260	4	S-DD	0.25	WT	0.06	WT	0.12	WT
	261†	16	S-DD	2	WT	0.5	WT	1	WT
	263*†	256	R	8	Non-WT	4	Non-WT	16	Non-WT
	268†	32	S-DD	0.5	WT	0.06	WT	0.25	WT
	273†	16	S-DD	1	WT	0.25	WT	1	WT
	274*†	32	S-DD	2	WT	0.25	WT	1	WT
	276*†	>256	R	>8	Non-WT	>8	Non-WT	>16	Non-WT
	277*	64	R	0.5	WT	0.25	WT	0.25	WT
	286*†	64	R	2	WT	1	Non-WT	1	WT
	290†	32	S-DD	2	WT	0.5	WT	1	WT
	310*†	>256	R	>8	Non-WT	8	Non-WT	>16	Non-WT
	313	32	S-DD	0.25	WT	0.12	WT	0.25	WT
	315†	16	S-DD	1	WT	0.25	WT	1	WT

Table 2. cont.

Origin	Isolate designation	MIC (μg ml^{-1}) and susceptibility category							
		FZ		PZ		VOR		IZ	
	316	8	S-DD	1	WT	0.25	WT	1	WT
Warsaw	1*†	16	S-DD	1	WT	0.25	WT	0.5	WT
	6*†	32	S-DD	2	WT	2	Non-WT	4	WT
	31*†	16	S-DD	1	WT	0.5	WT	0.5	WT
	38*†	128	R	1	WT	0.5	WT	0.5	WT
	61*†	8	S-DD	0.5	WT	0.12	WT	0.25	WT
	88*†	256	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	105*†	256	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	118	16	S-DD	1	WT	0.25	WT	1	WT
	137*†	16	S-DD	2	WT	0.5	WT	1	WT
	138*†	>256	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	209*†	256	R	>8	Non-WT	4	Non-WT	>16	Non-WT
	259†	32	S-DD	2	WT	0.5	WT	1	WT
	342	8	S-DD	1	WT	0.25	WT	0.5	WT
	373*†	256	R	>8	Non-WT	8	Non-WT	>16	Non-WT
	377*	256	R	>8	Non-WT	8	Non-WT	>16	Non-WT
	465*†	128	R	>8	Non-WT	2	Non-WT	>16	Non-WT
	513*	256	R	>8	Non-WT	4	Non-WT	>16	Non-WT
Szczecin	368	32	S-DD	2	WT	0.5	WT	1	WT
	385*†	>256	R	8	Non-WT	>8	Non-WT	>16	Non-WT
	402*†	16	S-DD	2	WT	0.5	WT	1	WT
	406†	8	S-DD	1	WT	0.25	WT	0.5	WT
	412*†	16	S-DD	1	WT	0.25	WT	0.5	WT
	413*†	32	S-DD	2	WT	0.5	WT	1	WT
	414*†	32	S-DD	2	WT	0.5	WT	1	WT
	435*†	8	S-DD	2	WT	0.25	WT	1	WT
	440*†	16	S-DD	2	WT	0.25	WT	1	WT
	444	16	S-DD	2	WT	0.5	WT	0.5	WT

*Sequencing of the *ERG11* gene was performed in these strains.
†Analysis of expression levels of the *CDR1*, *CDR2* and *ERG11* genes was carried out in these strains.

