ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 2/2015
247–251
http://dx.doi.org/10.18388/abp.2014_940

Regular paper

Selected mechanisms of molecular resistance of *Candida albicans* to azole drugs

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A phenomenon of increasing resistance of *Candida spp.* to azoles has been observed for several years now. One of the mechanisms of lack of sensitivity to azoles is associated with *CDR1, CDR2, MRD1* genes (their products are active transport pumps conditioning drug efflux from pathogen's cell), and *ERG11* gene (encoding lanosterol 14α-demethylase). Test material was 120 strains of *Candida albicans* (60 resistant and 60 susceptible to azole drugs) obtained from clinical samples. The first stage of experiment assessed the expression of *CDR1, CDR2, MDR1* and *ERG11* genes by Q-PCR. The impact of *ERG11* gene's mutations on the expression of this gene was analysed. The final stage of the experiment assessed the level of genome methylation of *Candida albicans* strains. An increase in the expression of *CDR2, MDR1* and *ERG11* was observed in azole-resistant strains of *Candida albicans* in comparison to strains sensitive to this class of drugs. Furthermore, 19 changes in the sequence of *ERG11* were detected in tested strains. Four of the discovered mutations: T495A, A530C, G622A and A945C led to the following amino acid substitutions: D116E, K128T, V159I and E266D, respectively. It has also been found that statistically five mutations: T462C, G1309A, C216T, C1257T and A945C affected the expression of *ERG11*. The applied method of assessing the level of methylation of *Candida albicans* genome did not confirm its role in the development of resistance to azoles. The results indicate however, that resistance of *Candida albicans* strains to azole drugs is multifactorial.

Key words: *Candida albicans*, drug resistance, azoles

Received: 19 November, 2014; revised: 23 March, 2015; accepted: 24 March, 2015; available on-line: 21 April, 2015

INTRODUCTION

Candida spp. are eukaryotic representatives of the fungi kingdom (Lim *et al*., 2012), which groups about 150–200 species (Neppelenbroek *et al*., 2014). These are mainly opportunistic microorganisms belonging to saprophytic flora inhabiting the gastrointestinal tract, urogenital system, skin, and mucous membranes of the airways (Lim *et al*., 2012).

Currently, the following 15 species of pathogenic *Candida* has been distinguished: *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida guilliermondii, Candida lusitaniae*, *Candida dubliniensis*, *Candida pelliculosa*, *Candida kefyr*, *Candida lipolytica, Candida famata*, *Candida inconspicua, Candida rugosa* and *Candida norvegensis* (Yapar, 2014).

Candida albicans is the most thoroughly investigated species and at the same time, the most frequent infection agent among fungi of the *Candida* genus (Ferreira *et al*., 2013; Vázquez-González *et al*., 2013).

Pathogenic fungi have developed a number of resistance mechanisms enabling their survival in the presence of toxic substances. According to the literature the following molecular mechanisms of drug resistance can be distinguished: modification of the drug target affinity, overproduction of enzymes that are targets to drugs, development of alternative metabolic pathways, active efflux of drugs from the cell, impermeability of the cell membrane to drug molecules, activity of enzymes inactivating the drug or degrading it outside the fungal cell (White *et al*., 1998; Mishra *et al*., 2007; Morschhäuser, 2010).

One of the mechanisms of resistance to azole drugs may result from over-expression of *CDR1*, *CDR2* and *MDR1* genes, whose products are active transport pumps conditioning drug efflux from the pathogen's cell (Sanglard, 2002).

These proteins are split into two groups on the basis of their structure and type of energy they use for the transportation of various molecules. The first group includes primary transporters using energy from degradation of ATP, which are proteins belonging to the family of ABC (ATP-binding cassette). The second group consists of secondary transporters drawing energy from the proton concentration gradient typical for biological membranes. This category is represented inter alia by MFS proteins (Major Facilitator Superfamily). In the case of yeast, the products of *CDR1* and *CDR2* are ABC proteins and products of *MDR1* are MFS-type transporters (de Waard *et al*., 2006; Del Sorbo *et al*., 2000; Piddock, 2006; Decottignies & Goffeau, 1997).