(MIC=4 μg ml^{-1}), the MIC values for this agent were relatively high (≥8 μg ml^{-1}). Fifteen of the fluconazole-resistant strains also revealed cross-resistance (classified as non-WT) against the other azoles tested: itraconazole, posaconazole and voriconazole (the MIC breakpoint values for these agents were 4, 2 and 0.5 μg ml^{-1}, respectively). A further seven isolates were classified as non-WT following analysis of susceptibility to voriconazole. One of these strains was cross-resistant to fluconazole, and thus two isolates were resistant only to fluconazole and six only to voriconazole (Table 2). Moreover, we examined the susceptibility to three echinocandins, anidulafungin, micafungin and caspofungin, as well as to two other compounds, 5-fluorocytosine and amphotericin B. All strains tested were found to be susceptible to echinocandins and to amphotericin B (the MIC values of all strains for amphotericin B were <1 μg ml^{-1}; the susceptibility MIC breakpoint value for classification of strains as non-WT is 2 μg ml^{-1}), and nearly all, except for five strains, were classified as WT in terms of susceptibility to 5-fluorocytosine (MIC breakpoint 0.25 μg ml^{-1}). Three of the five 5-fluorocytosine-resistant strains were able to grow at 5-fluorocytosine concentration as high as 64 μg ml^{-1}, which is rarely observed for *C. glabrata* clinical isolates. All the values of MIC breakpoints presented above are in agreement with the CLSI (2012) guidelines. The number of susceptible, S-DD and resistant strains, or WT or non-WT, to all chemotherapeutics tested are presented in Table 3.

ERG11 gene sequence analysis

Of the population of 81 isolates of *C. glabrata*, 41 were selected for sequencing of the *ERG11* gene. This group consisted of all strains that exhibited resistance to fluconazole (n=18), three strains resistant only to voriconazole and 20 isolates that were not resistant to any of the azoles tested. Analysis of the obtained nucleotide sequences revealed the existence of several cases of silent mutations (not resulting in changes in amino acid sequence of the gene product) within the *ERG11* gene. The strains carrying the

Table 3. Number of *C. glabrata* isolates from Gdansk, Szczecin and Warsaw classified as susceptible, S-DD and resistant, or as WT or non-WT

AND, anidulafungin; MF, micafungin; CAS, caspofungin; FC, 5-fluorocytosine; AB, amphotericin B; FZ, fluconazole; PZ, posaconazole; VOR, voriconazole; IZ, itraconazole.

		AND	MF	CAS	FC	AB	FZ		PZ	VOR	IZ
Gdansk	Susceptible	54	53	51	51	54		WT	48	42	48
	S-DD	0	1	3	2	0	46				
	Resistant	0	0	0	1	0	8	Non-WT	6	12	6
Szczecin	Susceptible	10	10	9	8	10			9	9	9
	S-DD	0	0	1	0	0	9	WT			
	Resistant	0	0	0	2	0	1	Non-WT	1	1	1
Warsaw	Susceptible	17	17	12	17	17			9	8	9
	S-DD	0	0	5	0	0	8	WT			
	Resistant	0	0	0	0	0	9	Non-WT	8	9	8
Total	Susceptible	81	80	72	76	81	0		66	59	66
	S-DD	0	1	9	2	0	63	WT			
	Resistant	0	0	0	3	0	18	Non-WT	15	22	15

silent mutations in the *ERG11* gene were present in groups, classified as resistant and S-DD (Table S1, available in the online Supplementary Material). However, none of these mutations resulted in changes in the amino acid sequence of the expressed enzyme (lanosterol 14-α demethylase). This indicated that mechanisms other than substitutions of amino acid residues within the lanosterol 14-α demethylase sequences were responsible for the observed lack of susceptibility to azoles.

Gene expression analysis

Mechanisms of azole resistance among non-*albicans candida* species are not as well recognized as those for *C. albicans*, the most common fungal pathogen. Besides mutations within the *ERG11* gene sequence, the most important factors causing a low level of activity of azoles against *C. albicans* are overexpression of genes encoding drug transporters, especially *CDR1*, and upregulation of genes encoding enzymes involved in biosynthesis of ergosterol, especially lanosterol 14-α demethylase, encoded by the *ERG11* gene. As has been presented above, our research on 41 clinical isolates of *C. glabrata* revealed the presence of only silent mutations within the *ERG11* gene sequences. In order to determine the reason for the observed high level of azole resistance among the strains tested, we also analysed the level of expression of the supposed resistance-associated genes *CDR1*, *CDR2* and *ERG11* in 71 isolates (including 14 strains classified as resistant to fluconazole and other azoles).

For the *CDR1* gene, we found that, in 13 out of 15 (86.6 %) resistant isolates, a significant increase in the level of expression was observed (Fig. 1). The level of expression increased from 2.07 to 1318.25 (*R* value) in comparison with the level of expression of this gene in the reference isolate Gd6. In the group of 56 isolates classified as S-DD to fluconazole, 16 (28.6 %) isolates showed a higher level of relative expression, which ranged from 2.27 to 36.13. Statistical analysis revealed the *P* value of <0.0001. The mean significant difference in the level of the *CDR1* gene expression in the group of resistant and non-resistant strains is shown in Fig. 3.

For the *CDR2* gene, overexpression was seen for 13 (22.8 %) of the non-resistant isolates and only five (35.7 %) isolates classified as resistant (Fig. 2). The values of the *R* parameter ranged from 0.06 to 18.81 (in general, and from 2.02 to 18.81 when only overexpression was concerned) in non-resistant strains and from 0.29 to 6.36 in resistant strains. It was also observed that the *R* parameter values were slightly higher in the group of resistant strains. When comparing this parameter with that of the *CDR1* gene, the expression levels as well as differences in the expression between both groups of isolates for the *CDR2* were much lower. The *P* value of the Mann–Whitney *U* test was 0.0681, so the two groups werere not significantly different (Fig. 3), which means that the level of expression of *CDR2* does not affect the low susceptibility of resistant strains to fluconazole and other tested azoles.

Unexpected results were obtained in the case of analysis of the *ERG11* gene expression. Thirty-three (58 %) of the 57 fluconazole non-resistant isolates expressed *ERG11* at higher levels than the susceptible control, isolate Gd6, displaying levels of *ERG11* transcript that increased from 2.00 to 22.62 (*R* value) compared with those detected in Gd6. In contrast, in the group of resistant isolates, such high-level upregulation of the *ERG11* gene was not observed, and the *R* values ranged from 0.64 to 3.28 compared with the reference. An *R* value of >2 was observed for the five strains not susceptible to fluconazole (Fig. S1). The statistical analysis did not reveal significant differences in the expression level of this gene between resistant and

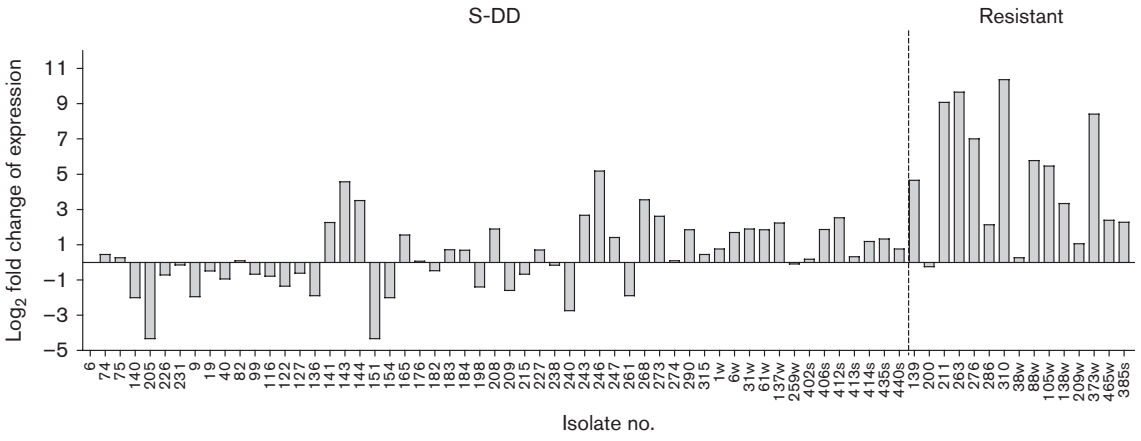

Fig. 1. Expression level of the *CDR1* gene in 71 isolates (15 classified as resistant and 56 classified as S-DD) in comparison with the reference strain Gd6 (recognized as S-DD with a relatively low MIC value for fluconazole of 8 μg ml^{-1}).

S-DD isolates (Fig. 3). This finding may be interpreted as evidence that moderate overexpression of *ERG11* does not contribute to the azole resistance of *C. glabrata*.