An example of phenomenon leading to modification of the drug target may be the occurrence of point mutations in genes encoding products, usually enzymes, to

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Abbreviations: 5-mc, 5-methylocytosine; ABC transporters, ATP-binding cassette transporters; cDNA, complementary DNA; CI, confidence interval; C_T, threshold cycles; FAM, 6-carboxyfluorescein; MIC, Minimal Inhibitory Concentration; MFS proteins, major facilitator superfamily proteins; Q-PCR, quantitative polymerase chain reaction; RQ, relative expression

Table 1. Criteria for division of *Candida albicans* strains into sensitive and resistant to azole drugs

Azole drugs	MIC [mg/ml]	
	C. albicans sensitive	*C. albicans* resistant
Fluconazole	≤ 8	≥ 64
Itraconazole	≤ 0.125	≥ 1
Voriconazole	≤ 1	≥ 4

be targeted by drugs (e.g. lanosterol 14-α-demethylase encoded by the *ERG11* gene), which are essential for the functioning of the microorganism's cell. These mutations can also lead to overexpression of such genes and, subsequently, the overproduction of the enzyme targeted by the drug (Morio *et al*., 2013). The regulation of gene expression may also occur by DNA methylation, which in the case of fungal cells has not yet been sufficiently investigated. Previous studies have shown that the level of DNA methylation varies depending on the species of fungus (e.g. in the case of *Neurospora* it is 1.5%; *Agaricus bisporus* ~ 4%; *Ustilago maydis* 2.3% and *Armillaria bulbosa* 4.3%). It is estimated that the level of DNA methylation of *Candida albicans* is only 0.5%. It is believed that DNA methylation plays in fungi a major role in silencing of repeated sequences (Mishra *et al*., 2011).

The aim of the study was to investigate the molecular mechanisms of resistance of *Candida albicans* strains to azole drugs through the study of *CDR1*, *CDR2*, *MDR1* and *ERG11* gene expression in *Candida albicans* strains sensitive and resistant to azole drugs; the effect of selected *ERG11* mutations on its expression; and to determine the level of DNA methylation of *Candida albicans* genome.

MATERIAL AND METHODS

Test material. The study was conducted on 120 strains of *Candida albicans* (60 sensitive and 60 resistant to azoles) obtained from a group of 658 strains from clinical specimens (sputum, pus, urine, blood, bronchial aspirates) derived from patients hospitalized in John Paul II Podhalański Specialist Hospital in Nowy Targ, Poland.

Isolation of *Candida albicans* strains. The isolation of *Candida albicans* was performed by culturing the clinical material on Sabouraud medium (Sabouraud's Agar with 4% glucose addition, BTL LLP, Poland) and on the differentiating medium Candida ID2 bioMerieux (France). ID 32C tests (bioMerieux, France) were used to confirm the identification.

Test of *Candida albicans* strains susceptibility to azole drugs. The test was performed by serial dilution method (MIC=Minimum Inhibitory Concentration) using ATB fungus2 system (bioMerieux, France) as recommended by CLSI and EUCAST. The criteria for the division of *Candida albicans* strains into sensitive and resistant to azole drugs were presented in Table 1.

Homogenization and RNA isolation. Strains of *Candida albicans* were homogenized by FastPrep®-24 homogenizer (MP Biomedicals, USA) using tubes with Lysing Matrix C silica beads (MP Biomedicals, USA) in the homogenization buffer (included in the RNeasy® Mini Kit). Subsequently, RNA was isolated using RNeasy® Mini Kit (Qiagen, Germany) according to the attached protocol. Additionally, digestion of samples was carried out by DNase I (included in the RNase Free DNase I Set; Qiagen, Germany).

Qualitative and quantitative assessment of isolated RNA. Quantification and quality assessment of RNA was performed using Agilent RNA 6000 Nano Kit (Agilent Technologies, USA), electrophoretic chip and Agilent 2100 Bioanalyzer (Agilent Technologies, USA) according to the attached protocol.

Synthesis of complementary DNA. In the next stage of the investigation complementary DNA - cDNA was synthesized from the isolated RNA, by the reverse transcription reaction using a set of High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA) according to the attached protocol.