DISCUSSION

C. glabrata is often isolated as a constituent of the natural flora of healthy individuals. For many years, it was not considered an important aetiological agent of serious infections in humans. However, during the last two to three decades, the frequency of mucosal and systemic infections caused by *C. glabrata* has increased significantly. There is no doubt that this is a consequence of the widespread and increased use of immunosuppressive therapies together with broad-spectrum antibiotic treatment (Dzierżanowska-Fangrat *et al.*, 2014; Hajjeh *et al.*, 2004; Rodrigues *et al.*, 2014).

C. glabrata is a haploid yeast, generally with an intrinsic low susceptibility to azole derivatives, although it can acquire resistance to azoles on exposure to these antifungals more frequently and rapidly than the diploid yeast *C. albicans*.

Low susceptibility to azoles was also observed in the case of the group of 81 strains investigated in this study. About 20 % were cross-resistant to all tested azoles and, even in the group of strains recognized as non-resistant (S-DD in the case of fluconazole and WT in the case of the other azoles), the MIC values were relatively high (e.g. $\geq$8 μg ml^{-1} for fluconazole, except for one strain). These observations are in agreement with the results of most of investigations concerning azole resistance of clinical isolates of *C. glabrata* collected in different geographical regions (Pfaller *et al.*, 2010). The basic phenotypic

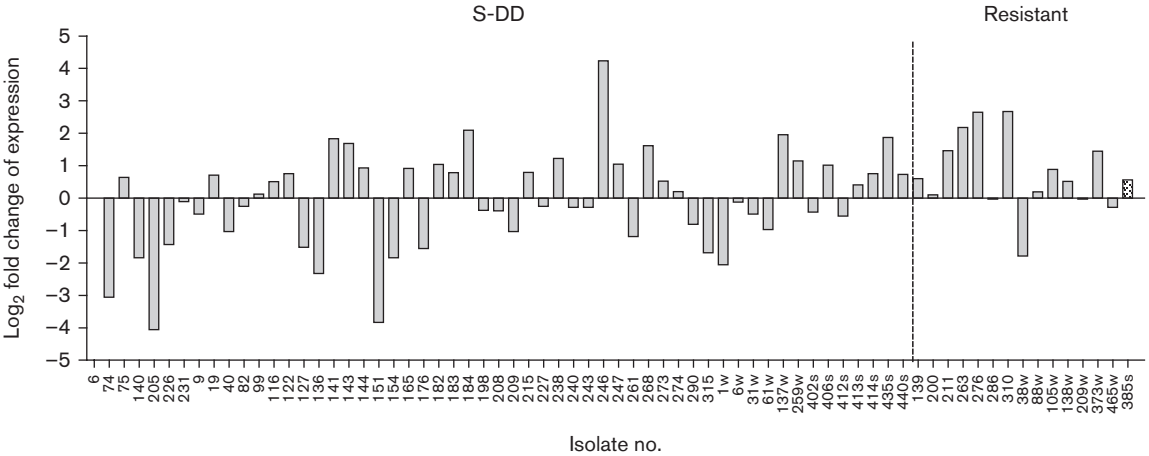

Fig. 2. Expression level of *CDR2* gene in 71 isolates (15 classified as resistant and 56 classified as S-DD) in comparison with the reference strain Gd6 (recognized as S-DD with a relatively low MIC value for fluconazole of 8 μg ml^{-1}).