Analysis of relative *CDR1, CDR2, MDR1* and *ERG11* gene expression. The relative gene expression of *CDR1*, *CDR2*, *MDR1* and *ERG11* was estimated by Q-PCR. Gene *ACT1,* encoding β-actin, was used as endogenous control. Calibrator (reference sample) was a reference strain of *Candida albicans* not treated with any drugs (obtained by the courtesy of Professor Piotr Kurnatowski, Medical University of Lodz). A set of primers and TaqMan probes, shown in Table 2 was used in the reaction (Custom TaqMan, Applied Biosystems, USA).

Q-PCR reactions were carried out in the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) on 96-well plates using SDS v1.4 software. Each sample, calibrator and the negative control (matrix-free), were analysed twice. Q-PCR reaction for the studied genes proceeded by the following steps:

1. activation of AmpliTaq DNA polymerase Gold® — 95°C, 10 minutes,
2. denaturation — 95°C, 15 seconds,
3. starter annealing and chain elongation — 60°C, 1 minute.

Steps 2 and 3 were repeated 40 times.

Measurement of the fluorescence was performed at a wavelength corresponding to FAM dye (λ_{max}=518 nm) that was attached to probes for tested genes.

The first stage of the analysis was to determine threshold cycles (C_T) for the amplification reaction of tested genes and endogenous control in test samples and the calibrator. Q-PCR reaction was repeated when the difference in C_T values in subsequent repetitions for each sample was larger than 0.5 of the cycle. The next stage was to determine the difference for all samples between C_T values of PCR performed on the matrix of tested genes and endogenous control, β-actin gene (ΔC_T). The normalized value of relative expression (RQ) of tested genes in relation to the calibrator was determined according to the formulas (after Tyburski *et al*., 2008):

Table 2. Sequences of primers and probes used in the Q-PCR method

Gene	Primer sequence	Probe sequence
CDR1	fwd CCGTTTTCGGTCAACTTGTAATGG rev AAACGATCCAGTGGTTTGACTAAGATT	ACACCGACGACAATAT
CDR2	fwd GGCTTATCAATTTTATTCTTCACACAAATGGA rev CAGTCAAGGCAACATAAACTCCTAAGA	ATGCAACGGTAATTCC
MDR1	fwd AACATTATTATATCGCAAGGCTAAAAGAT rev TCCTTCACTTGTGATTCTGTCGTT	CCGGTGATGGCTCTCA
ERG11	fwd ACCCTGAAGATTTTGATCCAACTAGATG rev CCCAAACCCATAATCAACTTCATCAGA	CTGCTGCCAAAGCTAA
ACT1	fwd TTGAGAGTTGCTCCAGAAGAACATC rev AGTCATCTTTTCTCTGTTGGATTTTGGA	TCGGTCAACAAAACTG

ΔC_T (test sample)=C_T test gene — C_T β-actin,
ΔC_T (calibrator sample)=C_T test gene — C_T β-actin,
$\Delta\Delta C_T$=Δ C_T (test sample) — Δ C_T (sample calibrator)
$RQ = 2^{-\Delta\Delta CT}$

The obtained C_T values were analysed and compared (test gene/endogenous control) by Relative Quantitation software (Applied Biosystems, USA).

Assessment of the effect of *ERG11* selected mutations on its expression. The results of previous studies carried out in the Department of Medical and Molecular Biology, published in *Acta Bioch Pol* (Strzelczyk *et al*., 2013) were used to evaluate the effect of selected mutations of *ERG11* on its expression. The test material consisted of the strains sensitive (61) and resistant (61) to azoles, which later was used to assess the expression of *CDR1*, *CDR2*, *MDR1* and *ERG11* genes by Q-PCR. MSSCP technique was used as a method for pre-selection of samples for sequencing. The analysis of *ERG11* sequencing was based on the reference sequences: X_13296 (NW_139492) at the DNA level, and XM_711729, XM_711668 at the mRNA level (Strzelczyk *et al*., 2013).

DNA isolation. DNA isolation was performed using Genomic Mini AX Yeast Kit (A&A Biotechnology, Gdynia, Poland) according to the attached protocol. In addition, the samples were digested using RNase I (included in the kit).

Qualitative and quantitative assessment of the isolated DNA. Qualitative and quantitative analysis of the isolated DNA was performed by spectrophotometry. The absorbance was measured by Biochrom WPA Biowave DNA UV/Vis Spectrophotometer (Biochrom, UK).

Quantitative determination of 5-methylcytosine (5-mC). 5-mC DNA ELISA Kit (Zymo Research Corporation, USA) was used for the quantitative determination of 5-mc according to the attached protocol. The procedure was as follows: thermic denaturation of DNA, coating the plate wells with the isolated DNA, blocking of unbound sites to exclude non-specific binding, reaction with an Anti-5-Methylcytosine and secondary antibody, washing, spectrophotometric analysis (measurement of absorbance at λ=405 nm). Spectrophotometric analysis was performed using VICTOR™ X3 Multilabel Plate Reader (Perkin Elmer, USA). Seven solutions of DNA containing 5-mC (0%, 5%, 10%, 25%, 50%, 75% and 100%) were used to determine the calibration curve. Each sample and standard solutions were analysed twice.

Statistical analysis. The assessment of normality distribution of data on *CDR1*, *CDR2*, *MDR1* and *ERG11* genes expression was performed by the Shapiro-Wilk's test and homogeneity of variance was assessed by Levene's test (Stanisz, 2006). T-test was used for independent data comparison in the groups used in the cases of normal distribution or after data normalisation. To examine the relationship between the occurrence of a mutation in *ERG11* and its expression in *Candida albicans* strains sensitive and resistant to azoles univariate and multivariate regression analysis were applied (stepwise backward regression model).

The Shapiro-Wilk's test was applied to assess normality distribution of the results on the methylation status of *Candida albicans* genome. The Mann-Whitney's test was used to compare data in groups (Stanisz, 2006).

Statistically significant parameters were considered variables for which the level of significance *p* was less than 0.05. Calculations were performed on STATISTICA 10.0 PL (StatSoft, Poland) and StataSE 12.0 (StataCorp LP, TX, USA) software.

RESULTS

The first stage of the experiment was to assess the relative gene expression of *CDR1*, *CDR2*, *MDR1* and *ERG11* in *Candida albicans* strains sensitive and resistant to azole drugs. The test results of the relative gene expression of *CDR1*, *CDR2*, *MDR1* and *ERG11* in strains sensitive and resistant to azoles are shown in Table 3.

Table 3. The results of relative expression of *CDR1, CDR2, MDR1* and *ERG11* genes in tested strains (average, relative expression; the significance level — *p*)

Gene	RQ (2^{-rrCT}) mean		*p*
	C. albicans sensitive	*C. albicans* resistant	
CDR1	28.4	30.9	0.8706
CDR2	0.2	0.3	0.0282
MDR1	0.6	0.9	0.0406
ERG11	0.9	2	<0.001

Sequence analysis of the *ERG11* gene in the studied *Candida albicans* strains revealed 19 different mutations: T462C, T495A, A504G, A530C, C558T, G622A, C216T, A354G, A1587G, T1617C, G1309A, T1143C, A1167G, A1173G, A1230G, C1257T, T1287C, A945C, T1203C. It should be marked that:

The occurrence of T495A, A530C, G622A and A945C mutations led to the following amino acid substitutions: D116E, K128T, V159I and E266D, respectively.

Mutations C558T and A354G were common only in azole-sensitive strains of *Candida albicans*.

Five changes in the sequence of *ERG11* occurred only in strains resistant to azoles (A530C, G622A, G1309A, A1167G and A1230G). The most frequently occurring mutation in this group of strains was G1309A (54%).

33% of strains resistant to azoles were characterised by the simultaneous presence of three mutations: A530C, G622A and A1167G.

Univariate regression analysis (stepwise backward regression model) showed that five mutations T462C, G1309A, C216T, C1257T and A945C are significantly associated with the expression of *ERG11* wherein it should be emphasized that the presence of two first increases and the other reduces the relative expression of *ERG11*.

The subsequent phase of work consisted of quantitative determination of 5-methylcytosine using the ELISA method. It has been found that strains of *Candida albicans* sensitive and resistant to azoles were characterised by the presence of 5-mC in the genome at levels of 0.635% and 0.596% (p=0.3334), respectively.