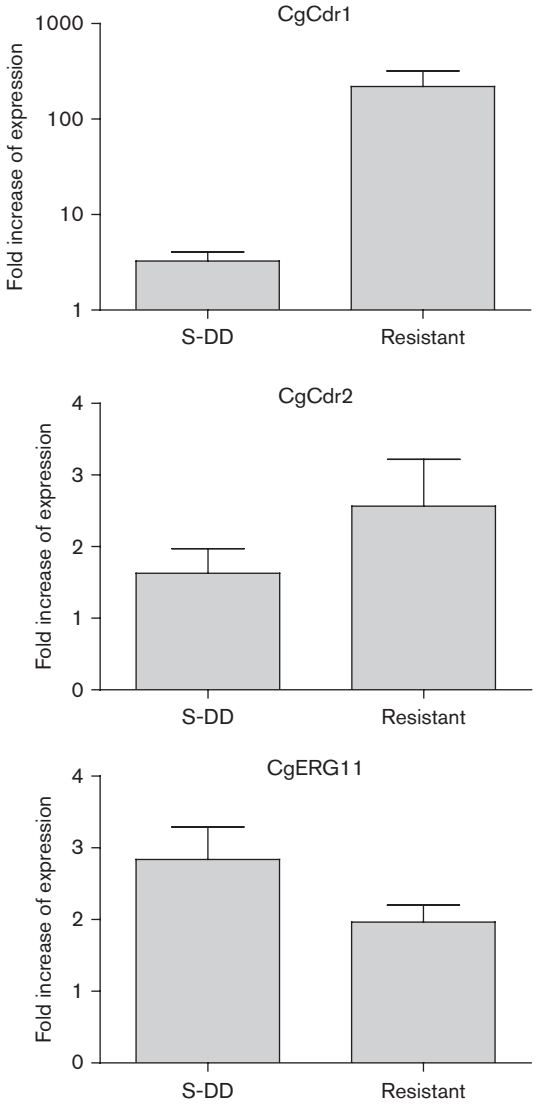

Fig. 3. Statistical analysis of the fold increase of expression for the *CDR1*, *CDR2* and *ERG11* genes in both groups: resistant isolates (n=15) and S-DD isolates (n=56). Results are shown as means ± SD.

properties of the azole-resistant strains, such as morphology and colour of the colonies grown on agar, and growth kinetics in liquid medium, were not different from those of the azole-susceptible strains. High susceptibility to other groups of antifungal agents – echinocandins (Pfaller *et al.*, 2013; Santhanam *et al.*, 2013), amphotericin B (Prażyńska & Gospodarek 2014; Santhanam *et al.*, 2013) and 5-fluorocytosine (Edlind & Katiyar 2010; Pfaller *et al.*, 2002; Santhanam *et al.*, 2013) – is also in agreement with the generally observed trends. However, three of the strains were characterized as extremely highly resistant to 5-fluorocytosine (MIC $\geq$64 μg ml^{-1}). The mechanisms for the lack of susceptibility to this agent developed by these isolates will be the subject of further investigations.

Mainly as a result of the widespread use of fluconazole in the treatment and prophylaxis of fungal infections, *Candida* spp. have evolved a number of mechanisms for resistance to azoles. As far as *C. albicans* is concerned, several mechanisms have been revealed. A very common mechanism is mutation in the *ERG11* gene. Data in the literature suggest that there may be more than 140 different amino acid substitutions within the sequence of the protein encoded by this gene. This high genetic polymorphism suggests that lanosterol 14-α demethylase is highly permissive to structural changes. Most of these substitutions are clustered into three hot-spot regions with aa 105–165, 266–287 and 405–488 (Morio *et al.*, 2010). In the population of *C. glabrata* studied here, no mutation in the *ERG11* gene resulting in alteration of the lanosterol 14-α demethylase amino acid sequence was found. This finding is in agreement with the results obtained by Sanguinetti *et al.* (2005), who did not identify any mutations in the *ERG11* gene among 29 clinical isolates of *C. glabrata* from the University Hospital in Rome, Italy. Actually, the only case of amino acid substitution (G315D) within the sequence of *C. glabrata* lanosterol 14-α demethylase contributing to azole resistance, which additionally resulted in cross-resistance to amphotericin B, was described by Hull *et al.* (2012). Additionally, single-nucleotide substitutions in the *ERG11* gene of *C. glabrata* clinical isolates from Slovakia have been identified, although most were classified as silent mutations, and the only amino acid substitution, E502V, found in some fluconazole-resistant isolates apparently did not contribute to their azole resistance (Berila & Subik, 2010; Berila *et al.*, 2009). In our opinion, our obtained results as well as analysis of the literature clearly indicate that mutation of the *ERG11* gene should not be considered as an important or common mechanism of resistance of *C. glabrata* to azole antifungal agents.

It is generally well documented that the most important factor affecting *C. glabrata* resistance to azole antifungal agents is upregulation of expression of the *CDR1* gene (Sanglard *et al.*, 1999, 2001; Sanguinetti *et al.*, 2005). This was also confirmed in our investigation. Thirteen out of 15 resistant strains revealed a significant increase in the level of expression of this gene, exceeding 1000-fold in the case of some isolates. This level of *CDR1* overexpression was even higher than the highest found so far (260-fold) by Sanguinetti *et al.* (2005). However, these authors also found that the azole resistance of the tested strains was importantly affected by upregulation of the *CDR2* gene. Eight of the 16 fluconazole-resistant isolates expressed this gene at higher levels than the susceptible control isolate DSY562. Our investigation did not confirm the influence of overexpression of the *CDR2* gene in the resistance of strains tested for azole antifungal agents. We also did not find any correlation between azole resistance and the level of expression of the *ERG11* gene. Although overexpression of the *ERG11* gene was found for some of the isolates, statistical analysis revealed that the mean level of upregulation in the group of resistant strains was not higher than in the

group of susceptible ones. In fact, statistically a slightly higher level of transcription of this gene was observed for non-resistant strains classified S-DD, thus confirming the previous observation of Sanguinetti *et al.* (2005), who demonstrated that there was no difference in transcription level between the population of resistant and susceptible clinical isolates from Italy. Upregulation of the *ERG11* gene was also investigated previously by Marichal *et al.* (1997), who noted an eightfold increase in *ERG11* mRNA levels in an azole-resistant clinical *C. glabrata* isolate due to amplification of the *ERG11* gene, which in turn resulted from chromosomal duplication. To date, there have been no more cases that could confirm the dependence of resistance of *C. glabrata* to azoles and upregulation of the *ERG11* gene.

In conclusion, the results obtained in the present study emphasize the role of upregulation of the *CDR1* gene encoding the ABC efflux transporter as the major mechanism of azole resistance in *C. glabrata*. From this point of view, *C. glabrata* is similar to *C. albicans*. In contrast, it is totally different as far as the *CDR2* and especially the *ERG11* genes are concerned. Neither point mutations nor upregulation at a transcriptional level of *ERG11* were observed. We also did not observe statistically important differences in the level of *CDR2* gene expression between resistant and susceptible isolates. This may suggest that drug resistance mechanisms can be very different, even within a genus of yeasts, and more studies are needed. In most publications concerning azole resistance among *Candida* spp., the authors mention *ERG11* gene mutations and overexpression of this gene, as well as upregulation of genes encoding the efflux pumps (*CDR1* and *CDR2*) as major causes of resistance. In our opinion, there is still not enough evidence, not only in this investigation but also in the literature, to confirm that point mutations of the *ERG11* gene as well as upregulation of the *ERG11* and *CDR2* genes can be considered important or common mechanisms of resistance among *C. glabrata*.

The molecular basis of azole resistance in two out of 15 of our fluconazole-resistant isolates not overexpressing *CDR1* remains unknown. This resistance might have resulted from overexpression of *SNQ2* encoding another ABC-type multidrug-transporter protein, overexpression of *TPO3* encoding the MFS-type drug efflux pump (Costa *et al.*, 2014) or mutations in *PDR1* encoding the transcriptional activator protein, responsible for controlling the level of expression of genes encoding drug efflux transporters including *CDR1* (Paul *et al.*, 2014) or any other mechanism. It should be noted that the slight overexpression of *SNQ2* found by Sanguinetti *et al.* (2005) in their *C. glabrata* azole-resistant clinical isolates not overexpressing *CDR1* or *CDR2* was not directly correlated with the high level of azole resistance, and no evidence for an important contribution of *PDR1* or *TPO3* upregulation in the azole resistance of *C. glabrata* clinical isolates has been reported so far. A detailed explanation of the mechanisms governing the overexpression of *CDR1*, including sequencing of *PDR1*, in the cells of the investigated resistant strains will be the subject of our future research.

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