DISCUSSION

A very important problem regarding the treatment of infections is the phenomenon of resistance of *Candida albicans* to many antifungal compounds. Decrease of the sensitivity of these pathogens to chemotherapeutics is a complex process, with many underlying mechanisms (White *et al*., 1998).

In this study, we investigated relative expression of *CDR1*, *CDR2*, *MDR1* and *ERG11* genes in azole resistant and sensitive strains of *Candida albicans*. The applied method of Q-PCR allowed for detection of statistically significant differences in the expression of *CDR2*

Table 4. Relation between the presence of mutations in *ERG11* and the relative expression of the gene (factor β, confidence interval -CI, the level of significance — *p*)

log_{10}(RQ)	Univariate		
Mutation ERG11	β	± 95% CI	*p*
T462C	0.676	0.174–1.778	<0.01
T495A	0.126	–0.652–0.393	0.633
A504G	–0.279	–0.820–0.261	0.307
A530C	0.371	–0.379–1.122	0.327
C558T	–0.187	–0.767–0.393	0.523
G622A	0.360	–0.363–1.084	0.325
C216T	–0.873	–1.55–0.196	<0.05
A354G	–0.396	–0.979–0.188	0.181
A1587G	–0.478	–0.99–0.357	0.068
T1617C	–0.417	–0.931-0.977	0.110
G1309A	0.742	0.11–1.283	<0.01
T1143C	–0.432	–0.942–0.78	0.096
A1167G	0.367	–0.313–1.046	0.268
A1173G	–0.432	–0.943–0.078	0.096
A1230G	0.287	–1.379–1.953	0.732
C1257T	–0.657	–1.154–0.16	<0.05
T1287C	–0.373	–0.556-0.481	0.887
A945C	–1.12	–1.629–0.612	<0.001
T1203C	–0.432	–0.943-0.078	0.096
log_{10}(RQ)	Multivariate		
A945C	–1.120	–1.629 0612	<0.001

(0.0282), *MDR1* (0.0406) and *ERG11* (<0.001) genes. It is worth emphasizing that the expression was studied using the Relative Quantitation software (RQ) for larger group of strains in comparison to studies by other authors (Chau et al., 2004; Cernicka & Subik, 2006; Goldman et al. 2004; Chen et al. 2010). Moreover, the same analyses were performed on strains sensitive and resistant to azole drugs.

The next stage of research centered on factors influencing changes in *ERG11* gene expression. Univariate regression analysis (stepwise backward regression model) revealed that five mutations T462C, G1309A, C216T, C1257T and A945C are significantly associated with the expression of *ERG11* wherein it should be emphasized that the presence of two first increased and the other reduced the relative expression of *ERG11*. When taking into consideration the division of strains into sensitive and resistant, only A945C mutation had a significant effect on the expression of *ERG11* gene, decreasing it.

The process of methylation in fungal cells has not yet been sufficiently investigated. There are only few papers regarding this issue. Mishra and coworkers (2011) studied 150 genes and demonstrated that *Candida albicans* genome methylation concerns only genes involved in morphogenesis, cell growth, phenotypic switching, iron metabolism, drug resistance, intercellular signaling, stress response, chromatin organisation, cell cycle and virulence. The authors believe that dynamic changes in the methylation profile may explain the morphological plasticity of *Candida albicans* cells. Although the applied method of assessing the level of *Candida albicans* genome methylation has not revealed its role in the development of resistance to azoles, it showed similar level of methylation, suggested by other authors (Mishra *et al*., 2011).

Our results showed that the mechanism of developing resistance to azoles is a complex process. It is associated both with genes that affect the functioning of active transport pumps participating in the drug efflux from the cell and the gene encoding lanosterol 14-α-demethylase, which is the target of azole drugs.

The observed increase in the expression of *CDR2* and *MDR1* genes can lead to increased active efflux of azole particles from the cell before they react with the target.

Presented results indicate also that the occurrence of drug resistance in the tested strains involves two mutations of *ERG11*: T462C and G1309A. These mutations affect an increase of the expression of this gene and may lead to modifications of lanosterol 14-α-demethylase affinity to azole drugs.

Acknowledgements

We would like to thank Professor Piotr Kurnatowski, Medical University of Lodz for providing us with a reference strain of *Candida albicans*.

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